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Thiamin requirement and carbohydrate metabolism in the nematode, caenorhabditis elegans

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THIAMIN REQUIREMENT AND CARBOHYDRATE METABOLISM IN THE NEMATODE, *CAENORHABDITIS ELEGANS*

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ABSTRACT

THIAMIN REQUIREMENT AND CARBOHYDRATE METABOLISM IN THE NEMATODE, CAENORHABDITIS ELEGANS

By Joi L. Augustin

Caenorhabditis elegans were cultivated in chemically defined media supplemented with two different energy sources, glucose and acetate, and five levels of thiamin (0, 0.0075, 0.075, 0.75, and 7.5 µg/ml). Optimal population growth occurred in both media at 0.075-7.5 µg/ml thiamin. At thiamin levels 0 and 0.0075 µg/ml, population growth and lactate to pyruvate (L:P) ratios were significantly reduced, while tissue pyruvate concentrations were significantly increased ($p < .05$), indicating severe thiamin deficiency. In acetate media, L:P ratio was significantly lower at 0.75 µg/ml thiamin, suggesting a higher thiamin requirement when acetate was used as the energy source. Results also suggest that optimal population growth and normal carbohydrate metabolism with respect to thiamin were achieved in C. elegans culture medium using glucose with 0.75 µg/ml thiamin and acetate with 7.5 µg/ml thiamin.

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PREFACE

The following is a publication style thesis. Chapter 2 is written in journal format according to 1992 guidelines and will be submitted to the Proceedings of the Society for Experimental Biology and Medicine. Chapters 1 and 3 are written according to guidelines outlined in the Publication Manual of the American Psychological Association (3rd. edition), 1990.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Caenorhabditis elegans (C. elegans) is a free-living, egg laying, microscopic nematode. It has a relatively short life-cycle, a rapid generation time, and the ability to be cultivated in large quantities for bioassay. For these reasons, it has become widely used as an animal model for biological (Croll & Matthews, 1977; Kenyon, 1988), genetic (Sulston & Brenner, 1974), and nutritional research (Lu et al., 1977,1978; Rothstein, 1965). While it is known that thiamin is required by the nematode, C. elegans (Nicholas et al., 1962), the amount of thiamin necessary for optimal population growth and maintenance is unknown. This information is necessary to complete the nutritional requirements of this animal model for nutrition research.

Thiamin is required by humans as well as by the nematode, C. elegans, for carbohydrate metabolism (Nicholas, 1984). Metabolism of glucose requires thiamin in the decarboxylation reaction which converts pyruvate to acetyl Coenzyme A (CoA). This reaction is theorized to be an important step in the energy producing glycolytic pathway in

the nematode, C. elegans. Acetate can also serve as fuel for energy production in C. elegans through the tricarboxylic acid cycle (TCA cycle) but not the glycolytic pathway described above. Because this conversion bypasses the pyruvate decarboxylase reaction, use of an experimental medium made with acetate as the energy source may provide different results with respect to thiamin requirement and population growth.

The first objective of this study was to quantitatively determine the thiamin requirement for optimal population growth of the nematode, C. elegans. This study was also conducted to investigate carbohydrate metabolism in C. elegans using experimental media containing two different energy sources. Finally, the levels of pyruvate and lactate in nematode tissues and culture media were determined to assess thiamin deficiency in the different experimental cultures of C. elegans.

Review of Literature

Thiamin

Metabolic functions and biochemical assay. Thiamin in its coenzyme form, thiamin diphosphate (TPP), participates in several reactions involved in the production of energy in humans (Figure A). The oxidative decarboxylation of pyruvate

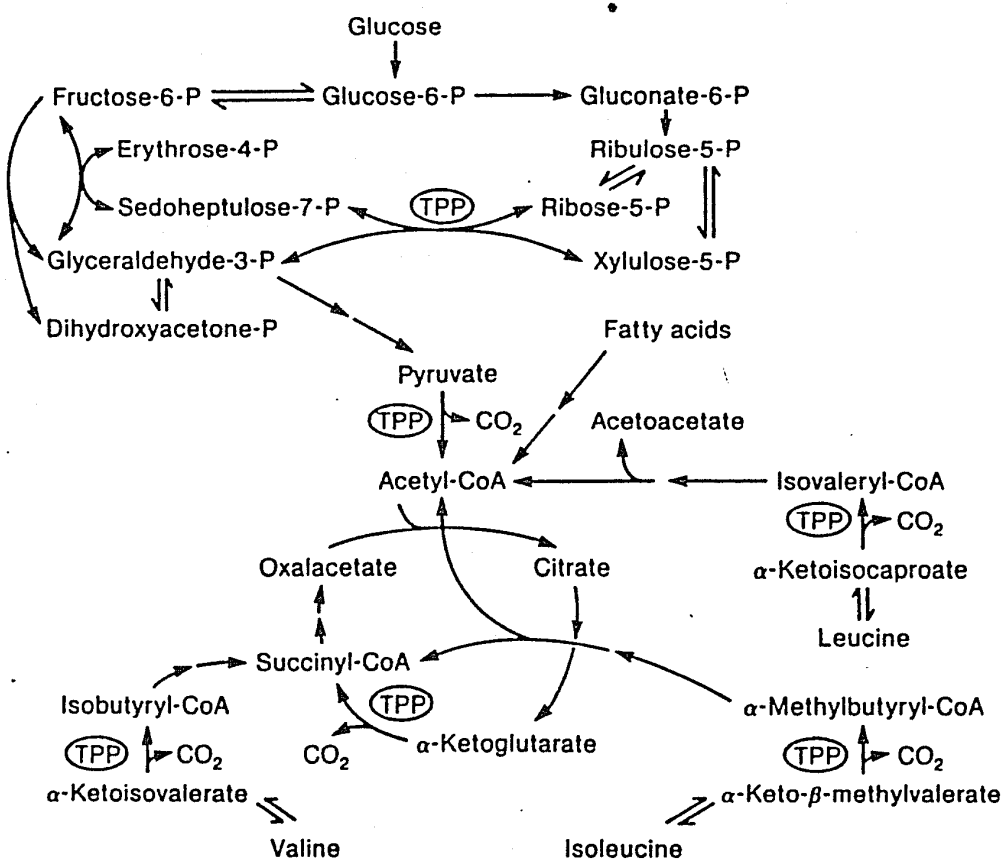

Figure A. Pathways dependent upon coenzyme forms of thiamin, TPP.

Note. From Modern Nutrition in Health and Disease (p. 359) by D.B. McCormick, 1988, Philadelphia: Lea & Febiger.

via glycolysis in the cytosol of cells and of ∂-ketoglutarate in the TCA cycle in the mitochondria are two of these reactions (Harper, 1979). Another oxidative decarboxylation in which TPP participates as a coenzyme is the transketolase reaction. In mammalian tissues, the main action of transketolase is related to the formation of ribose and dihydronicotinamide adenine dinucleotide phosphate (NADPH) from glucose; this is the mechanism for the synthesis of riboses, components of nucleotides, in humans (Sauberlich, 1967). The transketolase reaction is part of the pentose phosphate pathway and takes place in the cytosol of cells in various tissues of the body. The metabolism of branched chain amino acids also involves thiamin.

Biochemical tests of thiamin deficiency in humans include measurements of urinary thiamin level, urinary metabolites of thiamin, blood pyruvic and ∂-ketoglutaric acids, and erythrocyte transketolase activity (Sauberlich, 1967). The test which reflects the immediate functional level of TPP is a measurement of the ratio of lactic acid to pyruvic acid in the blood after a glucose load has been administered (Harper, 1979). One of the earliest observations of a biochemical abnormality in humans with thiamin deficiency was the accumulation of pyruvic acid in

the tissues and an increase of pyruvic acid concentration in the blood (Sauberlich, 1967). The most reliable method of determining thiamin status involves a measurement of transketolase activity in erythrocytes in vitro after addition of TPP (Brin, 1983). During thiamin deficiency, TPP levels decrease; therefore, the total activity of transketolase also decreases (Brin, 1983).

Deficiency and toxicity. Ommision of thiamin from basal media impairs normal growth and reproduction of C. briggsae (Nicholas et al., 1962). In humans, thiamin deficiency affects primarily the cardiovascular and peripheral nervous systems and the gastrointestinal tract. Beriberi is the acute disease resulting from thiamin deficiency in humans. It is characterized by mental confusion, anorexia, muscle weakness, ataxia, peripheral paralysis, muscle wasting, tachycardia, and cardiomegaly (Sauberlich, 1967). Wernicke's encephalopathy and the amnesic psychosis of Korsakoff syndrome result from changes in the central nervous system (CNS) in thiamin deficiency. Likewise, thiamin contributes to the composition and function of the CNS by essential reactions in energy production and in the biosynthesis of lipids and acetylcholine. Thiamin and its phosphate esters

are found in axonal membranes of nerve cells (McCormick, 1988).

Approximately 30 mg of thiamin are stored in the skeletal muscle and tissues of the brain, heart and kidney of humans; 80% of which is in the TPP form (McCormick, 1988). As a deficiency develops, there is a rapid loss of the vitamin from tissues.

There are several causes of thiamin deficiency, including inadequate dietary intake due to the consumption of refined, nonenriched grains or the ingestion of thiaminases (antithiamin factors) from raw fish and tea (Evans, 1975). Chronic alchoholism is a common contributor to thiamin deficiency due to decreased dietary consumption and impaired absorption. In addition, there are inborn errors of metabolism that relate to thiamin deficiency; lactic acidosis results from an absent or defective pyruvate decarboxylase enzyme (Scriver, 1973). Regardless of the cause of the deficiency, the symptoms or effects can be related to abnormal carbohydrate metabolism, i.e., a decrease in oxidative decarboxylation (Sauberlich et al., 1979).

Some diseases which result from thiamin deficiency are exacerbated by increased carbohydrate intake. For example, wet beriberi (with edema) results from increased physical

activity and high carbohydrate intake (Wilson, 1983). Watson et al. (1981) reported that carbohydrate loading hastens the occurrence of the peripheral neuropathy of Wernicke's Korsakoff syndrome. This disease develops as a result of severe thiamin deficiency often due to excessive consumption of alcohol and decreased consumption of food (Sauberlich, 1967).

Williams et al. (1943) found that plasma levels of lactic and pyruvic acids increased following a glucose load (100 gm) in subjects with moderate thiamin deficiency. In another study by Shigematsu et al. (1989), elevated plasma levels of branched chain amino acids and pyruvate were demonstrated in rats fed a thiamin deficient diet for four weeks.

When excess thiamin is consumed by humans, it is excreted via the urine. Williams et al. (1943) reported that rats fed 100 times the required level of thiamin for three generations exhibited no harmful side effects. Prolonged parenteral administration of pharmacological doses (greater than 400 mg daily) of thiamin caused nausea, anorexia, lethargy, and mild ataxia in humans (McCormick, 1988).

Nematode

Biology. C. elegans has a brief life cycle whereby the fertilized egg develops into the mature adult in 3.5 days

(Marx, 1984). Croll and Matthews (1977) reported that the longevity of C. elegans in axenic culture is 34 days at 18-22°C. Adults (Figure B) are sexually dimorphic, male and self-fertilizing hermaphrodite; it is the hermaphrodite through which most reproduction occurs (Edgar & Wood, 1977). The juvenile nematodes proceed through a series of four molts prior to reaching adulthood (Kenyon, 1988). A stage has been identified which occurs at the second molt; the "dauer" larvae has the capability of delaying maturation to withstand adverse conditions such as starvation (Croll & Matthews, 1977; Riddle, 1982).

Adult nematodes of C. elegans are approximately 1.0 mm long and 0.1 mm in width. One adult nematode has about 1000 somatic cells (Marx, 1984). The body is elongated, cylindrical, and tapered at both ends, a shape well suited for undulatory locomotion (Nicholas, 1984). A tough cuticle encloses the organism which is characterized by a tube within a tube; the outer tube represents the cuticle and longitudinal muscles, whereas the inner tube forms the alimentary tract composed of a buccal cavity, pharynx, intestine, and rectum (Nicholas, 1984).

Caenorhabditis briggsae (C. briggsae) and C. elegans are two closely related species of free-living nematodes which

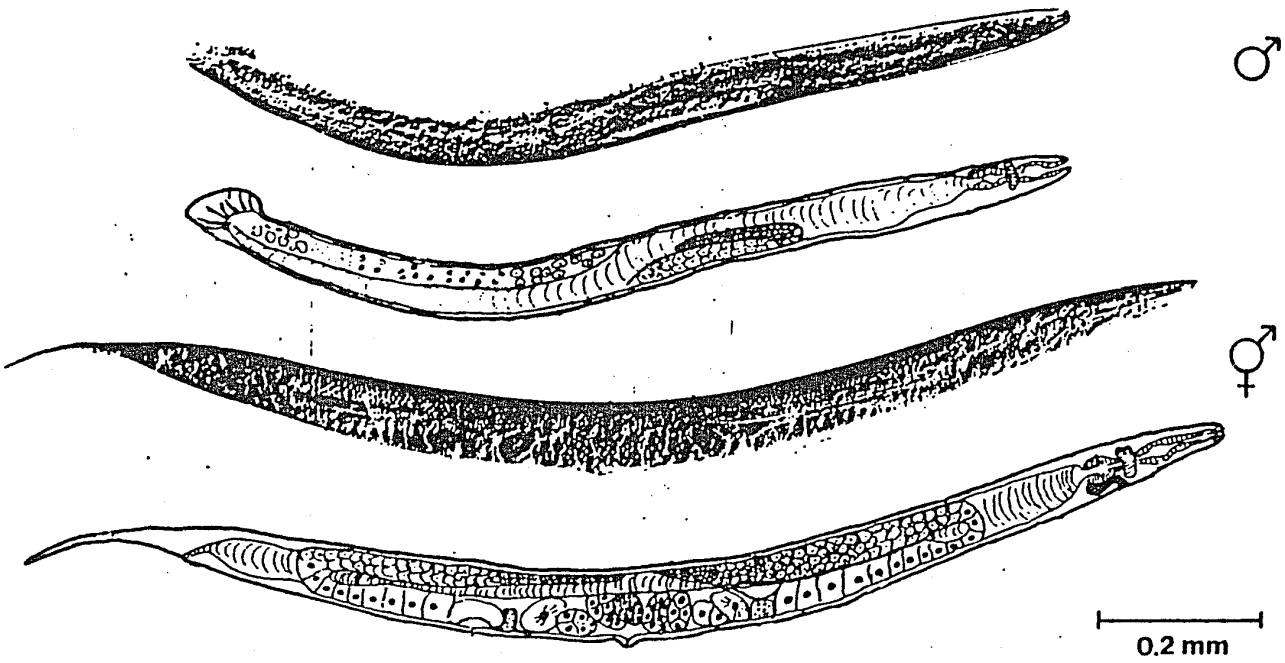

Figure B. Adults of Caenorhabditis elegans: male (above),hermaphrodite (below).

Note. From Nematodes as Biological Models: Volume I, Behavioral and Developmental Models (p. 7) by B.M. Zuckerman, 1980, New York: Academic Press.

differ only by genetic composition and the inability to interbreed (Friedman et al., 1977; Nicholas, 1984). The hermaphrodites of both species cannot be distinguished from one another by simple observation; therefore, prior to 1977, much confusion occurred regarding the identity of the specie being studied (Vanfleteren, 1980). Nicholas (1984) reported that, as a result of this confusion, some papers published on C. briggsae may in fact describe work on C. elegans.

Nutritional requirements. In nature, the nematode, C. elegans, lives in the soil and feeds on microorganisms (Kenyon, 1988). However, in the laboratory, C. elegans is cultivated axenically on a chemically defined medium consisting of C. briggsae Maintenance Medium (CbMM) and other defined compounds such as ß-sitosterol (sterol source), cytocrome c (heme source), and an energy source (acetate or glucose)(Lu et al., 1978). C. briggsae Maintenance Medium contains specific quantities of amino acids, nucleic acids, vitamins and growth factors, and minerals (Buecher et al., 1966).

Hansen and Buecher (1970) found that glucose and trehalose were the carbohydrate forms most readily utilized by the nematodes and that a lack of carbohydrate reduced reproduction in C. briggsae and C. elegans. Lu and Goetsch

(in press) tested various concentrations of glucose in basal media and reported that 32.5 mg/ml glucose supported maximal population at 80,000 nematodes/ml.

Amino acid requirements of C. briggsae were investigated by Vanfleteren (1973). Arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine, and valine are not synthesized by the nematode at levels to support reproduction and, therefore, are essential amino acids. The amino acids synthesized by C. briggsae and referred to as nonessential include alanine, asparagine, cysteine, glutamate, glutamine, glycine, proline, serine, and tyrosine.

Hieb and Rothstein (1968) demonstrated a sterol requirement in C. briggsae which could be met by cholesterol, ergosterol, or ß-sitosterol. Lu et al.(1977) found the optimal level of ß-sitosterol required by C. elegans to be 50 µg/ml. In 1978, Lu et al. reported that other lipid-related compounds--sodium oleate, sodium stearate, Tween 80, Tween 85, ethanol, n-propanol and potassium acetate--were also required for growth of C. briggsae.

The fatty acid composition of C. elegans and C. briggsae was determined by Hutzell and Krusberg (1982). Lipid accounted for 19.6 and 22.1% of the dry weight of C. elegans

and C. briggsae, respectively. Unsaturated fatty acids equal 78% of the total lipid dry weight of C. elegans. Rothstein (1969) reported that C. briggsae synthesized polyunsaturated fatty acids by the conversion of oleic to linoleic acid.

The requirement of heme (iron-containing porphyrin compound) by C. briggsae was first reported by Hieb et al. in 1970. Cytochrome c is the most effective heme source for promoting growth and reproduction in C. briggsae. Chang (1990) investigated the effects of six concentrations of cytochrome c and three surface area exposures of the culture medium on the population growth and ATP production of C. elegans. Optimal population growth was achieved with 200 µg/ml cytochrome c and a large surface area (12 cm^2) exposure. The author suggested that the effect of surface area exposure of the culture medium is more prominent than the concentration of cytochrome c (a heme source) on ATP production.

Nicholas et al. (1962) found that the ommission of thiamin, riboflavin, folic acid (pteroylglutamic acid), niacinamide, pantothenic acid, and pyridoxine from basal media (supplemented with tissue extract) impaired normal growth and reproduction of C. briggsae. They suggested that these B vitamins are essential nutrients for the nematode.

The effects of varying levels of pyridoxamine, pyridoxine, and pyridoxal phosphate (vitamin B_6) on the growth and reproduction of C. elegans was reported by Sun et al. (1986). Tryptophan metabolites (xanthurenic and kynurenic acids) accumulated in the culture medium of Vitamin B_6 deficient worms.

A folic acid requirement was demonstrated by Vanfleteren and Avau (1977) by the addition of aminopterin (a folic acid antagonist). Lu et al. (1974) found that formimino L-glutamic acid (FIGLU) accumulated in nematode tissue during folic acid deficiency, suggesting that folic acid is required for the catabolism of histidine. In 1976, Lu et al. demonstrated that both vitamin B_{12} and folic acid were required for the biosynthesis of methionine from homocysteine in C. briggsae. A biotin requirement was exhibited by adding avidin to the culture medium (Nicholas & Jantune, 1963).

Lu and coworkers (1983) induced mineral deficiencies of magnesium, sodium, potassium, manganese, calcium, and copper in the nematode, C. elegans, by deleting each mineral from a completely chemically defined medium. The quantitative requirements for each mineral were determined to be 73, 300, 530, 6.3, 1500, and 7.2 µg/ml, respectively. A zinc deficiency was not demonstrated.

Carbohydrate metabolism. Metabolism of carbohydrate by means of the Embden-Meyerhof pathway (Figure C) occurs in most nematode species (Nicholas, 1984). Rothstein (1963) first suggested the presence of a TCA cycle in C. briggsae through a radioisotope study of the excretion products of this nematode. Amino acids whose biosynthesis is associated with the transamination of keto-acid intermediates of the TCA cycle (Figure C) were present in the culture media. The tricarboxylic acid cycle is in the mitochondria of intestinal cells and completes the oxidation of carbohydrates and lipids, which enter the cycle as acetyl CoA. Murfitt et al. (1976) investigated metabolism and respiration of C. elegans and found that these systems are similar to those of various mammalian species (rat, dog, and human).

Another metabolic pathway utilized by the free-living nematode is the glyoxylate cycle or glyoxylate shunt (Rothstein & Mayoh, 1969). The glyoxylate cycle bypasses acetyl CoA from the TCA cycle and results in the synthesis of four-carbon compounds (malate, oxaloacetate) from two-carbon units (acetyl CoA, glyoxylate) rather than further oxidation to carbon dioxide (CO_2) via the TCA cycle (Figure D). This is important to organisms which utilize acetate as the only source of carbon for growth. Rothstein and Mayoh (1969),

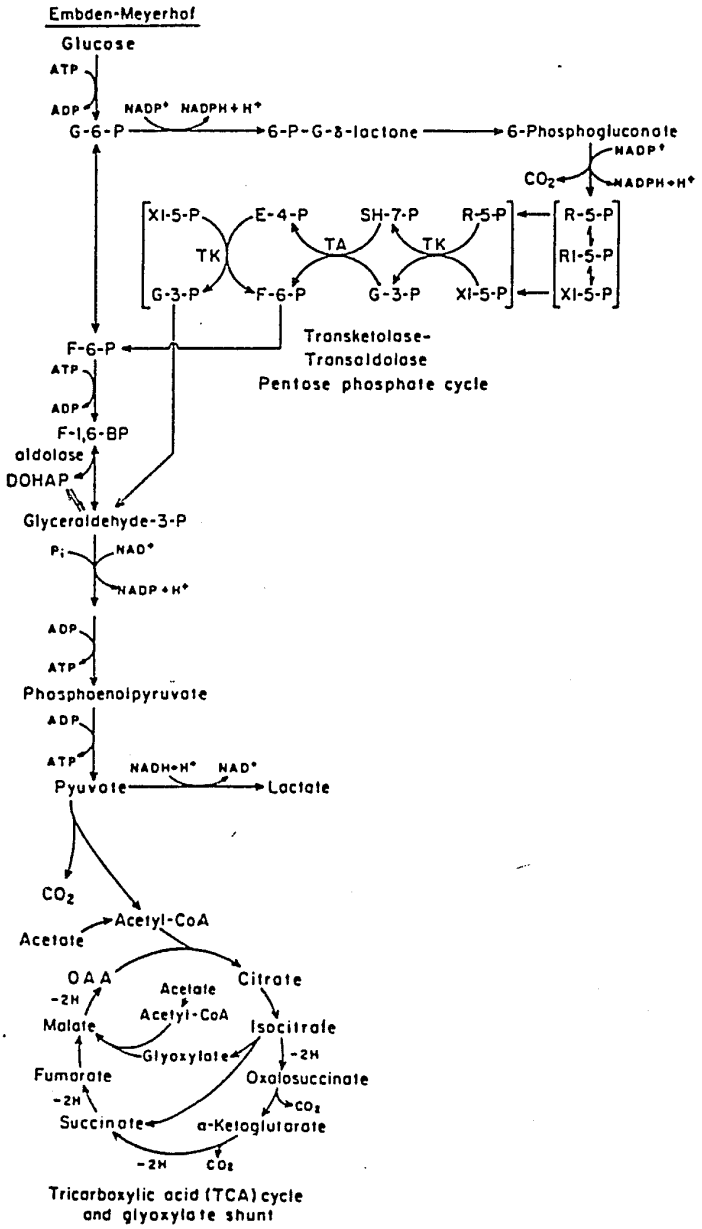

Figure C. Pathways of carbohydrate metabolism.

Note. Adapted from Microbial Physiology (p.26) by A.G. Moat and J.W. Foster, 1988, New York: John Wiley & Sons.

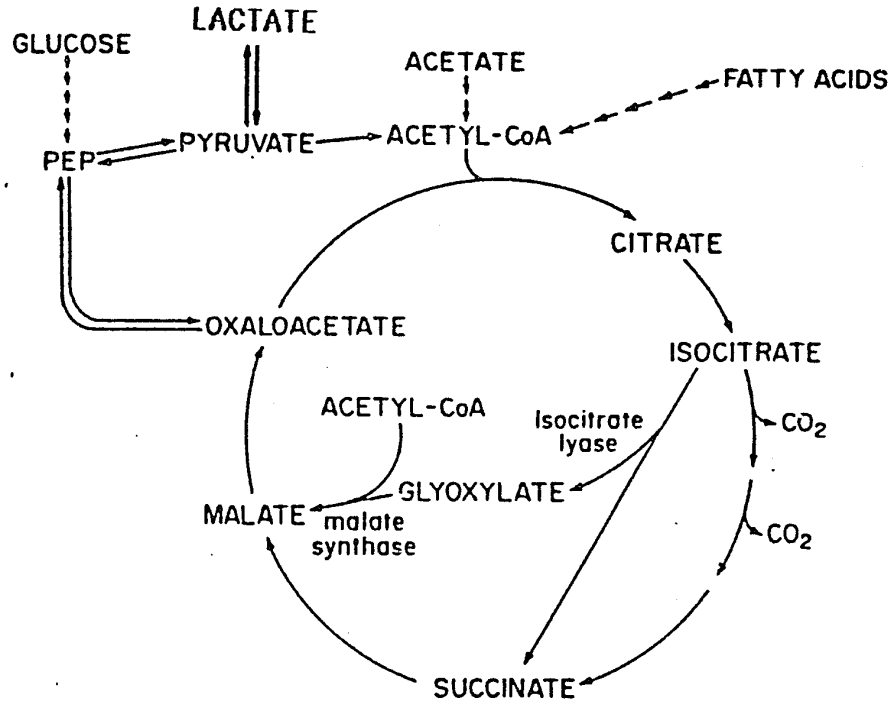

Figure D. The glyoxylate cycle or glyoxylate shunt.

Note. Adapted from Microbial Physiology (p.138) by A.G. Moat and J.W. Foster, 1988, New York: John Wiley & Sons.

Colonna and McFadden (1975), and Patel and McFadden (1978) identified the glyoxylate cycle enzymes, isocitrate lyase and malate synthase, in C. elegans. According to Moat and Foster (1988), the enzymes of the glyoxylate bypass system are repressed by the presence of glucose by a process known as catabolite repression.

Bolla (1987) suggested that nematodes such as C. briggsae are capable of altering the production of metabolic enzymes to fit the nutritional state of their environment. In a nutrient rich medium, the nematode shifts metabolism to a less efficient use of carbohydrate, such as glycolysis. According to Bolla (1987), a highly efficient and complete pathway requires the synthesis of additional enzymes at a large energy cost to the animal. Although some species of nematode may metabolize energy-producing carbon substrates completely to CO_2 and water, other species excrete partially metabolized products such as amino acids, carbohydrates, and intermediates of metabolic pathways (Bolla, 1987). Nicholas (1984) reported that lactic and pyruvic acids, which are end products of intermediate energy metabolism, are excreted by anaerobic parasitic nematodes and several plant parasitic nematodes, but usually not by free-living nematodes.

Phosphorus nuclear magnetic resonance and enzyme assays were used to determine changes in energy metabolism during larval development in C. elegans (Wadsworth & Riddle, 1989). Isocitrate lyase in C. elegans was examined as a measure of glyoxylate cycle activity. The activity is highest in embryos and decreases during the first and subsequent larval stages. Results suggest that there is a switch from the use of the glyoxylate cycle in embryos and first stage larvae to increased use of the TCA cycle in the second, third, and fourth stages. The tricarboxylic acid cycle is utilized in later stages of growth due to increased demand for energy and food intake (Wadsworth & Riddle, 1989).

Similar results were obtained by O'Riordan and Burnell (1989) when they investigated intermediary metabolism in the dauer larvae of C. elegans. The rate of flux of metabolites through the pathways of carbohydrate catabolism is reduced in dauer larvae compared to adults. Dauer larvae possess a considerable capacity to metabolize glycogen and to form oxaloacetate through the glyoxylate cycle, and the maximal rate of flux of metabolites through the TCA cycle are reduced. These metabolic changes form part of the adaptive response enabling dauer larvae to survive for several months without feeding (O'Riordan & Burnell, 1989).

CHAPTER 2

JOURNAL ARTICLE

Authors Title Page

THIAMIN REQUIREMENT AND CARBOHYDRATE METABOLISM IN THE NEMATODE *CAENORHABDITIS ELEGANS* [1]

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ABSTRACT

Caenorhabditis elegans were cultivated axenically in chemically defined media supplemented with two different energy sources, glucose and acetate (at 1:2 molar concentration ratio), and five levels of thiamin (0, 0.0075, 0.075, 0.75, and 7.5 µg/ml). Optimal population growth occurred at 0.075-7.5 µg/ml thiamin. Population growth was significantly reduced ($p < .05$) at thiamin levels 0 and 0.0075 µg/ml, indicating severe thiamin deficiency. Enzymatic determination of pyruvate and lactate in tissue extracts and culture media was conducted. Tissue pyruvate concentrations for nematodes cultivated in 0 and 0.0075 µg/ml thiamin were 1.6 and 1.8 ng/µg dry weight for glucose and 3.3 and 3.9 ng/µg dry weight for acetate. These concentrations were significantly higher ($p < .05$) from pyruvate concentrations (0.2-0.4 ng/µg dry wt) at thiamin levels 0.075-7.5 µg/ml, indicating pyruvate accumulation in nematode tissue, during thiamin deficiency. Lactate to pyruvate (L:P) ratios were computed from lactate and pyruvate concentrations in culture media. In glucose media, nematodes at 7.5 and 0.075 µg/ml thiamin had significant L:P ratios of 10 and 4.9, respectively. The L:P ratios in acetate media were significant at higher thiamin levels (7.5 and 0.75 µg/ml),

indicating a higher requirement for thiamin. This also suggests that mild thiamin deficiency may be present for nematodes in glucose media at 0.075 µg/ml thiamin and in acetate media at 0.75 µg/ml thiamin. Because there was no significant difference in population growth or tissue pyruvate between 0.075-7.5 µg/ml thiamin for nematodes cultivated in either glucose or acetate media, results suggest that L:P ratio could be used further to assess thiamin status in C. elegans.

INTRODUCTION

Caenorhabditis elegans (C. elegans) is a free-living, microscopic nematode. It has a relatively short life-cycle (34 days)(1), a rapid generation time (3.5 days), and the ability to be cultivated in large quantities for bioassay (2). Thus, it has become widely used as an animal model for nutrition research (3-7). While it is known that thiamin is required by C. elegans (8), the quantitative amount of thiamin necessary for optimal population growth and maintenance is unknown. This information is necessary to complete the nutritional requirements of this animal model. The axenic (germ-free) culture of C. elegans in a chemically defined media provides a good animal model for the investigation of metabolic interrelationships of a

multicellular organism under controlled nutritional conditions.

The objective of this study was to quantitatively determine the thiamin requirement for optimal population growth in C. elegans. Carbohydrate metabolism was also studied in C. elegans using experimental media containing two different energy sources: glucose (9) and acetate (5). Futhermore, thiamin status was assessed by the enzymatic determination of pyruvate and lactate in C. elegans tissues and culture media.

MATERIALS AND METHODS

Stock Media and Culture. The following culture methods are a modification of Lu *et al.*(3) and Pinnock *et al.*(10). C. elegans were cultivated axenically in 18 x 150 mm culture tubes in 5 ml of stock medium consisting of 4% Hi-soy (Sigma, St.Louis, MO), 1% yeast extract (Sigma, St.Louis, MO), and 10% heated liver extract (11). Cultures were incubated at 20°C on a tissue culture rotator for approximately 14 days. Nematodes were harvested by light centrifuging, washing (12), and resuspending in distilled water for use as innoculum for experimental cultures. Approximately 2500 nematodes in 0.1 ml were innoculated resulting in an initial population of approximately 500 nematode/ml in the experimental medium.

Experimental Media and Cultures. The basal experimental medium consisted of Caenorhabditis briggsae Maintenance Medium (CbMM)(13) less glucose and thiamin, cytochrome c (50µg/ml)(14), and ß-sitosterol (50µg/ml)(4,15). The basal medium was subsequently differentiated into two experimental media by the supplementation of energy source in the form of sodium acetate (6.95 mg/ml or 0.051 mole/liter) and glucose (4.6 mg/ml or 0.026 mole/liter) at a 2:1 molar ratio. Thiamin·HCl was also supplemented into the experimental chemically defined media at five levels (0 (control), 0.0075, 0.075, 0.750, and 7.5 µg/ml) to determine which level would promote optimal population growth. The level of thiamin present in CbMM is 7.5 µg/ml. Quadruplicate culture tubes for thiamin levels 7.5, 0.75, and 0.075 µg/ml, and twelve culture tubes for thiamin levels 0.0075 and 0 µg/ml were set up for each experimental media, for a total of 72 culture tubes.

Experimental cultures were incubated in 18 x 150 mm culture tubes at 20°C on a tissue culture rotator. A preliminary study of population growth was conducted for approximately 28 days to determine the average day of peak population growth among experimental cultures. Triplicate 0.1 ml samples of each experimental culture were counted on a

dissecting microscope by a simple tally of individual nematodes; counts took place each week to assess population growth. From the initial study, it was determined that day 18 represented the average day of peak population growth among nematode cultures. Therefore, experimental cultures were incubated for 18 days prior to determination of final population growth. For thiamin levels 0.0075 and 0 µg/ml, 12 culture tubes were pooled into four experimental cultures prior to population and dry weight determinations and tissue extraction. This was to ensure sufficient quantity of tissue extract for dry weight, and for pyruvate and lactate determination.

Dry Weight Determination. One culture from each thiamin level was washed and dried overnight in an oven at 100°C to obtain the dry weight of the nematode culture (1). The dry weight of average individual nematodes was calculated by dividing the total dry weight of the nematode culture by the number of nematodes in the culture.

Extraction and Measurement of Pyruvate and Lactate. Nematodes were separated from the culture medium by centrifugation and washed twice in distilled water. Culture medium (2 ml) was prepared for assay by vortex mixing with 8% perchloric acid (4 ml). Nematodes suspended in 2 ml

distilled water were homogenized in 8% perchloric acid (4 ml) for 10 minutes in an ice bath using a Potter Elvejehm Tissue Grinder powered by a Sears Craftsmen electric drill at 1200 rpm. Both tissue extracts and media were filtered using glass fiber filter paper (2.3 µm pore size) and stored frozen at 0° C for 1-4 days prior to lactate and pyruvate determination.

Enzymatic determination of pyruvate and lactate (16) in nematode tissue extracts and culture media was achieved using the Pyruvate and Lactate Determination Kits (Sigma, St. Louis, MO) and the Shimadzu UV Visible Recording Spectrophotometer. The procedure utilizes the enzyme lactate dehydrogenase (LDH) which catalyzes the reversible reaction of pyruvate and dihydronicotinamide adenine dinucleotide (NADH) to lactate and nicotinamide adenine dinucleotide (NAD). The amount of lactate and pyruvate present in the sample is determined by the measurement of the absorbance of NADH at 340 nm. The oxidation of NADH to NAD causes a reduction in absorbance and provides a measurement of the amount of pyruvate in the original sample. For the determination of lactate, the increase in absorbance at 340 nm is due to the reduction of NAD to NADH which provides a measure of the lactate originally present (16).

Statistical Analysis. One-way analysis of variance (ANOVA) with a Scheffe post hoc analysis was utilized to determine statistical significance of population growth, tissue pyruvate concentration, and lactate:pyruvate ratio of culture media at various levels of thiamin.

RESULTS

Population growth, dry weight, pyruvate, and lactate in C. elegans as affected by various concentrations of thiamin (0, 0.0075, 0.075, 0.75, and 7.5 µg/ml) and two different energy sources (glucose and acetate) are shown in Table I.

Quantitative Requirement of Thiamin. When C. elegans were cultivated in media containing no thiamin, population as well as dry weight of average individual nematodes were the lowest. In addition, nematodes appeared much smaller, curled, and lethargic as opposed to normal size and undulatory movement. In general, population growth increased as thiamin concentrations increased. The highest population growth (60 ± 8.5 nema x 10^3/ml) was supported by glucose test media with thiamin concentrations at 7.5 and 0.75 µg/ml. There was no significant difference ($p \geq .05$) between population growth at thiamin levels 0.075, 0.75, and 7.5 µg/ml in either glucose or acetate test media.

Effect of Energy Source on Dry Weight and Population Growth. Reduced population growth occurred in cultures of C. elegans grown in the acetate test media compared to the glucose test media. The dry weight of average individual nematodes as determined by the dry weight of the population varied with both concentration of thiamin and with media energy source. In acetate test media, the dry weight of average individual nematodes (72-82 ng) was about double that of nematodes cultivated in glucose test media (38-41 ng) for the thiamin levels 0.075-7.5 μg/ml. Dry weights of thiamin deficient nematodes (0 and 0.0075 μg/ml) were lower; however, they were similar for both glucose and acetate media at 12 and 24 ng and 14 and 21 ng, respectively.

Effect of Thiamin Deficiency on Pyruvate and Lactate Accumulation. There was no significant difference ($p \geq .05$) between concentration of tissue pyruvate (0.2-0.4 ng/μg) for thiamin levels of 7.5, 0.75, and 0.075 μg/ml in both experimental media. The level of thiamin had little or no effect on the concentration of pyruvate in the tissues except for the 0.0075 and 0 μg/ml levels which were significantly higher in both test media ($p < .05$). The concentration of pyruvate in thiamin deficient nematode tissues (0 and 0.0075 μg/ml thiamin) cultivated in glucose was approximately nine

times greater than base level pyruvate for nematodes grown in 7.5 µg/ml thiamin. For nematodes cultivated in acetate, the concentration of pyruvate was approximately 16-20 times greater in thiamin deficieny (0 and 0.0075 µg/ml thiamin) compared to nematodes grown in 7.5 µg/ml thiamin. Pyruvate concentrations in thiamin deficient tissues (0 and 0.0075 µg/ml thiamin) of C. elegans grown in the acetate test media (3.3 ± 0.3 and 3.9 ± 0.4 ng/µg) were approximately two times greater than the pyruvate of those grown in the glucose test media (1.6 ± 0.3 and 1.8 ± 0.3 ng/µg). It was not possible to determine lactate concentrations in C. elegans tissues; it is theorized that the levels present in the nematode tissues were below the sensitivity of the assay used in this study. However, lactate was determined for culture media.

Culture media levels of both pyruvate and lactate increased with a decrease in thiamin concentration. For thiamin deficient nematodes grown on glucose media and 0 µg/ml thiamin, there were approximately three times the pyruvate (170 ± 22 ng/µg dry wt) and lactate (180 ± 25 ng/µg dry wt) in culture media compared to those grown in 0.0075 µg/ml thiamin (57 ± 8.2 ng/µg dry wt pyruvate and 55 ± 9.5 ng/µg dry wt lactate). Furthermore, the pyruvate concentration for nematodes grown in 0 and 0.0075 µg/ml

thiamin was 1.5-2.0 times higher for acetate media (250 ± 3.7 and 120 ± 3.7 ng/µg dry wt) compared to glucose media (170 ± 22 and 57 ± 8.2 ng/µg dry wt). Generally, at all thiamin levels, the amount of pyruvate was higher for nematodes grown in acetate media; however, the amount of lactate was higher for nematodes grown in glucose media, with the exception of the 0 µg/ml thiamin level.

Lactate to pyruvate (L:P) ratios were computed from lactate and pyruvate concentrations of culture media. Nematodes cultivated in glucose test media with 7.5 µg/ml thiamin had a L:P ratio of 10. The nematodes grown in glucose test media at thiamin levels of 0 and 0.0075 µg/ml had a significantly reduced L:P ratio of 1. There was no significant difference between L:P ratios at 0.75 and 7.5 µg/ml thiamin (9.0 ± 1.4 and 10 ± 0.80, respectively) in glucose media. In acetate media, the L:P ratio at 0.75 µg/ml thiamin (2.2 ± 1.0) was significantly less than the L:P ratio at 7.5 µg/ml thiamin (4.0 ± 0.50). Furthermore, the lactate to pyruvate ratios for nematodes grown in acetate test media were different from the ratios in C. elegans grown in glucose media. The ratios at 7.5 µg/ml thiamin for glucose and acetate were 10 and 4, respectively. Generally, as thiamin

concentration decreased, the lactate:pyruvate ratios also decreased in both glucose and acetate media.

DISCUSSION

Nicholas *et al.* (8) found that population growth decreased when thiamin was omitted from culture medium. The quantitative requirement of thiamin (0-7.5 µg/ml) for population growth of C. elegans was demonstrated in the present study. Population growth was significantly decreased in C. elegans at thiamin levels of 0 and 0.0075 µg/ml, indicating severe thiamin deficiency. Optimal population growth was achieved in the glucose and acetate test media at 0.075 µg/ml and higher levels of thiamin; there was no significant difference ($p \geq .05$) between population growth at 0.075, 0.75, and 7.5 µg/ml thiamin. These results indicate that C. elegans requires a minimal thiamin concentration in culture media of 0.075 µg/ml for optimal population growth regardless of the energy source.

Results of this study show that glucose test media supported a much larger population of nematodes at thiamin levels 0.075, 0.75, and 7.5 µg/ml than the acetate culture media; however, nematodes cultivated in acetate were much larger in average dry weight. This suggests, that based on population growth and average dry weight, a similar quantity

of nematode tissue was present in the cultures regardless of the energy source. Interestingly, the levels of tissue pyruvate are similar for cultures grown in thiamin levels of 0.075, 0.75, and 7.5 µg/ml in both test media. However, at thiamin level 0 and 0.0075 µg/ml, tissue pyruvate in acetate media (3.3 ± 0.3 and 3.9 ± 0.4 ng/µg dry weight) is approximately two times that of the pyruvate concentration in nematode tissues from glucose media (1.6 ± 0.3 and 1.8 ± 0.3 ng/µg dry weight). A functioning glyoxylate cycle in C. elegans (17-19) may provide an explanation for the higher levels of pyruvate in nematodes from acetate media compared to those from glucose media. In the absence of glucose as an energy substrate, C. elegans relies primarily on acetate for energy production via the glyoxylate and TCA cycles. The glyoxylate cycle bypasses acetyl CoA from the TCA cycle and synthesizes four-carbon compounds (malate, oxaloacetate) from two-carbon units (acetyl CoA, glyoxylate) instead of continued oxidation to carbon dioxide and water by the TCA cycle. The gyloxylate cycle begins with acetate to produce malate which ultimately is converted to pyruvate via oxaloacetate and phosphoenolpyruvate as intermediates. Therefore, in culture medium containing acetate as the sole energy source, during thiamin deficiency it is possible that

this metabolic pathway would result in additional accumulation of pyruvate in nematode tissues and culture media.

Thiamin is required by humans as well as by the nematode, C. elegans, for carbohydrate metabolism (20). In humans, metabolism of glucose requires thiamin in the decarboxylation reaction which converts pyruvate to acetyl CoA. This reaction is reported to be an important step in the energy producing glycolytic pathway in the nematode, C. elegans. Inadequate thiamin nutrition results in abnormal carbohydrate metabolism, i.e., a decrease in oxidative decarboxylation (21). One of the earliest observations of a biochemical abnormality in thiamin deficiency in humans was not only accumulation of pyruvic acid in the tissues, but also an increased concentration in the blood (22). Platt and Lu (23) studied pyruvic acid accumulation in individuals with beriberi and found mean blood pyruvate concentrations were five times that of healthy controls. Thiamin deficient rats had mean levels of blood pyruvate two times that of the controls (24).

This is the first research to study the requirement of thiamin and carbohydrate metabolites in the nematode, C. elegans. In this study, tissue pyruvate concentration for

thiamin deficient nematodes (0.0075 µg/ml) grown in glucose test media is nine times that of nematodes cultivated in 7.5 µg/ml thiamin. Thiamin deficient nematodes (0 µg/ml thiamin) grown on glucose media showed approximately a hundredfold increase of pyruvate excreted into the culture media (170 ± 22 ng/µg dry wt) compared to the level retained in the nematode tissue (1.6 ± 0.3 ng/µg dry wt) suggesting that, in thiamin deficiency, the nematodes accumulate pyruvate in the tissues and excrete excess pyruvate into the culture media.

A specific biochemical index for diagnosing thiamin deficiency in humans and other animals is the ratio of lactic to pyruvic acid in the blood after administration of glucose. Williams and coworkers (25) reported abnormally high values for pyruvate and lactate in the blood of thiamin deficient individuals. A basal lactate to pyruvate ratio of 10 to 1 was reported normal in humans by Horwitt and Kreisler (26). The lactate to pyruvate ratios found in glucose and acetate culture media of C. elegans for normal thiamin level (7.5 µg/ml) were 10 to 1 and 4 to 1, respectively. For the thiamin deficient level at 0.0075 µg/ml thiamin, the same L:P ratios (1:1) were obtained in glucose and acetate media. It is hypothesized that nematodes grown on glucose media had a larger baseline L:P ratio as a result of the larger

population size. A larger number of nematodes has increased demand for oxygen; therefore, oxygen might be limited in the culture medium, and more lactate was produced via anaerobic metabolism. Nematodes grown on acetate test media were fewer in number, and oxygen demand might be lower; therefore, a higher level of pyruvate and a lower level of lactate would occur in the culture media.

Lactate to pyruvate ratios exhibit strong positive correlation with the level of thiamin in culture media. For the glucose test media, a normal L:P ratio of 10 was altered by thiamin deficiency to a ratio of 1. This change in the ratio of L:P was attributed to the difference in excretion of excess pyruvate from thiamin deficient nematodes compared to normal pyruvate excretion from nematodes grown with adequate thiamin. In thiamin deficiency, relatively larger quantities of pyruvate rather than lactate were presumably produced, and the excess was excreted into the media.

Results of this study also suggest that L:P ratio of culture medium is a more sensitive indicator of thiamin status in C. elegans than population growth, average dry weight, or tissue pyruvate. There was no significant difference in population growth or tissue pyruvate among thiamin levels 0.075, 0.75, and 7.5 µg/ml for nematodes

cultivated in either glucose or acetate test media. However, the L:P ratio (10:1) for nematodes cultivated in glucose test media and 7.5 µg/ml thiamin was significantly greater than the L:P ratio (4.9:1) of nematodes cultivated in glucose test media and 0.075 µg/ml thiamin. For the acetate test media, the L:P ratio (4.0:1) for nematodes cultivated in 7.5 µg/ml thiamin was significantly greater than the L:P ratio (2.2:1 and 1.2:1) for nematodes grown in 0.75 and 0.075 µg/ml thiamin, respectively. This suggests that thiamin deficiency may be present for nematodes grown in acetate test media as high as the thiamin level 0.075 µg/ml although population growth and average dry weight was not affected. Based on L:P ratio, 0.075 µg/ml thiamin may represent a mild thiamin deficiency for nematodes cultivated in glucose media. However, when acetate was used as the energy source, L:P ratio was signficantly reduced at 0.75 µg/ml thiamin, indicating that 0.75 µg/ml thiamin may result in a mild thiamin deficiency.

In conclusion, results indicate that optimal population growth and normal carbohydrate metabolism with respect to thiamin was achieved in C. elegans using glucose as the energy source with a minimum thiamin level of 0.75 µg/ml. When acetate was used as the energy source, a higher

concentration of thiamin, 7.5 μg/ml, provided optimal population growth and normal carbohydrate metabolism.

Table I. Effect of Thiamin Levels and Two Energy Sources on Dry Weight, Population Growth, and Pyruvate and Lactate Production in C. elegans on 18th day[a]

Energy Source	Thiamin (µg/ml)	Dry Wt/ Nema[b] (ng)	Population Growth[c] (nema/ml x 10^3)	Tissue Pyruvate[d] (ng/µg dry wt)	Culture Media Pyruvate (P) (ng/µg dry wt)	Culture Media Lactate (L) (ng/µg dry wt)	L : P Ratio[e]
	7.5	39 (1)	60 ± 8.3 (4)§	0.20 ± 0.10 (3)*	5.0 ± 0.70 (3)	51 ± 6.9 (3)	10 ± 0.80 (3)Ç
Glucose	0.75	38 (1)	60 ± 8.5 (4)§	0.30 ± 0.10 (3)*	4.9 ± 0.70 (3)	43 ± 5.9 (3)	9.0 ± 1.4 (3)Ç
(0.026	0.075	41 (1)	54 ± 7.6 (4)§	0.40 ± 0.10 (3)*	7.7 ± 2.1 (3)	38 ± 8.0 (3)	4.9 ± 0.30 (3)
mole/Liter)	0.0075	24 (1)	28 ± 3.3 (4)	1.8 ± 0.30 (3)*	57 ± 8.2 (3)	55 ± 9.5 (3)	1.0 ± 0.10 (3)∞
	0	12 (1)	10 ± 1.7 (4)	1.6 ± 0.30 (3)*	170 ± 22 (3)	180 ± 25 (3)	1.1 ± 0.20 (3)∞
	7.5	72 (1)	32 ± 4.5 (4)¶	0.20 ± 0.10 (3)=	7.6 ± 2.0 (3)	30 ± 8.1 (3)	4.0 ± 0.50 (3)
Acetate	0.75	82 (1)	26 ± 3.9 (4)¶¥	0.20 ± 0.10 (3)=	11 ± 2.8 (3)	22 ± 4.9 (3)	2.2 ± 1.0 (3)£
(0.051	0.075	77 (1)	26 ± 6.6 (4)¶¥	0.40 ± 0.10 (3)=	20 ± 1.4 (3)	25 ± 2.5 (3)	1.2 ± 0.20 (3)£-
mole/Liter)	0.0075	21 (1)	16 ± 1.5 (4)¥†	3.9 ± 0.40 (2)∫	120 ± 3.7 (3)	120 ± 10 (3)	1.0 ± 0.20 (3)£-
	0	14 (1)	12 ± 1.5 (4)†	3.3 ± 0.30 (3)∫	250 ± 3.7 (3)	70 ± 25 (3)	0.30 ± 0.10(3)-

[a] Values represent the mean ± SD in last 5 columns. Number of observations is indicated in parentheses.

[b] Values represent ng dry wt of an average nematode.

[c,d,e] Values in column are significantly different ($p<.05$) by one-way ANOVA. Means with same superscript symbol (§,¶,¥,†,*,*,=,∫,Ç,∞,£,-) are not significantly different ($p\geq.05$) by Scheffe post hoc analysis.

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CHAPTER 3

SUMMARY AND RECOMMENDATIONS

Summary

Thiamin requirement and carbohydrate metabolism were investigated in, C. elegans, cultivated axenically in chemically defined media. Test media were supplemented with two different energy sources, glucose and acetate (at 1:2 molar concentration ratio), and five levels of thiamin (0, 0.0075, 0.075, 0.75, and 7.5 µg/ml). Optimal population growth occurred at 0.075-7.5 µg/ml thiamin. Population growth was significantly reduced ($p < .05$) at thiamin levels 0 and 0.0075 µg/ml, indicating severe thiamin deficiency. Enzymatic determination of pyruvate and lactate in tissue extracts and culture media was conducted. Tissue pyruvate concentrations for nematodes cultivated in 0 and 0.0075 µg/ml thiamin were 1.6 ± 0.3 and 1.8 ± 0.3 ng/µg dry weight for glucose and 3.3 ± 0.3 and 3.9 ± 0.4 ng/µg dry weight for acetate. These concentrations were significantly higher ($p < .05$) from pyruvate concentrations (0.2-0.4 ± 0.1 ng/µg dry wt) at thiamin levels 0.075-7.5 µg/ml, indicating pyruvate accumulation in nematode tissue. Lactate to pyruvate (L:P) ratios were computed from lactate and pyruvate

concentrations in the media at each thiamin level. In glucose media, the L:P ratios at 7.5 and 0.075 µg/ml thiamin were significantly different (10 and 4.9, respectively). In acetate media, L:P ratios were significantly reduced at a higher thiamin level (0.75 µg/ml). This suggests that mild thiamin deficiency may be present for nematodes in glucose media at the thiamin level 0.075 µg/ml, while in acetate media at 0.75 µg/ml thiamin. Because there was no significant difference in population growth or tissue pyruvate concentrations between thiamin levels 0.075-7.5 µg/ml for nematodes cultivated in either glucose or acetate media, results indicate that L:P ratio may be a more sensitive and useful indicator of thiamin status in C. elegans.

Recommendations

To improve upon the experimental design of this study, the following recommendations are made.

1. Eight culture tubes instead of four tubes should be cultivated per thiamin levels 0.075-7.5 µg/ml, four tubes for dry weight and four tubes for lactate and pyruvate assay.

2. Twice the amount of deficient cultures (24 tubes instead of 12 tubes) should be cultivated at thiamin levels

0.0075 and 0 µg/ml to increase the tissue available for dry weight (6 tubes) and for the lactate assay (18 tubes).

3. Lactate assay of tissue extract should be conducted on the same day of extraction because of the instability of this compound during frozen storage.

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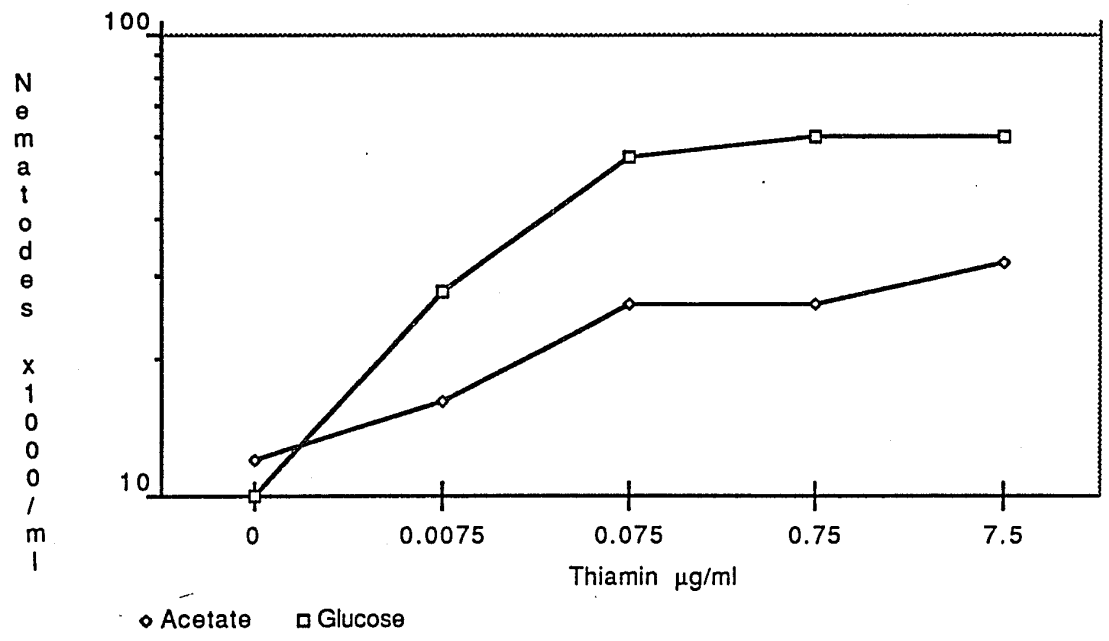
APPENDIX I

EFFECT OF THIAMIN AND ENERGY SOURCE ON POPULATION GROWTH OF C. ELEGANS ON 18TH DAY



APPENDIX II

COMPONENTS OF CAENORHABDITIS ELEGANS MAINTENANCE MEDIUM

		MW	gm*
I.	Vitamins & Growth Factors		
	N-Acetylglucosamine	222.2	0.01500
	Cyanocobalamine	1355.4	0.00375
	Niacinamide	122.1	0.00750
	Pantethine	554.7	0.00375
	Pantothenate(Ca)	238.3	0.00750
	Pyridoxamine•2HCl	241.1	0.00375
	Pyridoxine•HCl	205.6	0.00750
	Pyridoxal•PO_4	247.1	0.00375
	Riboflavin-5'-PO_4(Na)•$2H_2O$	514.4	0.00750
	Thiamin•HCl	337.3	0.00750
	Biotin	244.3	0.00375
	Niacin	123.1	0.00750
	Pterolylglutamic Acid	441.4	0.00750
	DL-Thioctic Acid	206.3	0.00375
	p-Aminobenzoic Acid	137.1	0.00750
II.	Salts		
	$CaCl_2•2H_2O$	147.0	0.2205
	$CuCl_2•H_2O$	170.5	0.0065
	$MnCl_2•4H_2O$	197.9	0.0222
	$ZnCl_2$	136.3	0.0102
	KH_2PO_4	136.1	1.2255
	K_3 Citrate•H_2O	324.4	0.4860
	$Fe(NH_4)_2(SO_4)_2•6H_2O$	392.2	0.0588
	$Mg(OH)_2$	58.3	0.1740
	Citric Acid•H_2O	210.1	0.6303
III.	Amino Acids		
	A. Essential Amino Acids		
	L-Arginine	174.2	0.9750
	L-Histidine	155.2	0.2830
	L-Lysine•HCl	182.6	1.2830
	L-Tryptophan	204.2	0.1840
	L-Methionine	149.2	0.3890
	L-Threonine	119.1	0.7170
	L-Leucine	131.2	1.4390
	L-Isoleucine	131.2	0.8610
	L-Valine	117.1	1.0200
	L-Phenylalanine	165.2	0.6230

APPENDIX II (continued)

COMPONENTS OF CAENORHABDITIS ELEGANS MAINTENANCE MEDIUM

	B. Non-essential Amino Acids	MW	gm*
	L-Phenylalanine	165.2	0.1800
	L-Tyrosine	181.2	0.2720
	L-Alanine	89.1	1.3950
	L-Aspartic Acid	133.1	1.6200
	L-Cysteine•HCl•H_2O	175.6	0.0280
	L-Glutamate(Na)•H_2O	187.1	0.5500
	L-Glutamine	146.2	1.4630
	Glycine	75.1	0.7220
	L-Proline	115.1	0.6530
	L-Serine	105.1	0.7880
IV.	Nucleic Acid Substituents		
	Adenosine-3'-(2')-Phosphoric Acid•H_2O	365.2	0.3652
	Cytidine-3'-(2')-Phosphoric Acid	323.2	0.3232
	Guanosine-3'-(2')-PO_4(Na)2•H_2O	425.2	0.3632
	Uridine-3'-(2')-Phosphoric Acid	324.2	0.3242
	Thymine	126.1	0.1261
V.	Other Growth Factors		
	Glutathione, reduced	307.3	0.2040
	Choline H_2 Citrate	295.3	0.0885
	myo-Inositol	180.2	0.0645
	Cytochrome c	12384.0	0.0500
	ß-Sitosterol	414.7	0.0500
VI.	Energy Source		
	D-glucose	180.2	32.5000
	Or K Acetate	98.1	5.0000
VII.	Solvents		
	KOH	56.1	**
	Triethanolamine (TEA)	149.2	0.0325
	Tween 80	1308.0	1.2500

*gm/500 ml (2X)

** needed for adjustment of pH to 5.9 ± 0.1