THE EFFECTS OF JUVENILE HORMONE ON MITROCHONDRIAL METABOLISM IN THE INDIAN MEAL MOTH, *Plodia interpunctella* (HÜBNER)

By

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By

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Investigation into the effects of synthetic *H. cecropia* juvenile hormone (methyl-10, 11-epoxy-7-ethyl-3, 11-dimethyl-*trans* 2, *trans* 6-tridecadienoate) on mitochondrial metabolism in larvae of the Indian Meal moth, *Plodia interpunctella* (Hübner) revealed that juvenile hormone affected citrate cycle oxidations, electron transport, heme synthesis and cytochrome synthesis.

Juvenile hormone inhibited all NAD-linked oxidations in the citrate cycle of isolated mitochondria. However, the flavoprotein-linked oxidation of α-glycerophosphate was not affected by juvenile hormone while succinate oxidation was stimulated.

NADH oxidation *in vitro* by aged and uncoupled mitochondria was also inhibited by juvenile hormone. Experiments

using ferricyanide to artificially accept electrons from NADH dehydrogenase indicated that juvenile hormone prevented electron transport at the nonheme iron level of complex I in the electron transport chain. Further experiments revealed that the inhibition was noncompetitive. These results indicated that the inhibition of NAD-linked oxidations in the citrate cycle was due to the effects of juvenile hormone in the electron transport chain.

Inhibition of NAD-linked substrate oxidations in the citrate cycle could result in an increase in cytochrome synthesis by a mechanism involving synthesis of malate from pyruvate and the reversal of reactions in the citrate cycle. Inclusion of juvenile hormone in the diet of the larvae resulted in an increase in the concentration of mitochondrial cytochrome. However, this stimulation was dependent upon some factor associated with the larval-larval molt. The concentration of juvenile hormone at the beginning of an instar affects the mitochondrial cytochrome concentration within that instar.

The results of the cytochrome analyses indicated that the hypothetical mechanism of control of cytochrome synthesis had a second level of control associated with the molt. Since cytochromes are composed of heme and apoprotein, the effects of juvenile hormone on the synthesis of just the heme portion of the molecule was investigated. Inclusion of juvenile hormone in the diet stimulated de novo

heme synthesis, but this effect was immediate and therefore presumably direct.

The data suggest a metabolic mechanism for juvenile hormone controlling insect growth and development by determining the maximum capacity of cellular energy production. Energy production levels may be limited by the concentration of electron transport chain components.

INTRODUCTION

Insect growth and metamorphosis are characterized by a series of sequential changes. These stepwise changes are necessitated by limits imposed by the insect's rigid exoskeleton. A number of reviews are available which discuss growth and give descriptions of development (Agrell, 1964; Whitten, 1968), the physiology of growth and metamorphosis (Wigglesworth, 1954, 1957, 1959a, 1965) and the histological and biochemical aspect of insect growth (Williams, 1951; Wigglesworth, 1959b; Wyatt, 1968).

The growth and development of insects are under hormonal control. During the growth and development of insects, characteristic changes occur in the quantity (Silhacek, 1967), morphology (Brosomer et al., 1963, Willis, 1966; Watanabe and Williams, 1953), phospholipid composition (Silhacek, 1967) and oxidative activities (Silhacek, 1967; Brosomer et al., 1963; Michejda, 1964) of mitochondria. Some investigators have linked these changes in mitochondrial metabolism with endocrine control. In an earlier study, DeWilde and Stegwee (1958) demonstrated that corpora allata, endocrine glands which produce juvenile hormone (JH), exerted a direct effect on the respiration of

Leptinotarsa decemlineata. A subsequent study (Stegwee, 1960) revealed that a JH-active extract from Hyalophora cecropia stimulated succinate oxidation in isolated mitochondria from L. decemlineata. Clarke and Baldwin (1960) also concluded that JH preparations affected the oxidative activity of isolated mitochondria from Locusta migratoria and Schistocerca gregaria. In contrast, the oxidative activities of mitochondria isolated from allatectomized L. migratoria (Minks, 1967) and Blaberus discoidalis (Keeley, 1971) were found to be similar to those of normal insects. The recent identification and synthesis of juvenile hormone permitted the direct testing of the effects of JH on isolated insect mitochondria in a defined medium.

It is evident that mitochondrial activities could play a central role in the processes responsible for growth and development in insects. However, our current knowledge is very limited and much data is contradictory. More information on the role of mitochondrial activities and juvenile hormone action and the relationship of these to other aspects of metabolism is required. The purpose of the present study is to elucidate the effects of juvenile hormone on mitochondria in vitro and in vivo, to determine the biochemical mechanism of action of JH in mitochondria and to determine the significance of the primary action of the hormone to other aspects of insect metabolism.

LITERATURE REVIEW

Hormonal Control of Growth and Development

The growth and development of insects is under hormonal control. The three hormones that appear to be important in this developmental regulation are prothoracicotropic hormone (brain hormone), ecdysone (molting hormone) and juvenile hormone (Neotenin). Prothoracicotropic hormone is produced by the median neurosecretory cells in the pars intercerebralis of the insect brain. It was shown that the prothoracicotropic hormone has no direct effect on molting but stimulates the prothoracic (ecdysial) glands to secrete ecdysone (Fukuda, 1944). Ecdysone, which initiates the molting process, is secreted shortly before each molt and disappears shortly afterward (Burdette, 1961; Karlson and Shaaya, 1964; Shaaya and Karlson, 1965). The crystalline hormone first prepared by Butenandt and Karlson (1954) was used in experiments on Chironomus tentans by Clever and Karlson (1960) and Clever (1961, 1964, 1965). These experiments indicated that injections of ecdysone induce a pattern of puffing at chromosome loci identical to that which occurs in normal metamorphosing last instar

larvae. The puffing of the chromosomes is thought to be a mechanism of exposing the DNA which is normally covered by protein and making it available to participate in RNA synthesis. Beermann and Clever (1964) found that the puffs induced by ecdysone were associated with rapid RNA synthesis. This resulted in the hypothesis (Karlson et al., 1964) that ecdysone acts directly on the genome and activates specific gene loci which results in the production of specific messenger RNAs and, consequently, specific proteins necessary for the physiological events of metamorphosis.

Kroeger (1963a) proposed a second hypothesis that ecdysone acts directly on the nuclear membrane causing an altered ionic balance of sodium and potassium which causes the activity described in the genome. Ito and Lowenstein (1965) found that nuclear membrane permeability to ions does change during development and that the changes in membrane permeability could be induced by ecdysone. Lezzi and Gilbert (1970) found that the chromosome puffs could be induced by altering sodium and potassium concentrations alone without ecdysone. However, work by Congote et al. (1969) indicates that ecdysone can stimulate RNA synthesis in a preparation of isolated nuclei in the presence of ecdysone without sodium or potassium ions. Congote et al. (1970) also show that in isolated nuclei the hormone-induced RNA differs from RNA isolated from control insects.

The third hormone, juvenile hormone (JH), is produced by the corpora allata. During development juvenile hormone titer is minimal at each molt but increases rapidly during the early part of the instar and decreases toward the end of the instar (Gilbert and Schneiderman, 1961b; Williams, 1963; Stephen and Gilbert, 1970). Juvenile hormone titer at the beginning of an instar is thought to affect the phenotype which will occur after the next molt (Wigglesworth, 1940; Clever, 1963; Kroeger, 1968), and may also affect the sequence of morphological changes in insect development (Gilbert, 1964). Juvenile hormone is thought to affect genetic regulation in such a way as to cause a larval-larval molt at high concentration, a larval-pupal molt at lower concentrations and a pupal-adult molt when it is absent (Bounhiol, 1938; Piepho, 1942, 1946, 1951; Fukuda, 1944; Nayar, 1954; Gilbert and Schneiderman, 1961b; Williams, 1961; Gilbert, 1963; Williams and Kafatos, 1971). However, the specific response to JH is modified by the ability of the specific tissues to react with JH (Piepho, 1942; Wigglesworth, 1948; Gilbert and Schneiderman, 1960).

Secretion of JH by the corpora allata may be under the control of nervous stimulation from the brain (Luscher and Englemann, 1960). Juvenile hormone titer decreases as larval age increases with the previously described cyclic variation occurring during each instar (Fukuda,

1944; Piepho, 1950b, 1951, 1952; Rehm, 1951; Williams, 1961; Johnson and Hill, 1973). The decrease in JH titer has been attributed to several different causes. One hypothesis states that because larval volume increases more rapidly than corpora allata volume there is a dilution of the hormone (Kaiser, 1949; Beljaeva, 1960; Novak and Cervenkova, 1960; Novak and Slama, 1962). A second hypothesis is that inactivation or degradation of JH becomes more intense in older larvae (Gilbert and Schneiderman, 1960). In support of the latter hypothesis, Weirich et al. (1973) has found the activity of esterase (an enzyme which inactivates JH) is higher in the fifth-larval instar in Manduca sexta than in the fourth-larval instar.

Williams and Kafatos (1971) have recently proposed a model which predicts three separate sets of genes, one set coding for larval characteristics, another set coding for pupal characteristics and a third set coding for adult characteristics. Only one of the three gene sets could be active at any one time and the titer of juvenile hormone would determine which one was active.

Insect growth and development is characterized by stepwise changes that are necessitated by limits imposed by the insect's rigid exoskeleton. In order to increase in size the insect must molt, shedding its exoskeleton and reforming another of a larger size. Insects may develop toward the adult form gradually or by distinct stages.

Insects that undergo gradual development (Hemimetabola) have immature stages (larvae) that are morphologically similar to the adult. Some structures show a gradual progression from the immature to the imaginal form as the molts progress. Immature insects that undergo complete metamorphosis (Holometabola) undergo extensive morphological alterations in transforming from the larvae to the adult stages. In holometabolous insects the larval molts permit increases in body size. An intermediate pupal stage is then required for the transition of larva to adult. During the pupal stage many larval tissues disappear, others are modified and new tissues are formed. The degree of tissue reorganization is dependent on the species of insect. Many of the imaginal tissues are derived from embryonic tissue (imaginal discs) which does not develop until the pupal stage.

Juvenile Hormone

Juvenile hormone was first demonstrated by Wigglesworth (1935, 1940) who showed by the use of parabiosis experiments that a substance carried in the hemolymph of Rhodnius prolixus was responsible for the retention of immature characteristics. An extract with strong juvenile hormone activity was prepared from abdomens of adult male Hyalophora cecropia moths by Williams (1956) and a purified

preparation of juvenile hormone was obtained by Williams and Law (1965). Several other procedures have been developed for the isolation and purification of juvenile hormone (Meyer and Ax, 1965; Röller et al., 1965, 1969).

The amount of JH per abdomen in the adult male H. cecropia moth has been determined to be between 6.0 µg (Metzler et al., 1971) and 0.5 µg (Meyer et al., 1965). Röller et al. (1967) identified the structure of juvenile hormone as methyl-10, 11-epoxy-7-ethyl-3, 11-dimethyl-trans 2, trans 6-tridecadienoate. This structural isomer of JH was also found to have the greatest effect of the eight possible isomers of juvenile hormone on several species (Röller and Dahm, 1968; Rose et al., 1968; Westermann et al., 1969; Wigglesworth, 1969; Pfiffner, 1971; Schweiter-Peyer, 1973). Several synthetic schemes have been developed for the production of juvenile hormone (Röller and Dahm, 1968b; Berkoff, 1969; Findlay and MacKay, 1969).

Two other naturally occuring juvenile hormones have been found. A second JH, methyl-10, 11-epoxy-3, 7, 11-trimethyl-2, 6-dodecadienoate has been found in H. cecropia by Meyer et al. (1968). Judy et al. (1973) found this JH along with a third juvenile hormone, methyl-10, 11-epoxy-3, 7, 11-trimethyl-2, 6-tridecadienoate, in Manduca sexta organ cultures. Schooley et al. (1973), using corpora allata cultured from M. sexta, found that

the biosynthesis of these juvenile hormones proceeds by a terpenoid pathway with biosynthesis of the carbon skeleton initiated through homomevalonate arising from propionate and acetate.

Prior to the extraction of juvenile hormone from H. cecropia, many investigators sought to correlate the molecular structures of a variety of substances with juvenilizing activity. These studies quantitate JH activity by correlating dosage to retention of juvenile morphological characteristics. Wigglesworth (1969b) discussed the correlation of 42 different compounds in relation to natural H. cecropia juvenile hormone. Other juvenilizing agents which have been studied include isoprenoid and straight chain alcohols (Bowers and Thomson, 1963; Schneiderman et al., 1965), other terpenoids (Schwarz et al., 1970), aromatic terpenoid ethers (Bowers, 1969; Kiouchi et al., 1974), terpenoid amines (Cruickshank and Palmere, 1971), acetals applied as a vapor (McGovern et al., 1971) and long chain fatty acids including oleic and linoleic acids (Slama, 1961, 1962). Although juvenilizing agents are generally nonspecific, one juvenile hormone analog, p-(1, 5-dimethyl-hexyl) benzoic acid was selective on Dysdercus sp. (Suchy et al., 1968). Farnesol and its derivatives have been the subject of many studies of JH activity (Karlson and Schmialek, 1959; Schmialek, 1961; Williams, 1961; Wigglesworth, 1961, 1962, 1963, 1969a, 1969b; Yamamoto and Jacobson, 1962;

Karlson, 1963; Schneiderman and Gilbert, 1964; Braun et al., 1968; Schwarz et al., 1969; Sonnet et al., 1969).

A study conducted by Slade and Wilkinson (1973) in which the enzymatic degradation of JH was quantitatively determined in the presence or absence of JH analogs indicated that the analogs prevented the inactivation of the natural hormone. This study casts some doubt as to whether the JH analogs have any juvenilizing effect or whether they only have a synergistic effect with the natural hormone.

Many of the studies to determine the physiological effects of juvenile hormone prior to the availability of isolated or synthetic JH were done by removal or implantation of the corpora allata, the glands which produce the hormone. It was shown that implantation of corpora allata would cause supernumerary molts and extend the immature life span of insects (Pflugfelder, 1937; Bodenstein, 1943; Srivastava and Gilbert, 1969). Other physiological effects attributed to JH include enhancing the regenerative abilities in Carausius morosus (Pflugfelder, 1939), induction of green coloration in larvae of Locusta under environmental conditions which would normally produce the brown, gregarious phase (phase polymorphism)(Staal, 1959, 1961) and influencing determination of caste polymorphism in social insects (Kaiser, 1955; Luscher, 1961; Rembold, 1974). Juvenile hormone is required for the maturation of the female reproductive organs and oöcytes in adult insects

(Ichikawa and Nishiitsutsuji-Iwo, 1959; Williams, 1959; Gilbert, 1964; Highnam, 1964; Wigglesworth, 1964; Postlethwait and Weiser, 1973; Sroka and Gilbert, 1972; Kambysellis et al., 1974). Although JH is required for egg maturation, application of exogenous JH to insect eggs can interrupt development (Riddiford and Williams, 1967; Riddiford, 1970). Implantation of corpora allata was also found to influence insect behavior in that it affects larval-larval premolt cocoon spinning in Galleria mellonella (Piepho, 1950) and the hormone induced mating behavior in giant supernumerary larvae or adultoids of Pyrrhocoris apterus (Zdarek and Slama, 1968). Juvenile hormone also stimulates sex pheromone production in Ips confusus (Borden et al., 1969) and Tenebrio molitor (Menon, 1970).

Adult diapause in Leptinotarsa decemlineata appears to be due to a deficiency in JH (DeWilde and Stegwee, 1958; DeWilde, 1959). However, larval diapause in Diatraea grandiosella appears to be induced by JH (Chippendale and Yin, 1973; Yin and Chippendale, 1973, 1974). The physiological functions of juvenile hormone have been reviewed by Scharrer (1953) Gilbert and Schneiderman (1961), Gilmour (1961), Wigglesworth (1962, 1964), Gilbert (1964), Novak (1966), and Slama et al. (1974).

Metabolic Effects of Juvenile Hormone

Although DNA synthesis in immature tissues is probably not stimulated by juvenile hormone (Novak, 1971), in the adult insects of some species DNA synthesis must be simulated by JH in order for the reproductive organs to develop (Hodkova, 1974). In larval insects juvenile hormone may play a role in determining which molecular species of RNA are synthesized (Williams and Kafatos, 1971), but may not have any effect on the overall quantity of RNA synthesized. Oberlander and Schneiderman (1966) found no stimulation of RNA synthesis in isolated pupal abdomens which lacked prothoracic glands during JH treatment. This was interpreted to mean that juvenile hormone does not have a direct metabolic regulatory role except on the prothoracic glands. More recent studies have shown that JH did stimulate RNA synthesis in silkworm wing disks (Patel and Madhavan, 1969) and in isolated fat body cell nuclei (Congote et al., 1969). However, in these systems simultaneous application of JH and ecdysone did not result in stimulation of RNA synthesis.

Because new proteins appear at different times during insect development, the production of different species of RNA must be under the control of JH. In some cases the effect may be stimulatory while in others inhibitory (Williams and Kafatos, 1971). Experiments by Ilan et al.

(1970) suggest that the synthesis of new species of transfer RNA and their activating enzymes (amino acyl-tRNA synthetases) which also require RNA synthesis are under the control of juvenile hormone. An increase in purine synthesis as an effect of JH is reliant on RNA synthesis and this increased purine synthesis results in a reduced uric acid excretion (L'Helias, 1956).

Following allatectomy (removal of the corpora allata) L'Helias (1956) found increases in uric acid excretion and free amino acids in the tissues with a decrease in tissue protein in larvae of Dixippus morosus. Allatectomy also caused an increase in hemolymph levels of amino acid (Minks, 1967). Drastic decreases in protein synthesis were found in all tissues, following allatectomy by Vandenberg (1963). Patel and Madhavan (1969) found that injection of JH induced protein synthesis.

The opposite effect was found for hemolymph protein concentrations in adult females. Allatectomy in adult females caused an increase in hemolymph protein (Hill, 1962, 1963; Highnam and Hill, 1963; Slama, 1964; Minks, 1963). This effect may be due to the continued synthesis of vitellogenic proteins without incorporation into the oöcyte whose development is controlled by juvenile hormone.

Specific proteins under juvenile hormone control include vitellogenic proteins (Englemann, 1969), which may be the same proteins found by Minks (1967) in L. migratoria,

tRNA acylases and cuticular proteins (Ilan et al., 1970) and esterases (Whitmore et al., 1972; Weirich et al., 1973). The induction of esterases is apparently a mechanism to deactivate the JH which can be degraded by hydrolysis of the methyl ester, hydrolysis of the epoxide group or conjugation with polar groups (Ajami and Riddiford, 1971; Siddall, et al., 1971; Slade and Zibitt, 1971a, 1971b; White, 1972). Another protein which binds and transports juvenile hormone (Trautmann, 1972; Whitmore and Gilbert, 1972; Emmerich and Hartmann, 1973) apparently serves to protect the hormone from degradation (Ferkovich, personal communication).

Both lipid and carbohydrate content undergo changes after the removal or implantation of corpora allata. In larvae of Dixxipus allatectomy caused an increase in glycogen concentration (L'Helias, 1955, 1964) and Sehnal and Slama (1966) and Sehnal (1971) found that implantation of corpora allata into last instar G. mellonella prevented accumulation of lipids but glycogen content increased. Studies relating the rate of lipid synthesis to JH during development (Stephen and Gilbert, 1969, 1970) indicate that high juvenile hormone titer may serve to inhibit lipid synthesis.

The metabolic effects of JH on adult insects is apparently due to stimulation of the reproductive system. These effects include stimulation of DNA synthesis in

developing ovarian tissue (Hodkova, 1974), increasing lipid and glycogen synthesis (Minks, 1967; Liu, 1973) and stimulation of incorporation of yolk precursors into oöcytes (Janda and Slama, 1965; Minks, 1967; Lanzrein, 1974).

A recent review by Slama et al. (1974) comprehensively discussed the morphological, physiological, and biochemical effects of the neuroendocrine system and the chemistry of juvenile hormone and other insect growth regulators in insects. Other recent surveys of the effects and chemistry of JH have been made by Novak (1966), Wyatt (1968), Slama (1971) and Pfiffner (1971).

Mitochondrial Oxidations

Mitochondria are subcellular organelles composed of two membranes, an outer relatively smooth membrane and an inner membrane which has many invaginations, called cristae (Lehninger, 1965). Mitochondria have some degree of autonomy from the control of the cell nucleus in that they can synthesize some of their own proteins under the direction of mitochondrial DNA (Beatie, 1971) and the biogenesis of mitochondria doesn't appear to be controlled by the nucleus (Ashwell and Work, 1970).

Insect flight muscle mitochondria have been studied because of an enormous increase in oxygen consumption above

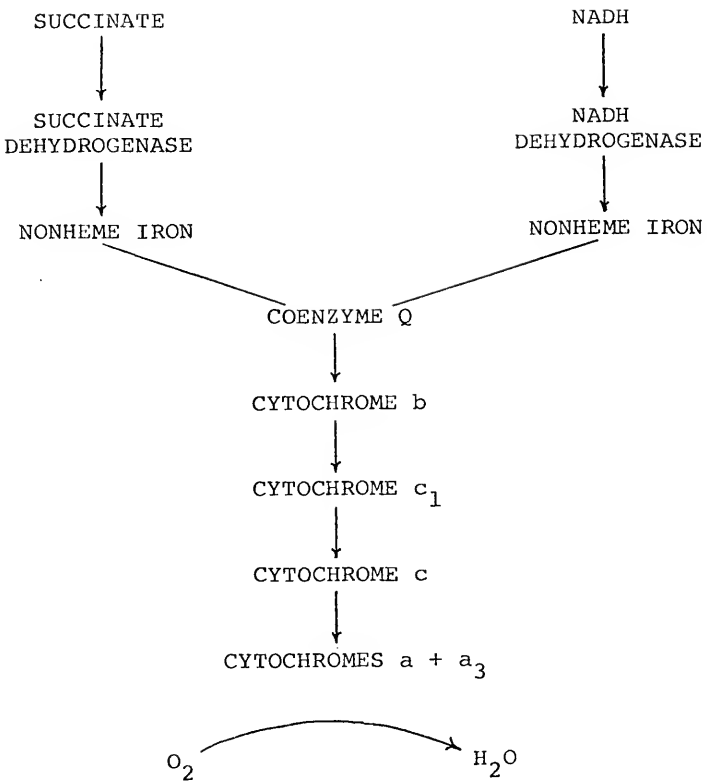
the basal rate in actively flying insects (Davis and Fraenkel, 1940). In the flight muscles of Phormia regina there are approximately 1.1×10^8 mitochondria per mg tissue which comprise 40% of the total muscle mass (Levenbook and Williams, 1956). Lehninger (1970) has calculated that active flight muscle mitochondria may have 10-times the inner membrane surface area of less active mammalian mitochondria.

There is a space between the inner and outer membranes and another space internal to and bounded by the inner membrane (matrix) resulting in four enzymatically distinct compartments; the outer membrane, the intermembrane space, the inner membrane and the matrix. Enzymes associated with the outer membrane include monoamine oxidase and rotenone-insensitive NADH:cytochrome c reductase (Reed and Sacktor, 1971). Adenylate kinase is located in the intermembrane space (Reed and Sacktor, 1971). The inner membrane is thought to contain the enzymes for coupled ATP synthesis (Hansford and Sacktor, 1971), flavoprotein-linked α-glycerophosphate dehydrogenase (Zebe and McShan, 1957; Reed and Sacktor, 1971), succinic dehydrogenase (Greville et al., 1965), proline dehydrogenase (Brosomer and Veerabhadrappa, 1965; Sacktor and Childress, 1967), trehalase (Reed and Sacktor, 1971), α-keto acid dehydrogenases and the enzymes of the electron transport chain (Hansford and Sacktor, 1971).

Enzymes found as soluble matrix proteins include citrate synthetase (Beenakkers et al., 1967), NAD and NAD)-linked isocitrate dehydrogenases (Goebell and Klingenberg, 1963, 1972), malate dehydrogenase (Delbruck et al., 1959; Reed and Sacktor, 1971), alanine and aspartate aminotransferases (Brosomer et al., 1963), 3-hydroxylacyl-CoA dehydrogenase (Beenakkers et al., 1967) and palmitoyl and carnitine acetyltransferase (Beenakkers and Henderson, 1967; Beenakkers et al., 1967; Childress et al., 1967).

Oxidative phosphorylation is defined as the addition of a terminal phosphate group to ADP to form ATP as a result of reactions coupled to the electron transport chain (Figure 1). The significance is that electrons derived from the oxidation of organic acids are passed to the electron transport chain which results finally in the reduction of oxygen to form water and the production of ATP, a form of chemically utilizable energy. The existence of oxidative phosphorylation in insects was first confirmed by Sacktor (1954) and Lewis and Slater (1954). Oxidative phosphorylation in insect mitochondria has been found to be coupled to the oxidation of pyruvate, citrate cycle intermediates (Sacktor, 1954; Gregg et al., 1960; Birt, 1961), α-glycerophosphate (Sacktor and Cochran, 1958; Van Der Bergh and Slater, 1960), amino acids (Rees, 1954; Sacktor and Childress, 1967) and fatty acids (Meyer et al., 1960; Beenakkers, 1963, 1965; Beenakkers and

Figure 1. The sequence of reactions in the electron transport chain (Hansford and Sacktor, 1971).


SUCCINATE
SUCCINATE DEHYDROGENASE
NONHEME IRON
NADH
NADH DEHYDROGENASE
NONHEME IRON
COENZYME Q
CYTOCHROME b
CYTOCHROME c_1
CYTOCHROME c
CYTOCHROMES a + a_3
O_2
H_2O

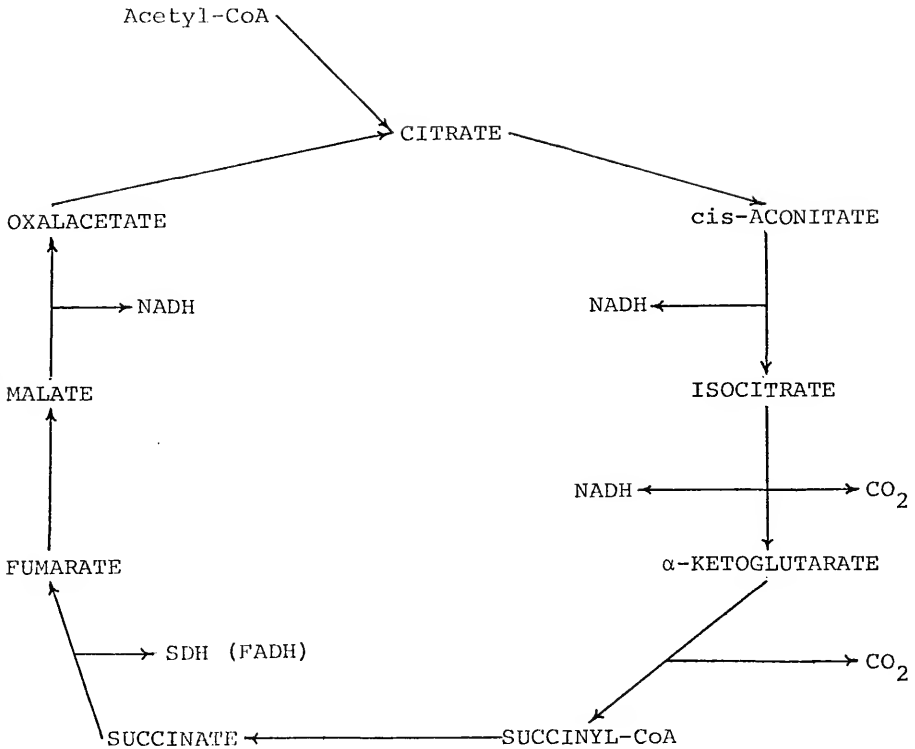
Klingenberg, 1964; Beenakkers and Henderson, 1967).

The conditions necessary for oxidative phosphorylation have been discussed by Sacktor (1953). Free fatty acids may uncouple oxidative phosphorylation (Wojtczak and Wojtczak, 1960; Wojtczak, et al., 1968) but serum albumin may afford some functional and structural protection.

The electron transport chain accepts electrons from NADH and various flavoprotein dehydrogenases. NADH is formed in the oxidation of pyruvate to acetyl-CoA, the oxidation of L-β-hydroxylacyl-CoA to L-β-ketoacyl-CoA in the β-oxidation of fatty acids and in several oxidative reactions of the citrate cycle (Kreb's cycle, tricarboxylic acid cycle)(Figure 2). The introduction of electrons into the electron transport chain through reduced flavoprotein dehydrogenases is the result of the oxidations of succinate, α-glycerophosphate and fatty acyl-CoA. Electrons from NADH enter electron transport at NADH dehydrogenase and those from flavoprotein dehydrogenases enter at coenzyme Q. The rate of substrate oxidation depends upon the permeability of the mitochondrial membrane to the substrate (Childress and Sacktor, 1966; Tulp et al., 1971) which appears to be facilitated by substrate ion translocators (Tulp and Van Dam, 1969; Tulp et al., 1971).

Mitochondrial enzyme activities as well as mitochondrial mass changed as development proceeded from one week prior to the imaginal molt to one week after in

Figure 2. The sequence of reactions in the citrate cycle showing the points of origin of NADH and succinate dehydrogenase (SDH)(Lehninger, 1965).


Acetyl-CoA
CITRATE
OXALACETATE
cis-ACONITATE
NADH
NADH
MALATE
ISOCITRATE
NADH
CO_2
FUMARATE
α-KETOGLUTARATE
SDH (FADH)
CO_2
SUCCINATE
SUCCINYL-CoA

L. migratoria (Brosomer et al., 1963). Michejda (1964) found similar results in developing flight muscles of H. cecropia. In adult flight muscle mitochondrial size increases as a function of age directly after adult emergence in Drosophila funebris and Phormia regina (Watanabe and Williams, 1953). Silhacek (1966) found changes in oxygen consumption correlated to age in the last two larvel instars of G. mellonella. Similar effects have been found in isolated mitochondria from Plodia interpunctella (Silhacek et al., 1974).

Leenders and Berendes (1972) and Leenders and Beckers (1972) found that inhibition of respiratory enzymes which increased the metabolic demand for the enzymes can activate certain gene loci in polytene chromosomes. The same effect is seen in dinitrophenol-treated larvae or larvae recovering from anaerobiosis (Berendes et al., 1965; Van Brenzel, 1966; Ashburner, 1970).

Keilin (1925) studied 40 species of insects and found light absorption bands in flight muscle. Three of the bands at approximately 605, 563, and 550 nm were designated to correspond to three hemochromagens, cytochromes a, b and c, respectively. Williams (1951) found high concentrations of the cytochromes in flight muscle and Sacktor (1953) found that within the muscle the cytochromes were restricted to the mitochondria. Individual cytochromes ranged from 0.5 to 1.5 μmoles/g protein in flight muscle

of several insects (Barron and Tahmisian, 1948; Levenbook and Williams, 1956; Chance and Sacktor, 1958; Bucher and Klingenberg, 1958; Klingenberg and Bucher, 1959; Stegwee and van Kammen-Wertheim, 1962; Slack and Bursell, 1972). The ratios of cytochromes are approximately 1:1.5:0.5 (a:c:b) (Chance and Sacktor, 1958; Bucher and Klingenberg, 1958; Stegwee and van Kammen-Wertheim, 1962).

Very little work has been done on the biosynthesis of cytochromes in insects. Soslau et al. (1971) found that exogenous δ-aminolevulinic acid would stimulate the synthesis of heme, a precursor of cytochrome. Hamdy et al. (1974) found incorporation of ^{14}C-glycine into heme. These studies indicated that heme biosynthesis proceeded as described for vertebrate systems (Burnham, 1969). Chan and Margoliash (1966) demonstrated the de novo synthesis of cytochrome c in Samia cynthia. Stimulation of the synthesis of cytochrome c was not inhibited by actinomycin D indicating that the stimulation of apoprotein synthesis was not a direct effect on the genome (Soslau et al., 1971; Williams et al., 1972).

Mitochondrial metabolism in insects has been reviewed by Sacktor (1965, 1970, 1974) and Hansford and Sacktor (1971).

Hormonal Control of Mitochondrial Oxidative Activities

Implantation of corpora allata increased the oxygen consumption of the recipient insect. The degree of stimulation was directly proportional to the activity of the implanted gland (Novak et al., 1959, 1962; Slama and Hrubesova, 1963). Allatectomy produced a decrease in oxygen consumption (Thomsen, 1949), however, these effects were weak and temporary. Others found that JH stimulated oxygen consumption only in female insects but had no effect on males (DeWilde and Stegwee, 1958; Sagesser, 1960; Novak and Slama, 1962; Slama, 1964). This effect on females has been attributed to the ability of JH to stimulate development of the female reproductive system and not due to a direct stimulation of respiratory activity (Pflugfelder, 1952; Thomsen, 1955).

Sehnal and Slama (1966) implanted corpora allata into larvae of G. mellonella and monitored the oxygen consumption of the intact insects through supernumerary larval instars. They concluded that the stimulatory effect on oxygen consumption is indirect and due to the increased mass of the insects. Clarke and Baldwin (1960) found that extracts of corpora allata stimulated oxygen consumption in tissue homogenates of L. migratoria but depressed oxygen consumption under the same conditions in Schistocerca gregaria. Stegwee (1960) found that oxygen

consumption by mitochondria isolated from diapausing L. decemlineata were stimulated by juvenile hormone. DeWilde (1961) confirmed this in vitro and found similar effects in vivo with implanted corpora allata. Both used sodium succinate as a substrate for in vitro mitochondrial oxidations. Stegwee (1959) has demonstrated that adult diapause is due to a deficiency in JH secretion. Diapause is characterized by low rates of oxygen consumption and very low cytochrome concentrations (Shappirio and Williams, 1957a, 1957b). This was detected in pupae of H. cecropia which undergo a pupal diapause. Cytochrome concentrations declined rapidly within hours of the larval-pupal molt. In contrast, nondiapausing pupae show little decline in cytochrome contents.

Minks (1967) found no effect on oxygen consumption of mitochondria isolated from flight muscles following allatectomy or implantation of corpora allata into adult L. migratoria. In addition, extracts or corpora allata had no effect on isolated mitochondria. However, an effect on oxidative phosphorylation was detected when "cecropia oil" was added to the mitochondria. At concentrations approaching 10^{-5} (v/v) and above pyruvate-malate oxidation was inhibited and P/O ratio decreased (Minks, 1967). Keeley (1970, 1971) also found hormonal control of insect mitochondrial oxidative activity, but the control was exerted by the corpora cardiaca and no effect was noted

after allatectomy in adult Blaberus discoidalis. He found that cardiatectomy resulted in reduced oxygen consumption and lowered cytochrome c reductase and cytochrome oxidase activities. However, the addition of corpora cardiaca to isolated mitochondria had no effect (Keeley, 1971; Keeley and Wadill, 1971). Treatment of cardiatectomized insects in vivo with an extract of the corpora cardiaca for several days resulted in restoring oxidative enzyme activities (Keeley and Wadill, 1971). The effects of cardiatectomy were duplicated by severing the nerves from the brain to the corpora cardiaca. The factor appears to be a polypeptide and appears to be produced in the brain and released by the corpora cardiaca (Keeley and Wadill, 1971). Keeley (1972) found that the corpora cardiaca hormone is a controlling factor in the biogensis of mitochondria in the adult cockroach.

Conclusion

The preceding literature review has dealt primarily with the endocrine control of growth and development, the physiological and biochemical effects of juvenile hormone and the biochemistry of insect mitochondria. It is evident that mitochondrial activities could play a central role in the processes responsible for growth and development in insects. However, our current knowledge is very limited

and much data is contradictory. More information on the role of mitochondrial activity and juvenile hormone action and the relationship of these to other aspects of metabolism is required.

MATERIALS AND METHODS

Insect Rearing Methods

Successive generations of the Indian-meal moth, Plodia interpunctella, were reared by the standardized method of Silhacek and Miller (1972) in a 2.4 m x 2.4 m x 2.4 m walk-in incubator (American Instrument Company, Silver Springs, Md.) maintained at 30° C ± 1/2°C and 70% R.H. ± 1%. A photoperiod consisting of sixteen hours light and eight hours dark was supplied by four Westinghouse high output daylight lamps (Westinghouse, 96t12) controlled by an electric time clock.

For egg collection, 23-day-old adult insects were first anesthetized with carbon dioxide and placed in a container with an 18-mesh screened bottom. The container of moths was returned to the incubator and set on a piece of black construction paper at the onset of the dark period. The eggs which accumulated on the construction paper were removed after one hour and cleaned. Forty-four mg of cleaned eggs and 450 g of loosely packed growth medium were placed in each of twelve 12.7 cm x 17.8 cm x 10.2 cm polystyrene plastic pans with plastic lids having

a 6.4 cm diameter screened hole. The eggs were thoroughly mixed into the rearing medium and the pans were then incubated as previously described for the required time.

The medium for rearing P. interpunctella was essentially the same as that described by Silhacek and Miller (1972). Medium components were refrigerated at 4°C to prevent insect contamination. The medium was prepared by mixing 620 g ground Gaines Dog Pellets, 255 g ground rolled oats, 1,665 g white corn meal, 1,480 g whole wheat flour, 160 g wheat germ and 325 g of brewers yeast. The dry components were first mixed and then a mixture of 1,000 g glycerol and 900 g of honey was added. The fresh medium was placed in sealed containers and allowed to stand 24 hours. It was then ground in a Viking hammer mill to give a particle size which passed through an American Standard 8-mesh sieve and stored at room temperature until it was used.

Test Diet Preparation

Test diets were prepared by adding 0.5 ml of an acetone (Mallinckrodt, A.R.) solution of the hormone to 700 mg of the mixture of dry food components. The acetone was removed under a stream of dry nitrogen while stirring in order to uniformly distribute the hormone throughout the food. Three hundred milligrams of

honey:glycerol (1:1 v/v) were then thoroughly mixed into each 700-mg portion of test food. Acetone solutions of 0.2 and 1.0 mg/ml of Cecropia JH (methyl cis-10, 11-epoxy-7-ethyl-3, 11-dimethyl-trans, trans-2, 6-tridecadienoate) were used to provide final hormone concentrations of 0.10 and 0.50 mg/g of food, respectively. After adding the honey:glycerol the diets were routinely held at room temperature overnight and stirred the next day before use to break any clumps of food.

Selection of Insects

Small amounts of food containing larvae were placed on a brown paper towel and larvae were removed with mosquito forceps. Fourth- and fifth-instar larvae could be recognized by the relative sizes of the head capsules.

Larval ages are presented as -2, -1, 0, +1, +2, +3, +4 and +7 days with respect to days prior to (minus) or following (plus) the molt into the fifth larval instar which occurred just prior to collection of 0-day insects. Minus-two-day larvae were early-four-instar larvae approximately two days prior to the larval-larval molt. For in vivo JH treatment, -2-, 0- and +2-day larvae were obtained from pans which were seeded with eggs eight, ten and twelve days earlier, respectively. As they were

collected, two hundred to one-thousand larvae were placed on approximately 100 g of test diet in a 12.5 cm x 6.6 cm x 6.6 cm polystyrene plastic pan with a plastic lid having a 5.7 cm screened hole. The pans containing JH diet and larvae were then placed in the incubator at standard rearing conditions.

After holding on test diets for appropriate intervals, the insects were collected from the test diets and used for mitochondrial isolation. Control insects were collected from stock culture pans.

Isolation of Mitochondria

Mitochondria were isolated by differential centrifugation in a refrigerated centrifuge fitted with a 3.6 x spindle speed attachment (Model PR-6, International Equipment Co., Needham, Mass. Rotor #859)(Firstenberg and Silhacek, 1973). Larvae were placed in a small breaker on ice prior to mitochondrial isolation. One to two grams of larvae were required for isolation of mitochondria. A 5% homogenate was prepared by grinding a group of test insects of known weight in a ground glass homogenizer with an isolation medium containing 0.5 M recrystallized mannitol, 10^{-2}M triethanolamine (TEA), 10^{-3}M EDTA and sufficient HCl to give a pH of 7.4 (Silhacek, 1967).

Mitochondria are defined as that fraction which sedimented between 500 x g for 10 minutes and 600 x g for 10 minutes. Mitochondria were resuspended in fresh isolation medium and resedimented to provide a twice-washed mitochondrial pellet. Mitochondria were then resuspended in a volume equal to the original tissue weight (1.0 ml/g tissue) in a suspension medium consisting of 0.45M mannitol, 10^{-3}M $MgSO_4$, 6 x 10^{-3}M ATP (pH 7.4 with TEA) and 5 mg/ml bovine serum albumin (BSA). All procedures during isolation were carried out at 0-4°C.

Polarographic Enzyme Activity Determinations

Mitochondrial oxidative activities were determined using an oxygraph (Model KM, Gilson Medical Electronics Inc., Middleton, Wisc.) equipped with a vibrating platinum electrode and a jacketed cell maintained at 30°C to determine oxygen uptake. The basic incubation medium contained 600 µmoles recrystallized mannitol, 15 µmoles $MgSO_4$, 6 µmoles ADP and 0.25 mg cytochrome c in 1.55 ml of water. To this medium was added either 0.5 ml BSA (25 mg/ml) or 0.5 ml BSA-JH (BSA containing 250 µg Cecropia JH (a mixture of isomers of methyl-10, 11-epoxy-7-ethyl-3, 11-dimethyl-2, 6-tridecadienoate/ml). The BSA-JH was prepared by mixing 25 µl of JH solution (10 mg JH/ml of acetone) per 25 mg of dry BSA, drying under nitrogen and making up

to volume (1.0 ml) with water. Lower concentrations of JH in the cell were obtained by substituting equivalent volumes of BSA for BSA-JH. One-tenth of a ml of mitochondrial suspension was then added to the cell and allowed to preincubate for 5 minutes. After the preincubation period 30 μmoles of each substrate being tested in a total volume of 0.2 ml were added to the cell contents. The substrate systems used were: pyruvate-malate, pyruvate-glutamate, pyruvate, malate, glutamate, α-ketoglutarate, succinate, α-glycerophosphate, ascorbate and NADH. Oxygen consumption was monitored for 3 to 6 minutes. After the addition of 50 μmoles of inorganic phosphate (P_i) in 0.05 ml (pH adjusted to 7.8 with TEA), a second rate of oxygen consumption was measured. Respiratory control ratios (RC) were calculated by dividing the rate of oxygen consumption after addition of P_i by the rate prior to the addition.

One-tenth milliliter volumes of 10^{-3}M antimycin A, 10^{-3}M oligomycin and 1.5×10^{-6}M rotenone were used in ethanol solution to inhibit mitochondrial reactions in some experiments. The effects of ethanol were determined prior to the use of inhibitors.

Three experiments consisting of duplicate enzyme assays were run with different mitochondrial preparations. For some experiments it was necessary to maximize the permeability of the mitochondrial membranes to NADH by

aging the mitochondrial suspension at 30°C for 45 minutes prior to storage on ice for experimentation.

Assay of NADH Dehydrogenase Activity

NADH dehydrogenase was assayed in a system using ferricyanide as an electron acceptor. For the assay 0.1 ml volumes of 10^{-3}M antimycin A, 0.5M P_i, 5 x 10^{-2}M $K_3Fe(CN)_6^{+3}$, 0.04M NADH and aged mitochondria were added to 0.5 ml BSA or BSA-JH plus 1.55 ml basic incubation medium in a 3.0 ml cuvette. The rate of ferricyanide reduction at 30°C was monitored at 420 nm on a Gilford recording spectophotometer (Model 2000, Gilford Laboratories Inc., Oberlin, Ohio) with a Beckman monochromator (Model DU, Beckman Instruments Inc., Fullerton, Calif.). The initial rate of decrease in the optical density (O.D.) is a measure of the NADH dehydrogenase activity.

Mitochondrial Nitrogen Determinations

Mitochondrial nitrogen content was determined by micro-Nesslerization (Minari and Zilversmit, 1962). Duplicate 0.05 and 0.10 ml samples of each mitochondrial preparation and 0.10 ml samples of the suspension medium were put into 50 ml Nessler's tubes. One milliliter of dilute sulfuric acid (1:4, H_2SO_4:H_2O) was added to each tube. Tubes were covered with parafilm and stored at

-18°C for later determination. Tubes were warmed to room temperature and placed in a Kjeldahl digestion apparatus to char organic residue. Two to three drops of H_2O_2 were then added to the tubes and the contents were allowed to reflux until all of the peroxide was removed. After cooling, approximately 35 ml of water was added to each tube. After mixing, 7.5 ml of Nessler's reagent (Sigma Chemical Co., St. Louis, Mo.) was added, the volume adjusted to 50 ml with water, and the contents were mixed by inversion. The O.D. at 490 nm was read against a blank on a Bausch and Lomb colorimeter (Spectronic 20, Bausch and Lomb Inc., Rochester, N.Y.). The O.D. for the suspension medium was subtracted from the mitochondrial sample O.D.s and the resulting value was compared to a standard curve to give nitrogen concentrations of the mitochondrial suspensions. The standard curve was prepared each day Nessler's tests were run from duplicate tubes containing 0, 150 and 300 mg nitrogen (0.0, 0.75 and 1.5 ml of a 571.4 µg/ml NH_4NO_3 solution, respectively).

Assay of Mitochondrial Cytochromes

Mitochondrial cytochrome concentrations were determined using a variation of the Chance and Williams method (1955). Isolated mitochondria were suspended in approximately 10 ml of suspension medium and 1.0 ml aliquots were placed in each of two 1.0 cm light path, 1.0

ml black wall cuvettes. Analysis was accomplished with a two-wavelength double beam scanning spectrophotometer (model 356, Perkin Elmer Corp., Norwalk, Conn.) operated in the split beam mode. A baseline was established by scanning the cuvettes from 650 nm to 500 nm. A difference spectrum was then obtained by adding 10 µl 10^{-1}M $K_3Fe(CN)_6^{+3}$ to the reference cuvette to fully oxidize the sample and 10 µl 0.05 M $Na_2S_2O_4$ to the sample cuvette in order to fully reduce the sample. The cuvettes were then rescanned from 650 nm to 500 nm and maxima at 605 nm, 564 nm and 551 nm corresponding to cytochromes a, b and c, respectively were revealed. Corresponding minima occurred at 630 nm, 575 nm and 540 nm. Subtraction of minimal O.D.s from maximal O.D.s yielded a measure of cytochrome concentration. Millimolar extinction coefficients for cytochromes a, b, and c are 16.0, 20.0, and 19.0, respectively (Chance and Williams, 1955).

Assay of Mitochondrial Hemes

Mitochondrial hemes were extracted from mitochondria and analyzed using the method of Reiske (1963). In this procedure mitochondria were extracted with acetone, centrifuged at 6000 x g for 15 minutes (International PR-6 centrifuge, rotor #859), extracted with chloroform-methanol (2:1 v/v), and then with acetone to remove lipids. The supernatants were discarded. Three extractions with

acetone-HCl (2.5 ml 36% HCl in 100 ml acetone) removed hemes a and b from the protein precipitate and left heme c in the precipitate. The precipitate was dissolved in approximately 10 ml alkaline pyridine (50 ml 1.0M NaOH in 50 ml pyridine) and a difference spectrum for heme c was obtained as described for cytochrome analysis. The pooled extracts from the acetone-HCl extractions were flash evaporated to near dryness in a rotary vacuum evaporator at 0°C and alkaline pyridine was added. All procedures during the isolation of hemes were carried out at 0-4°C in subdued light. A difference spectrum for heme b was obtained as described earlier. A difference spectrum for heme a was obtained by scanning dithionite reduced alkaline pyridine extract against water. Maxima of 587 nm, 556 nm, and 550 nm were obtained for hemes a, b and c, respectively. Millimolar extinction coefficients for hemes a, b, and c are 24.0, 30.0, and 19.1, respectively (Reiske, 1963).

A quantity of hemoglobin from a hemolysate of human blood was determined spectrophotometrically by the method of Hainline (1958) and was used as a standard to determine extraction efficiency. Admixing hemoglobin to a mitochondrial suspension (100 mg hemoglobin/ml mitochondria yielded about a 50% recovery of the added heme). Quantitative mitrochondrial heme determinations were adjusted for extraction efficiency.

Assay of De Novo Heme Synthesis

De novo synthesis of hemes was determined isotopically. Early-fourth-instar insects 2 days before the last larval-larval molt were placed on either a control or JH-treated diet prepared to give a uniform distribution of 2-^{14}C-glycine (specific activity in the diet 2.0 μCi/g diet). The 2-^{14}C-glycine (Schwarz Mann Radiochemicals, Orangeburg, N.Y.) was added to the diet in acetone solution. The acetone was removed by drying under a stream of nitrogen. Insects were removed from the diet after four days, and mitochondria were isolated and hemes a and b were extracted from the mitochondria. The hemes were solubilized in soluene 100 (Packard Instrument Co.) and were assayed by scintillation counting in a toluene based scintillation fluid in a refrigerated scintillation counter (model Tri-Carb #3003, Packard Instrument Co., Downers Grove, Ill.). The difference in radioactivity between the control and JH-treated tests was proportional to the quantity of JH-stimulated de novo heme synthesis.

Electron Microscope Studies

Early-fifth-instar larvae placed on either a control diet or a 0.1 mg/g JH test diet for four days were dissected and their midguts were removed. The midguts were fixed with glutaraldehyde and osmium tetroxide

(Venable and Coggeshall, 1965). The fixation required sequential dehydration in ethanol-water solutions of increasing concentrations and finally acetone. Embedding was in an Epon-Araldite mixture (Mollenhauer, 1965). Sectioning was performed on a Sorvall ultramicrotome (model MT-2B, Ivan Sorvall Inc., Norwalk, Conn.). Only silver or gray sections were placed on the 200 mesh grids. Specimens were viewed on a Hitachi electron microscope (model HU-125E, Hitachi Ltd., Tokyo, Japan). Photomicrographs were analyzed for mitochondrial size, number, shape, dispersion, surface area, volume and general condition.

RESULTS

Electron Microscopy

As a basis for subsequent biochemical studies, I examined the ultrastructure of midgut mitochondria from control and JH-fed larvae. Midgut mitochondria of control larvae were relatively small, roughly spherical and were concentrated on the hemocoel side of the midgut (Figure 3). In JH-fed larvae (Figure 4) the mitochondria were more irregular, twice as large as control mitochondria and there were only half as many as in the control. Also, they were more dispersed through the cytoplasm than mitochondria in midguts of control larvae (Table 1). Mitochondria in control larvae had a higher surface area to volume ratio than mitochondria in JH-fed larvae. However, the total mitochondrial volume within the midguts were essentially equal for JH-fed and control larvae.

Effects of In Vivo JH Treatment on Enzyme Activities of Mitochondria Isolated from JH-fed Insects

These cytological differences in mitochondria suggested altered metabolism. Experiments to test this hypothesis established that the rate of pyruvate-malate

Figure 3. Electron micrograph of midgut tissue from control larvae (magnification, 15,000 X).

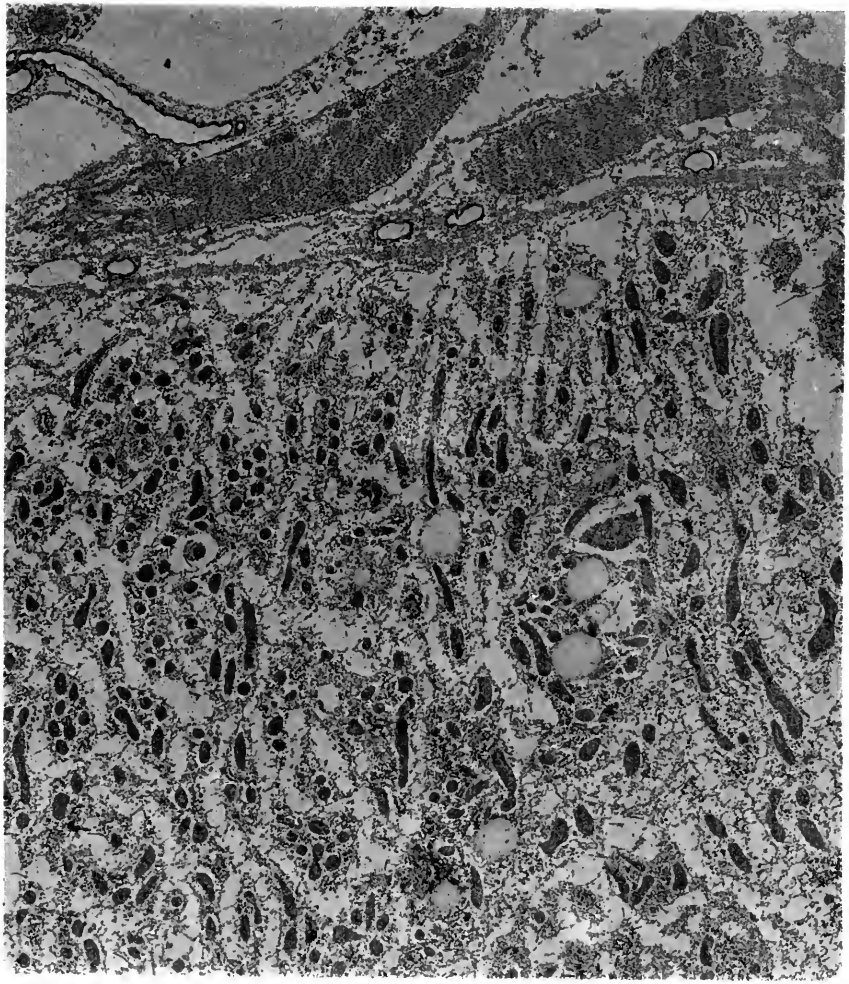


Figure 4. Electron micrograph of midgut tissue from JH-fed larvae (magnification, 15,000 X).

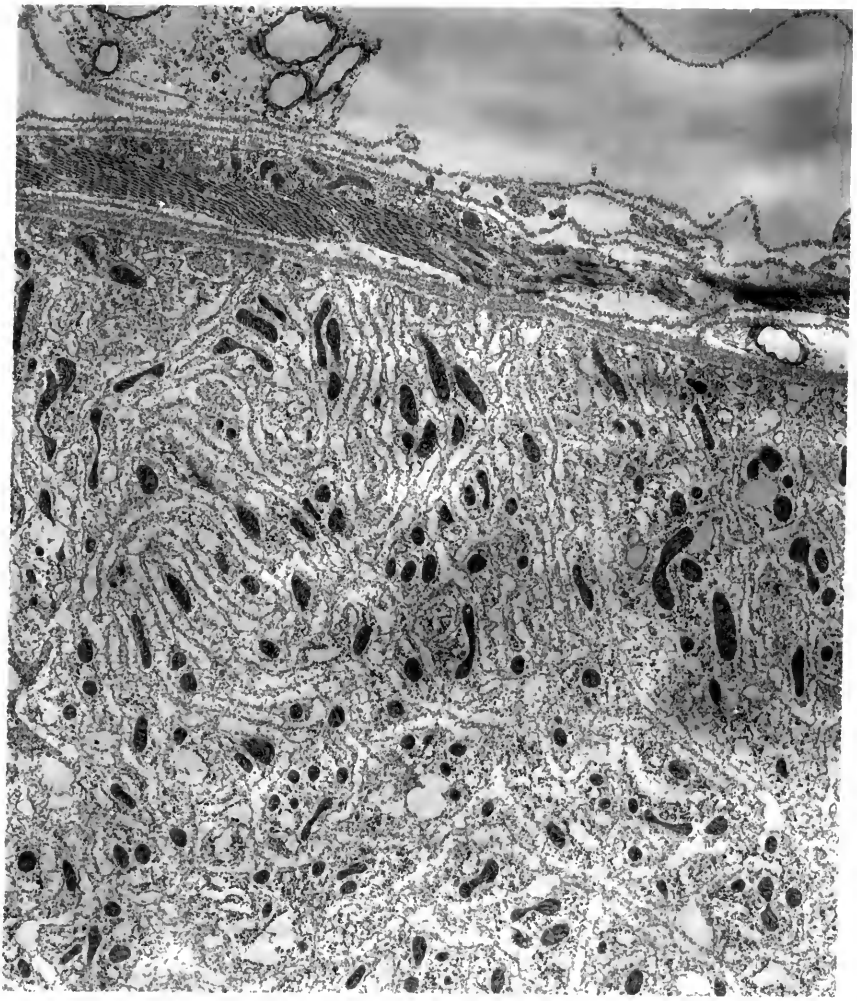


Table 1. A Comparison of Mitochondria in Midguts of Control and JH-Fed Larvae

	JH-Treated	Control
Mean volume (μm^3)	7.8×10^{-3}	3.0×10^{-3}
Mean surface area (μm^2)	.203	.093
Surface area to volume ratio	23.66	34.96
Mean concentration in midgut columnar epithelium cells (mitochondria/μm^3)*	.522	1.33
Per cent of mitochondria within 10 µm of the midgut wall (hemocoel)	48.4	62.1

*Mitochondria were counted in one sagittal section from each of three midgut columnar epithelium cells.

oxidation by mitochondria isolated from +4-day larvae treated *in vivo* for four days with JH (treatment initiated on 0-day) was higher than the rate by mitochondria isolated from the same age control larvae (Table 2). Rates of pyruvate-malate oxidation by mitochondria isolated from +4-day control larvae and +4-day JH-treated larvae were lower than that of 0-day control larvae. The rate of pyruvate-malate oxidation by mitochondria isolated from larvae fed a diet containing 0.5 mg JH/g for 4 days was not significantly different from the rate observed for mitochondria from larvae fed a diet containing 0.1 mg JH/g for 4 days. The +4-day control larvae may not offer a good comparison with JH-treated insects because these insects had stopped feeding while the JH-treated insects were still actively feeding (Firstenberg and Silhacek, in press). Mitochondria from insects fed on the diet containing 0.1 mg JH/g for 7 days oxidized pyruvate-malate at a lower rate than those from insects similarly treated for 4 days. Mitochondria from insects fed on 0.5 mg/g diet for 7 days oxidized pyruvate-malate at a lower rate than mitochondria from larvae fed on the 0.1 mg/g diet for 7 days. In all cases mitochondria from larvae treated *in vivo* with JH were inhibited more by JH treatment (1.77×10^{-4}M) *in vitro* than control insects. In addition, mitochondria from JH-treated larvae had slightly lower respiratory control ratio values than mitochondria

Table 2. Rates of Pyruvate-Malate Oxidation in Isolated Mitochondria from Plodia interpunctella treated in vivo with Juvenile Hormone

Duration of in vivo Hormone Treatment* Days	Hormone Concentration MG/G	Additions	Mean $Q_{O_2(N)}$***	Mean R.C.
0 (Control)	0	None	335.6**	5.27
		JH	11.5	0.74
4 (Control)	0	None	87.1	2.37
		JH	3.3	-
4	0.1	None	171.0	3.92
		JH	1.8	-
4	0.5	None	163.9	4.42
		JH	0.8	-
7	0.1	None	104.0	3.91
		JH	1.6	-
7	0.5	None	62.4	4.25
		JH	0.5	-

*Treatment began with 0-day larvae
**Values represent duplicate runs on each of three replicates
***Units of $Q_{O_2(N)}$ are µatoms oxygen consumed/hour/mg mitochondrial nitrogen.

from the 0-day control larvae but higher values than from +4-day control larvae. Since control insects stopped feeding by the 4th day and pupated by the 7th day there were no suitable mitochondria to serve as controls.

The results of the tests for succinate oxidation by mitochondria (Table 3) indicated that there was a small decrease in their ability to oxidize succinate in +4-day control larvae as compared to 0-day control larvae. Mitochondria isolated from both 0-day and +4-day control larvae had higher rates of succinate oxidation when treated with JH (1.77×10^{-4}M) *in vitro*. Mitochondria isolated from larvae fed on either the 0.1 mg/g or the 0.5 mg/g JH diets for 4 days had greater rates of succinate oxidation than either of the control mitochondrial preparations. Mitochondria from insects fed the 0.1 mg/g JH diet for 7 days had a lower rate of succinate oxidation than those mitochondria from larvae fed for 4 days on the same diet. Mitochondria from larvae fed on the 0.5 mg/g JH diet for 7 days had an even lower rate of succinate oxidation. None of the mitochondrial preparations from larvae treated with JH responded appreciably to JH (1.77×10^{-4}M) *in vitro*. These results indicated that the mitochondria in JH-treated larvae differ from the controls in their capacity to be stimulated by JH *in vitro*. The only apparent effect of *in vitro* JH treatment of isolated mitochondria from JH-treated larvae was a small decrease in

Table 3. Rates of Succinate Oxidation in Isolated Mitochondria from Plodia interpunctella treated in vivo with Juvenile Hormone

Duration of in vivo treatment* Days	Hormone Concentration MG/G	Additions	Mean $Q_{O_2(N)}$***	Mean R.C.
0 (Control)	0	None	121.4**	1.23
		JH	160.8	1.20
4 (Control)	0	None	94.1	1.39
		JH	130.9	1.29
4	0.1	None	157.1	1.43
		JH	137.4	1.12
4	0.5	None	136.6	1.62
		JH	137.7	1.14
7	0.1	None	110.0	1.59
		JH	119.9	1.08
7	0.5	None	61.6	1.36
		JH	53.2	1.04

*Treatment began with 0-day larvae

**Values represent duplicate runs on each of three replicates

***Units of $Q_{O_2(N)}$ are µatoms oxygen consumed/hour/mg mitochondrial nitrogen.

respiratory control ratios when succinate was used as the substrate. The +4-day controls in the succinate tests had the same deficiencies as noted in the pyruvate-malate experiments. More recent results indicated that the stimulation of succinate oxidation in mitochondria from +4-day control larvae by JH treatment *in vitro* may be erroneous and that the capacity of JH to stimulate succinate oxidation may be lost as a consequence of larvae aging (Silhacek and Kohl, unpublished data).

Effects of In Vitro JH Treatment on Mitochondrial Enzyme Activities

The *in vivo* data demonstrated an inhibitory effect by JH on both pyruvate-malate and succinate oxidation. The inhibition of pyruvate-malate oxidation and the stimulation of succinate oxidation by JH *in vitro* in mitochondria isolated from untreated larvae suggested that JH may have a direct effect on mitochondrial metabolism. This was tested by determining the oxidative activities of isolated mitochondria with various citrate cycle intermediates in the presence and absence of JH.

Inhibition of pyruvate-malate, pyruvate-glutamate, malate, α-ketoglutarate, and glutamate oxidations occurred in mitochondria isolated from 6 to 8 mg larvae treated *in vitro* with 1.77×10^{-4}M JH (Table 4). Ascorbate and α-glycerophosphate oxidations were not affected, while

Table 4. Effect of Juvenile Hormone on the Rates of Substrate Oxidation by Isolated Mitochondria from Plodia interpunctella

Substrate	$Q_{O_2(N)}$** Additions	
	None	JH*
Pyruvate-Malate	335.6	11.5
Pyruvate-Glutamate	303.2	20.6
Malate	55.7	7.6
α-Ketoglutarate	100.0	23.2
Glutamate	64.1	10.2
Succinate	121.4	160.8
α-Glycerophosphate	314.4	295.7
Ascorbate	69.9	72.0

*JH concentration during incubation = 1.77×10^{-4} M
**Units of $Q_{O_2(N)}$ are µatoms oxygen consumed/hour/mg mitochondrial nitrogen.

succinate oxidation was stimulated. It was noted that only those substrates requiring NAD as a cofactor for oxidation were inhibited. These results indicated that the inhibition probably occurred in the NADH dehydrogenase complex, complex I, of the mitochondrial electron transport chain.

NADH was used as a substrate to localize the site of JH action in the electron transport chain. Fresh mitochondria oxidized NADH very slowly due to low permeability of the mitochondrial membrane to NADH. The mitochondrial membrane was rendered permeable to NADH by aging the mitochondria at 30°C. Mitochondria aged for 45 minutes and then placed on ice gave almost maximal NADH dehydrogenase activity which was realtively stable for 2.0 hours (Figure 5). Mitochondria aged less than 45 minutes did not reach maximal oxidative activity. Those aged more than 45 minutes were unstable with a marked decrease in oxidative activity occurring between 100 and 120 minutes of aging.

Aged mitochondria were less susceptible than fresh mitochondria to inhibition by oligomycin, an inhibitor of oxidative phosphorylation (24.1% versus 71.0%), indicating that aging uncoupled oxidative phosphorylation (Table 5). Since JH was effective inhibiting NADH oxidation in both fresh and aged mitochondria then it must be acting in the electron transport chain.

Inhibition of pyruvate-malate oxidation in fresh mitochondria and inhibition of NADH oxidation in aged

Figure 5. The relationship between NADH dehydrogenase activity and time of aging of isolated mitochondria from larvae of Plodia interpunctella.

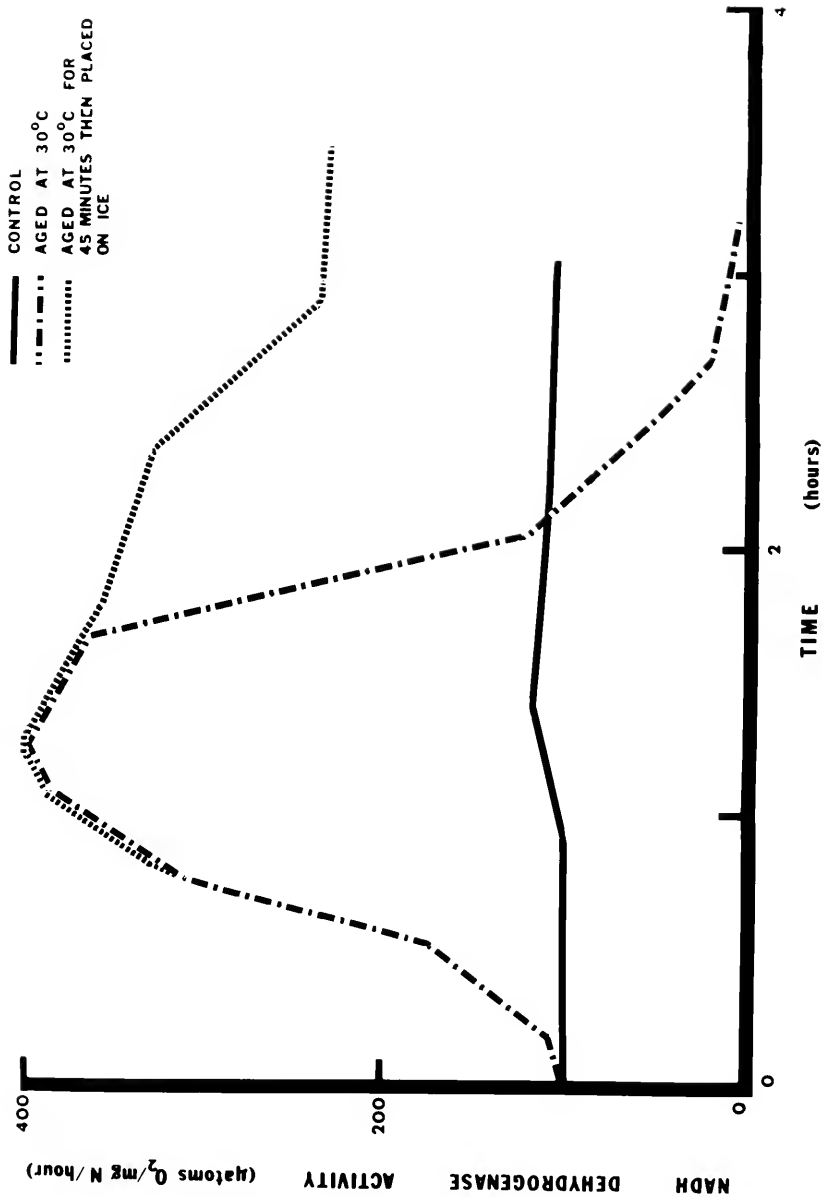

CONTROL
AGED AT 30°C
AGED AT 30°C FOR 45 MINUTES THEN PLACED ON ICE
NADH DEHYDROGENASE ACTIVITY (μatoms O2/mg N/hour)
400
200
0
0
2
4
TIME (hours)

Table 5. Effect of Inhibitors and Aging on NADH Oxidation by Mitochondria from Plodia interpunctella

Mitochondrial Aging Time (min)	Inhibitor	$Q_{O_2(N)}$ [4]
0	None	107.2
30	None	288.0
45	None	414.5
60	None	448.6
0	Oligomycin [1]	31.1
45	Oligomycin	315.0
0	JH [2]	55.9
45	JH	120.7
45	Oligomycin + JH	43.3
45	Rotenone [3]	21.5

[1] Oligomycin concentration during incubation = 4.10×10^{-5} M

[2] JH concentration during incubation = 1.77×10^{-4} M

[3] Rotenone concentration during incubation = 6.25×10^{-8} M

[4] Units of $Q_{O_2(N)}$ are µatoms oxygen consumed/hour/mg mitochondrial nitrogen.

mitochondria were related to the concentration of JH in the same way (Figure 6). Minimal inhibition was detected by a JH concentration of approximately 3×10^{-5}M. Maximal inhibition was reached by 1.41×10^{-4}M JH. A plot of NADH concentration divided by reaction rate versus substrate concentration at JH concentrations of 0, 7.1×10^{-5}M, 1.06×10^{-4}M and 1.77×10^{-4}M (Figure 7) revealed that NADH oxidation was noncompetitively inhibited (K_i = 0.1069 mM).

These results indicated that the inhibition definitely did occur in the electron transport chain. Since electrons from succinate and α-glycerophosphate oxidations enter the electron transport chain at coenzyme Q and since these oxidations were not inhibited by JH, then the inhibition must occur between NADH and coenzyme Q in the electron transport chain.

Sodium ferricyanide was used as an electron acceptor from NADH dehydrogenase to assay the activity of NADH dehydrogenase. The rate of reduction of $Fe(CN)_6^{-3}$ by aged mitochondria was not significantly altered by the presence of 1.77×10^{-4}M JH. When JH was present at a rate of 742.6 µmoles $Fe(CN)_6^{-3}$/hour per mg mitochondrial nitrogen was obtained compared to 762.8 µmoles/hour per mg mitochondrial nitrogen when JH was absent. These rates accounted for 92% of the oxygen uptake observed in polarographic experiments and indicated that juvenile hormone did not inhibit

Figure 6. The relationship of juvenile hormone concentration to the percent inhibition of NADH and pyruvate-malate oxidations.

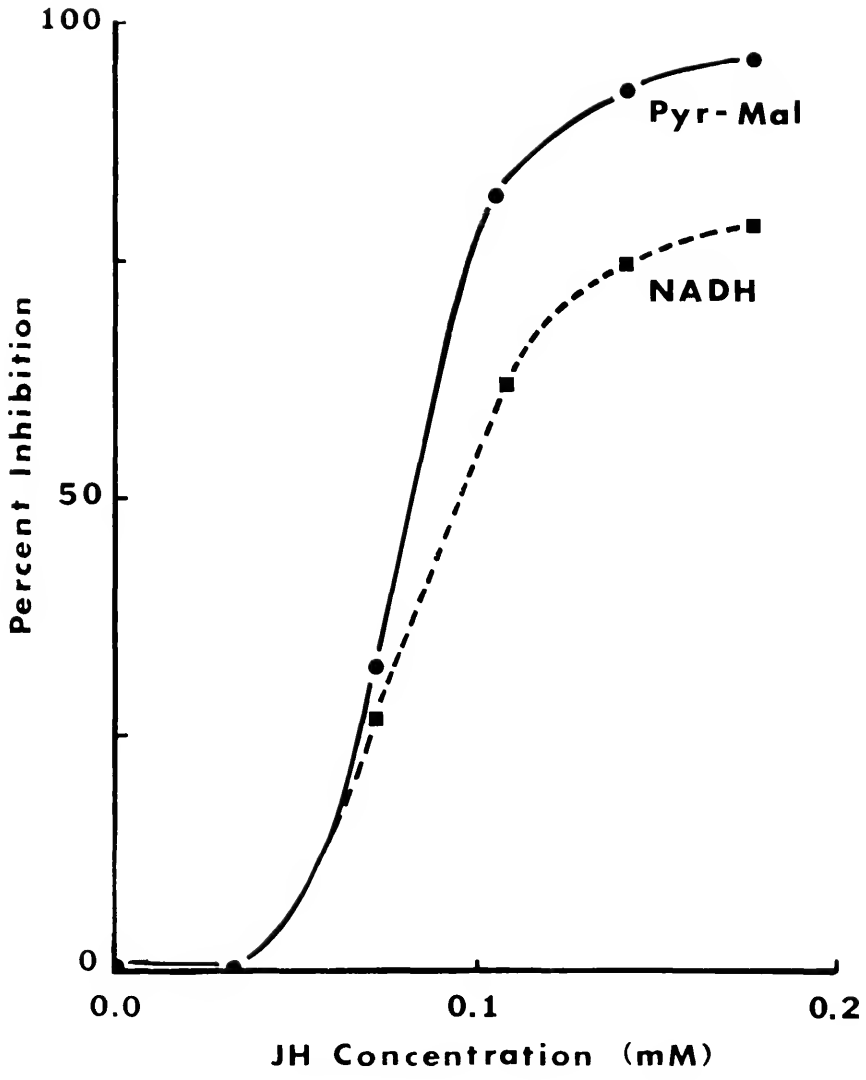
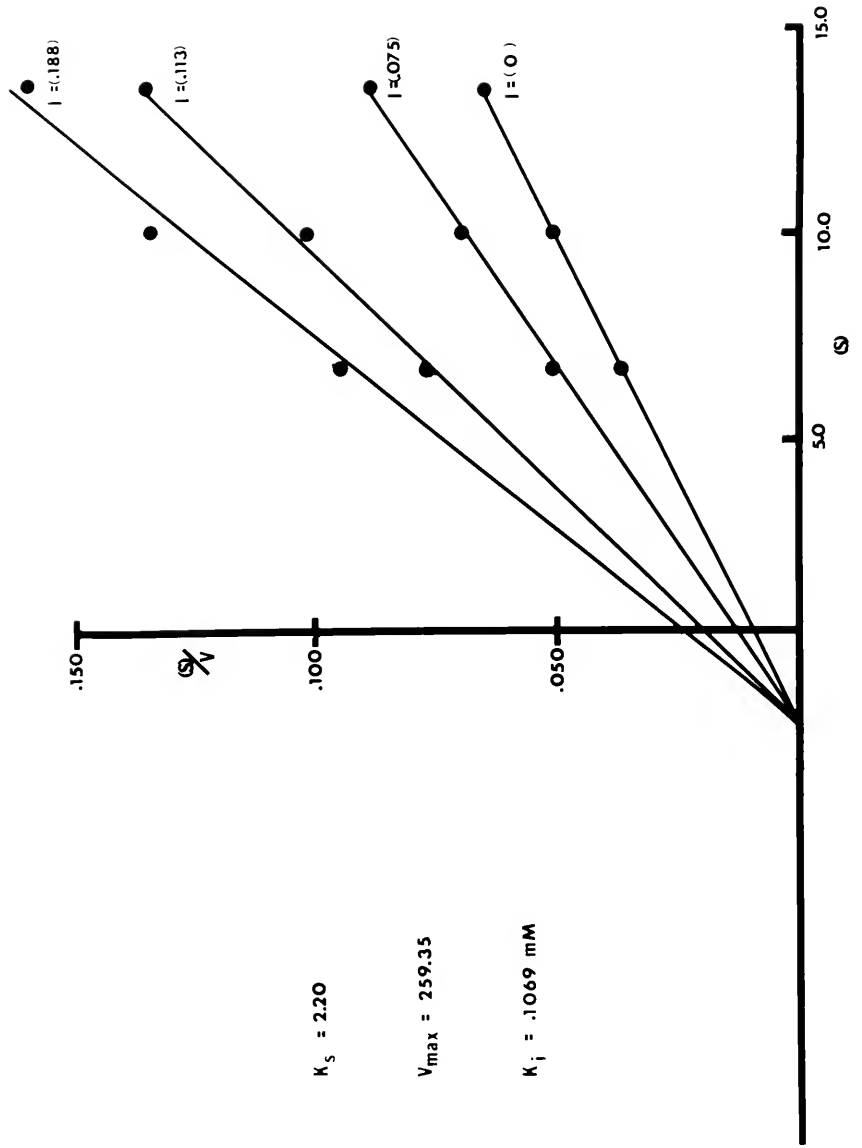

100
50
0
Percent Inhibition
Pyr-Mal
NADH
0.0
0.1
0.2
JH Concentration (mM)

Figure 7. Plot of (S)/V versus (S) ((S) is the NADH concentration and V is the reaction rate). The substrate constant (K_s), inhibitor constant (K_i) and the maximum velocity of the reaction (V_{max}) are shown.


I =(.188)
I =(.113)
I =(.075)
I =(0)
.150
.100
.050
S/v
5.0
10.0
15.0
(S)
K_s = 2.20
V_{max} = 259.35
K_i = .1069 mM

at the level of NADH dehydrogenase. This experiment localized the inhibition by JH to the nonheme iron protein region of the electron transport chain (Figure 1). The mechanism of this inhibition was not investigated.

Cytochrome Analyses

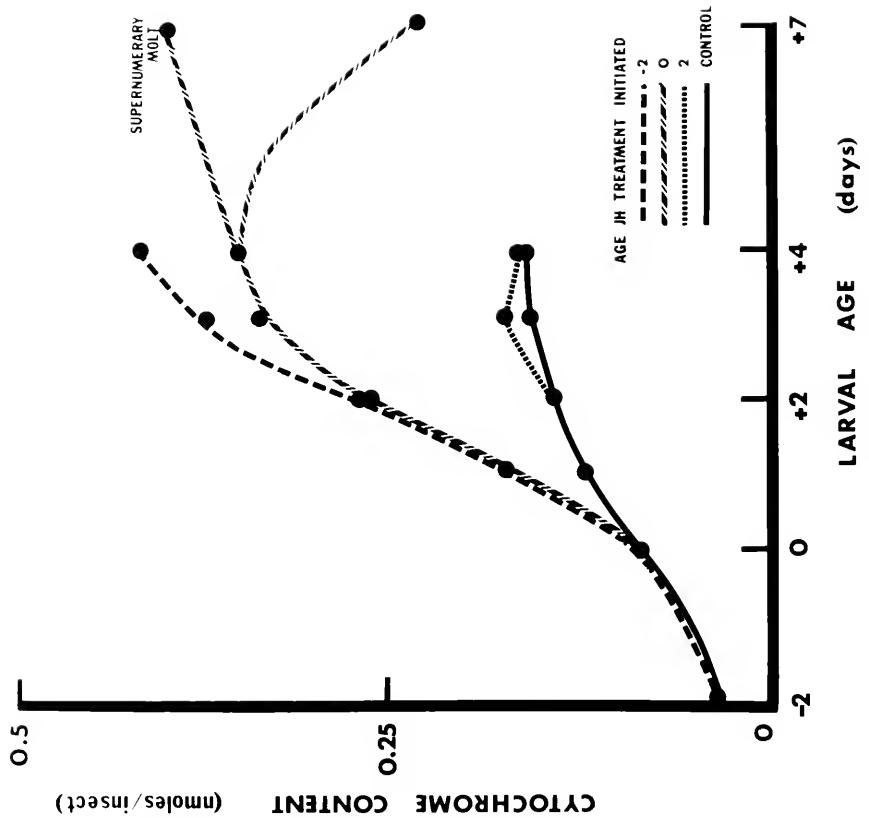
Mitochondria isolated from larvae treated *in vivo* with JH were red to purple, while mitochondria from control insects were tan. Such coloration may be the result of differences in cytochrome content. Therefore, I investigated the cytochrome content of mitochondria from control and JH-fed larvae.

Newly molted 5th-instar larvae (0-day) had a mitochondrial cytochrome content of approximately 88.7 pmoles/insect. The cytochrome content increased to 158.2 pmoles/insect in +4-day larvae. When 0-day larvae were fed JH for 4 days the mitochondrial cytochrome content increased to approximately 418.6 pmoles/insect. Preliminary experiments indicated that the amount of stimulated cytochrome synthesis was identical with either 0.1 mg/g or 0.5 mg/g JH diet so all subsequent tests utilized the 0.1 mg/g diet. Mitochondrial cytochrome concentration (nmoles/g tissue) was higher in 0-day insects than in older larvae.

These observations conclusively demonstrated that JH participated in the stimulation of cytochrome synthesis,

but did not eliminate the possibility that some other event associated with the molt was also necessary. 0-, -2- and +2-day larvae were placed on JH-treated diet, removed at one day intervals and assayed for mitochondrial cytochrome. The mitochondrial cytochrome content (pmoles/insect) of both the control and JH-fed larvae increased throughout the test (Figure 8). However, juvenile hormone treatment did not stimulate cytochrome synthesis until after the last larval-larval molt. At +1-day the JH-fed larvae had a significantly higher cytochrome content than the controls. The mitochondrial cytochrome contents for larvae which were placed on JH diet at -2- days were identical to those for larvae which were placed on JH diet at 0-days. Compared with comparably aged control insects, the two JH-fed larval groups showed approximately a 40% increase over controls in mitochondrial cytochrome content (pmoles/insect) by +1-days, a 113% increase by +2-days, a 200% increase by +3-days with no additional increase by +4-days. The mitochondrial cytochrome content of the larvae which were placed on JH diet at +2-days did not differ significantly from the controls. These results indicated that JH stimulated cytochrome synthesis, but the stimulation was dependent upon some event associated with the molt. Figure 9 shows a comparison of the changes in mitochondrial cytochrome contents (pmoles/insect) from -2- to +4-days of larvae fed JH starting at -2-days and of control larvae. Cytochrome synthesis in control larvae

Figure 8. The relationship of larval age and age at initiation of JH treatment to the mitochondrial cytochrome content.


SUPERNUMERARY MOLT
AGE JH TREATMENT INITIATED
-2
0
2
CONTROL
0.5
0.25
0
CYTOCHROME CONTENT (nmoles/insect)
-2
0
+2
+4
+7
LARVAL AGE (days)

proceeded from -2- to +4-days with most of the synthesis occurring before +1-day. The mitochondrial cytochrome content of JH-fed larvae also increased throughout the test period at a rate of 6 times faster after the molt. This resulted in a total cytochrome content per insect 3 times greater in JH-fed as control insects by +4-days.

Figure 9 shows cytochrome concentrations as nmoles/g tissue. Cytochrome concentration in control larvae was highest in -2-day larvae, decreased in -1-day larvae, increased in 0-day larvae and then decreased to the end of the experiment (+4-days). Again there was no difference in cytochrome content of JH-fed larvae and control larvae until after the last larval-larval molt. After the molt cytochrome concentrations of the controls dropped sharply while that of the JH-fed larvae remained high and dropped more slowly. Approximately 30% of the larvae fed JH diet had undergone a supernumerary molt by the seventh day on the diet (treatment began with 0-day larvae). The larvae which molted an additional time had a higher mitochondrial cytochrome concentration than non-molting JH-fed larvae (Table 6).

Cytochrome a, b and c concentrations (pmoles/insect) of JH-treated and control larvae are shown in Figure 9. In mitochondria from control larvae cytochrome $a + a_3$ concentration increased 10-fold, cytochrome b

Figure 9. The relationship of larval age to cytochromes a, b and c concentration per insect for JH-treated and control insects.


CYTOCHROME a
CYTOCHROME b
CYTOCHROME c
CONTROL
JH-TREATED
500
250
0
CYTOCHROME CONCENTRATION (pmoles/insect)
-2
-1
0
+1
+2
+3
+4
LARVAL AGE (days)

Table 6. Comparison of Mitochondrial Cytochrome Concentrations of 7-day-old JH-fed Larvae which had undergone a Supernumerary Molt with those from Larvae which had not molted

	pmoles/insect		nmoles/g tissue	
	control	JH-treated	control	JH-treated
Cytochrome a	157.9	255.5	5.46	7.18
Cytochrome b	23.4	61.6	0.81	1.73
Cytochrome c	45.4	83.0	1.57	2.33
Total Cytochrome	226.7	400.1	7.84	11.24

concentration increased 4-fold and cytochrome c concentration increased 3-fold during the period from -2- to +4- days. In JH-treated larvae the corresponding increases were 30-fold for cytochrome a + a_3, 11-fold for cytochrome b and 4-fold for cytochrome c.

Cytochromes a + a_3, b and c concentrations (nmoles/g tissue) are shown in Figure 10. The concentration of the individual cytochromes followed the same general trend as noted for total cytochrome content. There was no difference between control and JH-fed insects in cytochromes a + a_3 and c concentrations prior to the last larval-larval molt but after the molt the concentrations of these cytochromes decreased rapidly in control larvae while the concentration was maintained at a level 3-times higher in JH-treated larvae. On the other hand, cytochrome c concentration was essentially identical in control and JH-treated larvae throughout the experiment. The concentrations of cytochromes a + a_3, b and c were higher in insects which underwent a supernumerary molt than in corresponding non-molting larvae (Table 6).

The ratios of cytochrome b to cytochromes a + a_3 decreased throughout the experiment and were essentially the same for control and JH-fed insects (Table 7). The ratio of cytochrome c to cytochromes a + a_3 decreased throughout the experiment in both control and JH-fed insects but the decrease in the ratio was greater in JH-fed

Figure 10. The relationship of larval age to cytochromes a, b and c concentration per gram tissue for JH-treated and control insects.

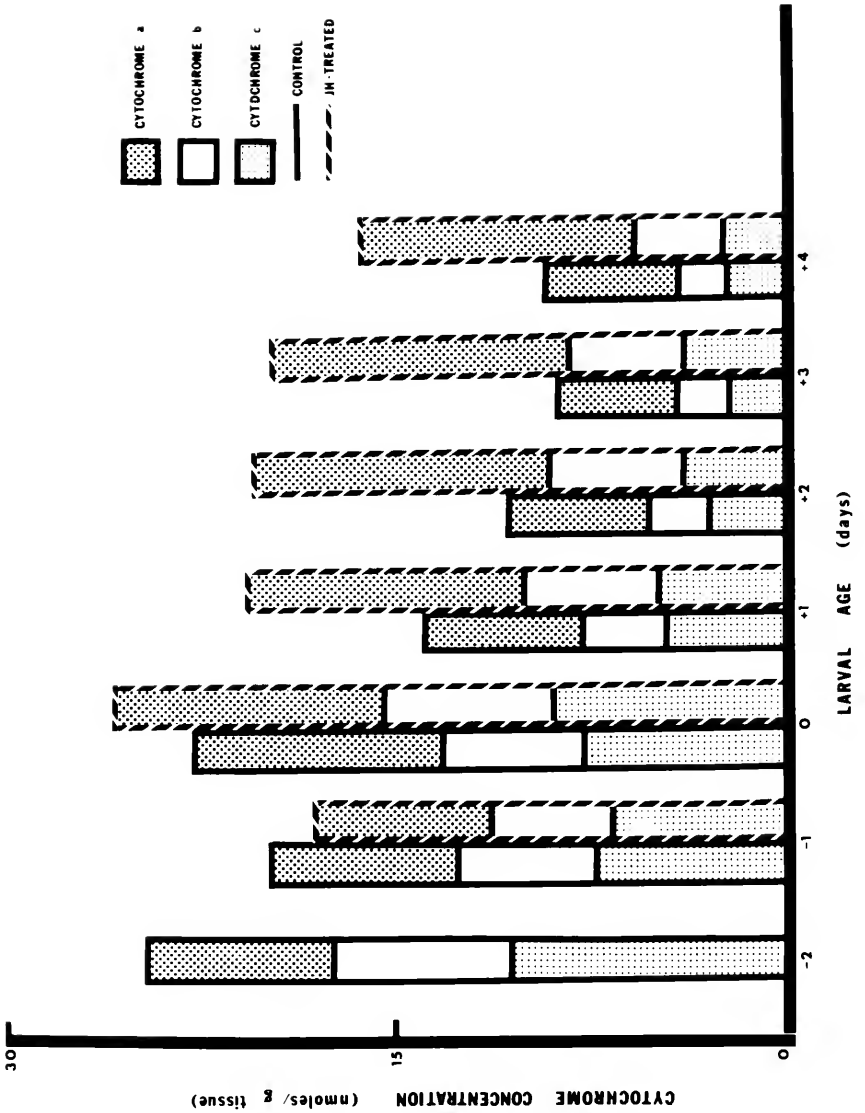

CYTOCHROME a
CYTOCHROME b
CYTOCHROME c
CONTROL
JH-TREATED
30
15
0
CYTOCHROME CONCENTRATION (nmoles/ g tissue)
-2
-1
0
+1
+2
+3
+4
LARVAL AGE (days)

Table 7. Ratios of Cytochrome $A+A_3$, B and C Concentrations to Cytochrome $A+A_3$ Concentration for Mitochondria isolated from Control and JH-treated Larvae

Larval Age	Control Larvae			JH-Treated Larvae		
Days	Cyt. $a+a_3$	Cyt. b	Cyt. c	Cyt. $a+a_3$	Cyt. b	Cyt. c
-2	1.0	0.97	1.50	---	---	---
-1	1.0	0.76	1.01	1.0	0.72	0.96
0	1.0	0.59	0.80	1.0	0.66	0.87
+1	1.0	0.58	0.75	1.0	0.51	0.45
+2	1.0	0.47	0.49	1.0	0.44	0.34
+3	1.0	0.41	0.44	1.0	0.36	0.33
+4	1.0	0.36	0.40	1.0	0.35	0.22

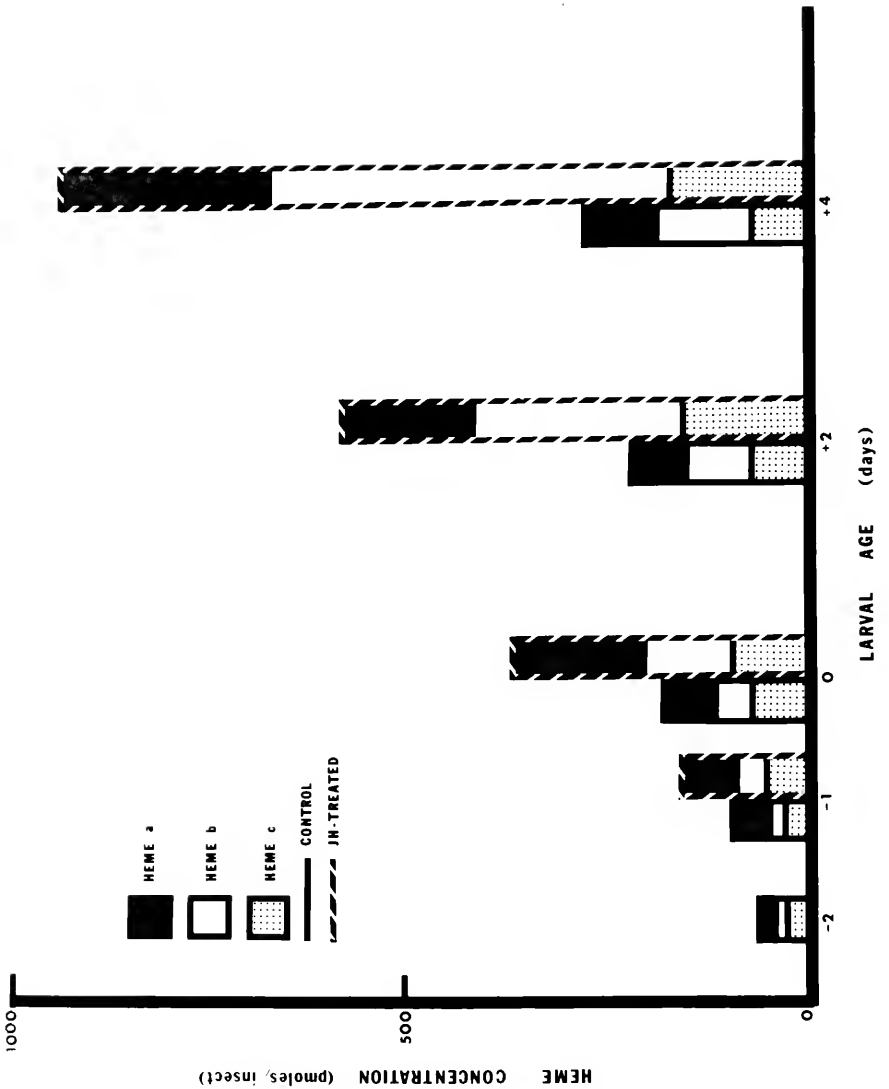
insects. The greater decrease in this ratio may be attributed to loss of cytochrome c from the mitochondria during isolation because cytochrome c is not bound to the mitochondrial membranes as tightly as the other cytochromes.

Assay of Mitochondrial Hemes

Because the stimulation of cytochrome synthesis was dependent on both JH and some factor associated with the last larval-larval molt, it was decided to determine whether the two controls were exerted at the same site or at different sites in the cytochrome synthetic pathway. Since cytochromes are composed of heme and apoprotein, the effects of JH on the synthesis of just the heme portion of the molecule was investigated.

The total mitochondrial heme concentration (pmoles/insect) increased 4-fold from -2- to +4-days in control larvae. It increased 14-fold in insects fed on a JH diet (0.1 mg/g JH). A difference in heme concentration between controls and JH-treated larvae was observed within one day after initiating JH treatment 2 days before the molt into the fifth instar (Figure 11). Heme content of JH-fed insects increased 63% above the control value within the first day of the test and had doubled the control value by the time of the last larval-larval molt (0-day). The

Figure 11. The relationship of larval age to hemes a, b (protoheme) and c concentration per insect for JH-treated and control insects.


HEME a
HEME b
HEME c
CONTROL
JH-TREATED
1000
500
0
HEME CONCENTRATION (pmoles/ insect)
-2
-1
0
+2
+4
LARVAL AGE (days)

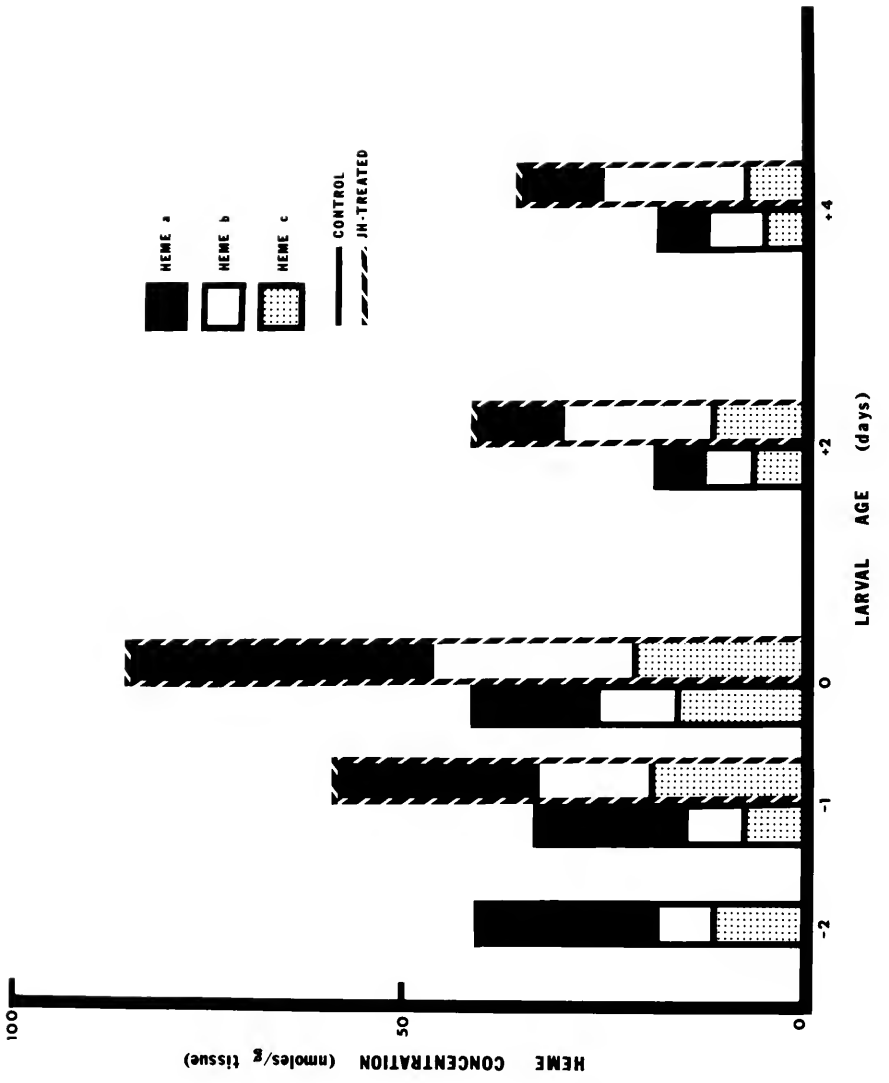
increase approached 230% by the termination of the experiment at +4-days. The concentration per insect increased linearly after a short lag bewteen -2- and -1-days in both control and JH-fed insects.

Heme concentrations per gram tissue for control larvae decreased initially but rose to a peak just after the molt (Figure 12). It decreased to a low level in +2-day larvae and remained stable to the conclusion of the experiment. Heme concentrations of the JH-fed larvae increased immediately reaching a peak in 0-day larvae and then decreased to a level 2-fold the control concentration until the termination of the experiment at +4-days.

Concentrations of hemes a, b and c per insect are shown in Figure 11. In control insects heme a increased 2.5-fold, heme b increased 12-fold and heme c increased 2-fold during the period from -2- to +4-days. In JH-fed larvae the corresponding increases were 12.5-fold for heme a, 50-fold for heme b and 6-fold for heme c. Hemes b and c were synthesized at a linear rate after a short lag between -2- and -1-days. However, the rate of heme a synthesis was higher in younger larvae and decreased toward the end of the experiment.

In control larvae the ratios of concentrations of heme a to heme b to heme c at the beginning of the experiment were 1.00:0.49:1.25 but by the end of the experiment the ratios were 1.00:1.36:0.70. The same ratios at the

Figure 12. The relationship of larval age to hemes a, b (protoheme) and c concentration per gram tissue for JH-treated and control insects.


HEME a
HEME b
HEME c
CONTROL
JH-TREATED
HEME CONCENTRATION (nmoles/g tissue)
100
50
0
-2
-1
0
+2
+4
LARVAL AGE (days)

end of the experiment in JH-fed larvae were 1.00:1.89:0.63 indicating a shift in heme synthesis toward heme b and away from hemes a and c.

Assay of De Novo Synthesis of Hemes

I could not determine from the heme analyses whether the increase in mitochondrial hemes was due to de novo synthesis, to a sequestering of hemes by mitochondria or to a partial synthesis from sequestered intermediates in heme synthesis. Incorporation of a carbon-14 labeled glycine was used to determine if de novo synthesis of hemes did occur.

Insects placed on a medium containing 2-^{14}C-glycine (2.0 uCi/g diet) from -2- to +2-days incorporated the ^{14}C-label into hemes. During that period control larvae incorporated 213 cpm/hr./insect into 6.9 nmoles of hemes a and b. The corresponding values for JH-fed larvae were 1,140 cpm/hr./insect incorporated into 23.9 nmoles hemes a and b per insect. Comparing the rates of heme synthesis indicates that JH-fed larvae accumulated 3.46-times as much heme as control larvae during the period from -2- to +2-days. During the same period, the incorporation of radiolabeled glycine into hemes was 3.58-times greater in JH-fed larvae than in the controls. The similarity of these two ratios indicated that hemes a and b were

synthesized de novo in both control and JH-fed larvae and that the sequestering of preformed hemes or heme precursors did not play an important role in the increased concentration of mitochondrial heme.

DISCUSSION

The results presented in this dissertation establish that juvenile hormone (JH) acts directly on the mitochondria of Indian meal moth larvae. Juvenile hormone affects citrate cycle oxidations, electron transport, heme synthesis and cytochrome synthesis. Juvenile hormone inhibits electron transport by the nonheme iron protein in complex I of the mitochondria which results in lower oxidation rates for citrate cycle intermediates requiring NAD as a cofactor for oxidation. Minks (1967) found that relatively high concentrations of a crude extract of Hyalophora cecropia, a potent source of JH, (10^{-3}%) depressed pyruvate-malate oxidation in mitochondria from adult Locusta migratoria. The effect was very weak and was attributed to "toxic effects" at high concentrations of hormone. Firstenberg and Silhacek (unpublished observations) have shown that inhibition by JH of oxidation of NAD-linked substrates in isolated mitochondria did not change throughout the last larval instar. Silhacek and Kohl (unpublished observations) have also shown that the response to H. cecropia JH (JH-1, Röller et al., 1967) is greater than the response to the other two known insect juvenile hormones (JH-II, Meyer

et al., 1968, and JH-III, Judy et al., 1973) indicating that the inhibition is probably physiological and not pharmacological. It may be noted that several substances with insecticidal properties, rotenone, amytal and piericidin A, also inhibit electron transport by the noneheme iron protein (Hatefi, 1968; Horgan and Singer, 1968). However, none of the other effects of JH on isolated mitochondria reported in this dissertation have been reported for rotenone, amytal or piericidin A.

This dissertation also establishes that juvenile hormone stimulates succinate oxidation in isolated mitochondria from P. interpunctella larvae. Clarke and Baldwin (1960) noted stimulated succinate oxidation with mitochondria isolated from adult L. migratoria when incubated with preparations of corpora allata. However, the JH stimulation of succinate oxidation did not occur when the same procedure was done with larvae of Schistocerca gregaria and, in fact, a slight inhibition was noted. Their experiments were unreplicated experiments and the effects were weak. DeWilde and Stegwee (1958) demonstrated that removal of the corpora allata from diapausing Leptinotarsa decemlineata adults resulted in reduced succinate dehydrogenase activity when oxygen consumption was measured in tissue homogenates. DeWilde (1959) subsequently demonstrated that both corpora allata and extracts of Hyalophora cecropia stimulated succinate oxidation in tissue homogenates

from diapausing L. decemlineata. Stegwee (1960) confirmed results of earlier studies by treating isolated thoracic muscle mitochondria from L. decemlineata with H. cecropia extract. Keeley (1970, 1972, 1973) and Keeley and Wadill (1971) found that neither allatectomy nor corpora allata extract affected succinate oxidation in isolated mitochondria from adult Blaberus discoidalis fat body.

Interpretations of previous studies are difficult because the adult tissues studied may not be targets of JH action, JH degradation may occur in crude tissue preparations and the active principle in crude hormone preparations may not be JH. Early studies on the effects of corpora allata or juvenile hormone indicated that JH stimulation of oxygen consumption in adult insects was due to the stimulation of ovarian development. Since that time investigators have placed their research emphasis on either male or ovariectomized female adult insect, thereby avoiding tissues which are sensitive to juvenile hormone. The overall result of this emphasis was that JH was tested on mitochondria from tissues or insects in which no function for JH is known. The exception to this is the research on diapausing adult L. decemlineata in which diapause appears to be a result of deficiency in JH secretion. Recent work by Silhacek and Kohl (unpublished observations) indicates that stimulation of succinate dehydrogenase is dependent on larval age. Their experiments

demonstrated that the ability of JH to stimulate succinate dehydrogenase is lost in older larvae. These results emphasize the need to study juvenile hormone effects in tissues which are responsive to JH.

The present study utilized a twice-washed mitochondrial preparation isolated from larval tissues which are responsive to juvenile hormone and a chemically defined juvenile hormone preparation. The titer of juvenile hormone in P. interpunctella has not been determined and it is not known whether the concentrations of JH used in this study are physiological; however, the JH concentrations used are similar to those determined in vivo in H. cecropia adults by Meyer et al. (1965, 1968) and Bieber et al. (1972).

The results of in vivo JH treatment on mitochondrial enzyme activities presented in this dissertation are inconclusive because the controls stopped feeding. However, the results do not disagree with the effects of in vitro JH treatment of isolated mitochondria.

The evidence presented here shows that heme synthesis, a topic having received little attention in insects, is immediately and therefore presumably directly stimulated by juvenile hormone. Since the occurrence of hemes is general in nature a similar biosynthetic pathway is assumed. Shemin and his coworkers (1952, 1953), Dresel and Falk (1956a, 1956b, 1956c), Granick and Mauzerall (1958), Mauzerall and Granick (1958) and Granick (1958) have

studied porphyrin and heme biosynthesis in duck and chicken erythrocytes. They found that the initial reaction in heme biosynthesis is associated with citrate cycle enzymes in mitochondria and involves an enzymatic condensation of succinyl CoA and glycine to form δ-aminolevulinic acid. The next series of reactions involving cytosol enzymes begins with the condensation of two molecules of δ-aminolevulinic acid to form porphobilinogen which condenses to form coproporphyrinogen, a cyclic tetrapyrrole. The third enzyme group located in the mitochondria converts coproporphyrinogen to protoporphyrin. The final enzyme in heme biosynthesis, ferrochelatase, is located on the inner surface of the mitochondrial inner membrane and participates in the insertion of iron into the protoporphyrin ring (Jones and Jones, 1969). Hamdy et al. (1973) have demonstrated incorporation of radiolabeled glycine and succinate into hemes in a tick, Dermacentor andersoni, lending support to the occurrence of this pathway in arthropods. The incorporation of ^{14}C-glycine into hemes in the present study indicates that the mechanism of heme biosynthesis in P. interpunctella is similar to that found in duck and chicken erythrocytes and in D. andersoni.

Two mechanisms are known for controlling the early reactions of heme biosynthesis. Granick (1966) proposed that induction of δ-aminolevulinic acid synthetase controls heme synthesis in chickens by resulting in increased levels

of δ-aminolevulinic acid which disturbs the steady-state equilibrium to favor increased heme synthesis. Granick (1966) demonstrated that δ-aminolevulinic acid synthetase is inducible by several substances. Muthukrishnan et al. (1972) have shown that a mold, Neurospora crassa, the second enzyme in heme biosynthesis, δ-aminolevulinic acid dehydrase, is inducable by iron. Its induction results in increased heme biosynthesis. A further level of control in the heme biosynthetic pathway involves end-product feedback inhibition. Muthukrishnan et al. (1972) and Burnham and Lascelles (1962) have demonstrated that δ-aminolevulinic acid dehydrase is inhibited by coproporphyrinogen III. Burnham and Lascelles (1962), Scholnick et al. (1971) and Whiting and Elliott (1972) demonstrated that δ-aminolevulinic acid synthetase is inhibited by hemin.

In P. interpunctella the increase in heme synthesis may be a result of the effects of JH on electron transport and succinate dehydrogenase. As a result of my experiments, I have proposed that inhibition of complex I of electron transport could affect the citrate cycle and related metabolism which would result in the production of succinyl-CoA by a conversion of pyruvate to malate and a reversal of the citrate cycle (Silhacek, Firstenberg and Kohl, in press). Malic enzyme, the enzyme which catalyzes the conversion of pyruvate to malate is active during the early part of the last larval instar in P. interpunctella

(Silhacek, unpublished data) when juvenile hormone titer is thought to be high. Another explanation is that JH could induce either δ-aminolevulinic acid synthetase or dehydrase resulting in increased heme synthesis. One or both of these mechanisms may contribute to the stimulation of heme synthesis in P. interpunctella larvae. High concentrations of the end-product, hematin, could exert a feedback inhibition on succinate dehydrogenase (Keilin and Hartree, 1947) and/or δ-aminolevulinic acid synthetase (Burnham and Lascelles, 1963).

Another possible function of the juvenile hormone inhibition in electron transport is to provide a reducing environment for conversion of ferric (Fe^{+3}) to ferrous ion (Fe^{+2}) by increasing intramitochondrial concentrations of NADH. Barnes et al. (1972) confirmed that the conversion of Fe^{+3} to Fe^{+2} is facilitated by NADH. Porra and Jones (1963) earlier determined that the enzyme, ferrochelatase, which participates in the insertion of iron into porphyrins, utilizes iron only in the ferrous form.

The synthesis of cytochromes depends on the coordination of heme and cytochrome apoprotein syntheses. My results indicate that cytochrome apoprotein synthesis is stimulated by JH. This stimulation depends upon an event associated with larval molting. Therefore, the stimulation of cytochrome apoprotein synthesis may be due in part to ecdysone. Indeed, Patel and Madhavan (1969) have

demonstrated a general stimulation of protein synthesis associated with ecdysone titer in imaginal wing disks of Samia cynthia ricini. The same study also indicated that JH had a stimulatory effect on protein synthesis. However, ecdysone is not the only factor that may be involved. Recently, Keeley and Wadill (1971) found a corpora cardiaca factor which stimulates cytochrome oxidase activity. Secretion of this corpora cardiaca factor could also be the event associated with the molt which stimulates cytochrome synthesis.

Soslau et al. (1971) found that cytochrome c synthesis was stimulated in developing adult Antheraea polyphemus following injection of pupae 24 hours earlier with δ-aminolevulinic acid. It was also found that actinomycin D an inhibitor of RNA synthesis, had no effect on the stimulation of cytochrome c synthesis indicating formation of the messenger RNA for cytochrome c apoprotein prior to the administration of the inhibitor.

An interesting correlation occurs in the case of diapause. DeWilde (1959) demonstrated that diapause was a result of JH deficiency in L. decemlineata. If diapause were caused by the lack of JH, one would expect diapausing insects to have low cytochrome concentrations. This is exactly what was found in induced diapausing Antheraea pernyi by Shappirio (1965) who also found that non-diapausing insects had little decrease in cytochrome content.

The current information (Kiese et al., 1958; Sinclair et al., 1967) indicates that hemes a and c are the product of a conversion from heme b. The data presented in this dissertation indicates that this mechanism is probably operative in P. interpunctella since the ratios of the individual hemes follow the same pattern in JH-fed larvae as in control insects. This result would be expected if heme b were the precursor of the other two hemes and the enzymes controlling the conversion were unaffected by JH.

It must be emphasized that my experiments do not preclude a direct interaction of juvenile hormone with the genome. However, the results do indicate that JH can affect development under certain conditions by altering metabolic reactions without interacting directly with the genome.

My results also indicate that the titer of juvenile hormone at the beginning of an instar can affect the metabolism of the insect within the same instar. This is contrary to the classical view which states that JH titer within an instar will control the morphology (Wigglesworth, 1940; Clever, 1963) and metabolism (Kroeger, 1968) of the insect in the following instar.

Since cytochrome levels are regulated by JH titer and cytochrome concentrations could be rate limiting in energy (ATP) production, juvenile hormone can control

cellular energy levels. It is therefore possible that JH, through the mechanisms presented in this dissertation, determines the levels of metabolic processes in developing insect tissues.

These experiments provide a basis for a hypothesis of metabolic control of insect development. I hope that the information provided can be useful in designing further experiments to elucidate the metabolic mechanisms of insect hormones during growth and development.

CONCLUSIONS

It can be concluded from the results presented in this dissertation that juvenile hormone directly inhibits the NADH dehydrogenase complex of the electron transport chain and stimulates succinate dehydrogenase activity in mitochondria from larvae of Plodia interpunctella. Juvenile hormone also stimulates the de novo synthesis of hemes. However, the incorporation of heme into cytochrome requires the intervention of the molt, suggesting the participation of ecdysone in cytochrome apoprotein synthesis as a second level of control.

The increase in heme synthesis may be a result of the inhibition of NAD-linked oxidation and stimulation of succinate dehydrogenase activity. The inhibition of NAD-linked oxidations could result in the production of succinyl-CoA from malate. The malate is generated by converting pyruvate to malate in the cytosol and reversing the citrate cycle (Silhacek, Firstenberg and Kohl, in press). An increase in succinyl-CoA pool size could result in an increase in heme synthesis by altering the steady state equilibrium of the reactions in the heme biosynthetic pathway to favor the formation of hemes. In

addition, the inhibition of NADH oxidation in electron transport could provide an intramitochondrial reducing environment favorable for the insertion of iron into porphyrin to form heme.

Juvenile hormone could exert its metabolic effects without an interaction with the genome. However, it is possible that cytochrome synthesis is mediated by ecdysone interacting with the genome. Juvenile hormone through its effects on mitochondrial metabolism could regulate the intensity of metabolism by controlling maximum levels of intracellular cytochromes. Therefore, I speculate that the main function of juvenile hormone is to establish the upper limit for mitochondrial oxidations in developing insect tissues.

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BIOGRAPHICAL SKETCH

Donald Elliott Firstenberg was born September 13, 1946 at New Brunswick, New Jersey. He is married to the former Ilene Lois Sager. They have one child, Michael Harrison Firstenberg.

Donald Elliott Firstenberg graduated from Scotch Plains-Fanwood High School in June, 1964. In May, 1968, he received the degree of Bachelor of Science with a major in biochemistry from Rutgers University. In September, 1968, he enrolled in the Graduate School of the University of Florida. From January, 1969, until December, 1974, he worked as a graduate assistant in the Department of Entomology and Nematology while pursuing his work toward the degrees of Master of Science and Doctor of Philosophy. In August, 1971, he received the degree of Master of Science.

Donald Elliot Firstenberg is a member of the Entomological Society of America, The American Chemical Society and Phi Sigma National Biological Honorary Society.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Basic Biology Research Laboratory

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James L. Nation, Co-Chairman
Professor of Entomology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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