Juvenile Hormone Regulation of Oxidative Metabolism in Isolated Insect Mitochondria

During the growth and development of insects, characteristic changes occur in the quantity[1], morphology[2–4], phospholipid composition[1], and oxidative activities[1,2,5] of mitochondria. Some investigators have linked these changes in mitochondrial metabolism with endocrine control. In an early study, DE WILDE and STEGWEE[6] demonstrated that corpora allata, endocrine glands which produce juvenile hormone (JH), exerted a direct effect on the respiration of *Leptinotarsa decemlineata*. A subsequent study[7] revealed that a JH-active extract from *Cecropia* stimulated succinate oxidation in isolated mitochondria from *Leptinotarsa*. CLARKE and BALDWIN[8] 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*[9] and *Blaberus discoidalis*[10] were found to be similar to those of normal insects. The recent identification and synthesis of JH permitted us to test directly the effects of JH on isolated insect mitochondria in a defined medium. In this paper, we report that JH not only stimulated succinate oxidation but also inhibited NAD-linked oxidations in isolated insect mitochondria.

Table I. Effect of juvenile hormone on the rates of substrate oxidation by isolated mitochondria from *Plodia interpunctella*

Substrate	Additions	$Q_{O_2}(N)$	RC
Pyruvate-malate	None	335.6	5.27
	JH[a]	11.5	0.74
Pyruvate-glutamate	None	303.2	3.95
	JH	20.6	1.00
Malate	None	55.7	1.65
	JH	7.6	0.91
α-Ketoglutarate	None	100.0	2.15
	JH	23.2	0.91
Glutamate	None	64.1	2.23
	JH	10.2	1.34
Succinate	None	121.4	1.23
	JH	160.8	1.20
α-Glycerophosphate	None	314.4	1.68
	JH	295.7	1.07
Ascorbate	None	69.9	0.99
	JH	72.0	0.93

[a] JH concentration during incubation = $1.77 \times 10^{-4} M$.

Table II. Effect of inhibitors and aging on NADH oxidation by mitochondria from *Plodia interpunctella*

Mitochondrial aging time (min)	Inhibitor	$Q_{O_2}(N)$	RC
0	None	107.2	0.99
30	None	288.0	1.04
45	None	414.5	0.96
60	None	448.6	1.00
0	Oligomycin[a]	31.1	0.75
45	Oligomycin	315.0	0.95
0	JH[b]	55.9	0.84
45	JH	120.7	1.07
45	Oligomycin + JH	43.3	–
45	Rotenone[c]	21.5	0.93

[a] Oligomycin concentration during incubation = $4.10 \times 10^{-5} M$. [b] JH concentration during incubation = $1.77 \times 10^{-4} M$. [c] Rotenone concentration during incubation = $6.25 \times 10^{-8} M$.

Materials and Methods. The test insects were larvae of the Indian meal moth, *Plodia interpunctella* (Hübner), reared at the standard conditions previously described[11]. A 5% homogenate of actively feeding last instar larvae (6–8 mg average weight) was prepared in 0.5 *M* recrystallized mannitol containing 10^{-2} *M* triethanolamine (TEA), 10^{-3} *M* EDTA, and sufficient HCl to give a pH of 7.4[12]. Twice-washed mitochondria were isolated by differential centrifugation (defined as those particles sedimenting during 10 min at forces between 500 and $6000 \times g$) and resuspended in a volume equal to the original tissue weight in a medium consisting of 0.45 *M* mannitol, 10^{-3} *M* $MgSO_4$, 6×10^{-3} *M* ATP (pH 7.4 with TEA) and 5 mg/ml bovine serum albumin (BSA). All manipulations during isolation were conducted at 0–4 °C.

The mitochondrial oxidative activities were determined on a Gilson oxygraph equipped with a vibrating platinum electrode and a jacketed cell maintained at 30 °C. The basic incubation medium placed in the cell contained 600 μmoles recrystallized mannitol, 15 μmoles $MgSO_4$, 6 μmoles ADP and 0.25 mg cytochrome c in 1.55 ml H_2O. To this medium was added 0.1 ml of mitochondrial suspension and either 0.5 ml of BSA (25 mg/ml) or 0.5 ml of BSA-JH [BSA containing 250 μg *Cecropia* JH[12] (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 1 μl of JH solution (10 mg JH/ml of acetone) per mg of dry bovine serum albumin, drying under nitrogen, and making up to volume with H_2O. Other concentrations of JH in the cell were obtained by substituting equivalent portions of BSA for BSA-JH.

After a 5-min preincubation period, 30 μmoles of each substrate being tested in a total volume of 0.2 ml were mixed into 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 min, then 50 μmoles of inorganic phosphate (P_i) in 0.05 ml (pH 7.8 with TEA) was added, and a second rate of oxygen consumption was measured. Respiratory control (RC) values were calculated by dividing the rate of O_2 consumption before P_i addition into

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the rate of oxygen consumption after P_i addition. Mitochondrial oxidative activity is expressed as μatoms of O_2 consumed per h per mg mitochondrial nitrogen [Q_{O_2}(N)]. All determinations were replicated twice with different mitochondrial preparations. Mitochondrial nitrogen was determined by micro-Nesslerization[13].

Results and discussion. Results of these experiments (Table I) showed that those substrate systems which have NAD-linked oxidations (α-ketoglutarate, glutamate, malate, pyruvate-malate, and pyruvate-glutamate) were strongly inhibited by JH. Those substrate oxidations that enter the respiratory chain on the oxygen side of NADH dehydrogenase at coenzyme Q (succinate, α-glycerophosphate) or at cytochrome C (ascorbate) were not inhibited by JH. Since JH inhibited the oxidation of only the NAD-linked substrates, it was apparently inhibiting the transfer of electrons in the region between NAD-linked substrate oxidations and ubiquinone of the respiratory chain.

For further studies of the mechanism of JH inhibition, it was necessary to minimize substrate permeability as a variable. This was accomplished by preincubating the mitochondrial suspension at 30 °C for 45 min and then storing on ice until needed. These aged mitochondria oxidized NADH at a considerably faster rate than did fresh mitochondria (Table II). However, pyruvate-malate, but not succinate, oxidation was almost completely lost during the 45-min preincubation period. These observations indicated that aging at 30 °C altered mitochondrial permeability so that NADH entered more readily but also enabled some cofactors (e.g., those for pyruvate-malate oxidation) to be lost[14].

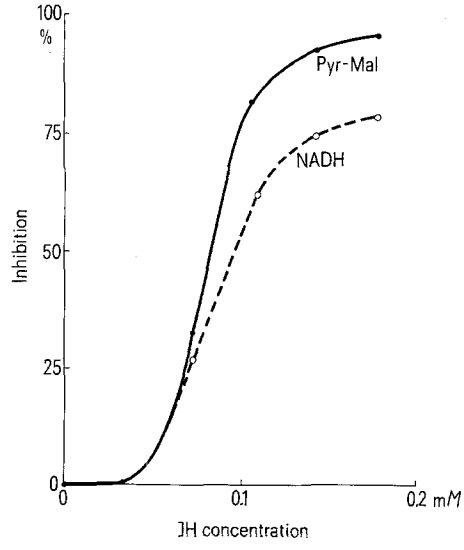
Although the aged mitochondria showed no respiratory control (RC = 1.0) with NADH as substrate, some coupling of electron transport and oxidative phosphorylation was indicated by the inhibition of respiration that occurred when oligomycin (specifically inhibits oxidative phosphorylation[15]) was added (Table II). When JH was added to aged mitochondria, either in the presence or absence of oligomycin, NADH oxidation was inhibited to the same degree as was observed with pyruvate-malate oxidation by fresh mitochondria. These results clearly indicated that JH was inhibiting electron transport between NADH and ubiquinone and eliminated the possibilities that JH was acting by altering permeability of the mitochondrial membrane to NAD-linked substrates or by inhibiting coupled phosphorylation in the NADH region of the respiratory chain.

More precise localization of JH inhibition was demonstrated in an experiment in which ferricyanide was used as the electron acceptor[16]. For this assay, 0.10-ml volumes of 10^{-3} *M* antimycin A, 0.5 *M* P_i, 5×10^{-2} *M* $K_3Fe(CN)_6$, 0.04 *M* NADH and aged mitochondria (ca. 0.1 mg N) plus 0.50 ml of either BSA or BSA-JH were added to 1.55 ml of basic incubation medium. The rate of ferricyanide reduction at 30 °C was monitored at 420 nm on a Gilford recording spectrophotometer. The results showed that the rate of ferricyanide reduction was not altered by substituting BSA-JH for BSA (742.6 vs. 762.8 μmoles $Fe(CN)_6^{-3}$ reduced/h per mg mitochondrial N, respectively), indicating that NADH dehydrogenase was not inhibited by JH. Therefore, it was concluded that JH inhibits electron transport in the nonheme iron region between NADH dehydrogenase and ubiquinone. Whether or not the mechanism of JH inhibition is similar to that of rotenone, amytal, deoxycortocisterone, diethyl stilbestrol, progesterone, and piercidin A, which have been reported to inhibit electron transport in the same region[15,17] will require further study.

With pyruvate-malate as substrate, respiratory activity of fresh mitochondria was maximally inhibited by a JH concentration of ca. 2×10^{-4} *M* and minimally inhibited by a concentration of ca. 10^{-5} *M*. A similar range of effective JH concentrations was found when NADH was oxidized by aged mitochondria (Figure). A plot of substrate concentration divided by reaction rate vs. substrate concentration at different JH concentrations revealed that the NADH oxidation was noncompetitively inhibited by JH (K_i = 0.11 m*M*).

Also, succinate oxidation by fresh mitochondria was stimulated by the addition of JH (Table I) although α-glycerophosphate and ascorbate oxidation were not. It appeared that this JH effect did not involve the electron transport chain but was only associated with the succinic dehydrogenase step of the oxidation. Experiments in progress indicate that JH does not stimulate succinate oxidation indirectly by inhibiting the NAD-linked formation of oxaloacetate (an inhibitor of succinic dehydrogenase). Further elucidation of the actual site at which JH affects succinate oxidation is currently under investigation.

Our data conclusively demonstrate that isolated *Plodia* mitochondria respond to changes in JH concentration. The concentrations of JH which are effective are of the same order of magnitude as the JH concentrations found in vivo in the adults of the *Cecropia* moth[18–20]. We do not know if similar concentrations of JH occur in the larvae of *Plodia*, nor do we know the concentrations of JH that are accessible to mitochondria in vivo. Therefore, the



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physiological significance of these JH effects on mitochondrial metabolism cannot be fully assessed until further studies relate the observations in vitro to processes in vivo[21].

Zusammenfassung. Nachweis, dass das Juvenilhormon in isolierten Mitochondrien der Getreidemotte *Plodia interpunctella* die NAD-gekoppelte Oxydation hemmt und die Succinat-Oxydation stimulieren kann. JH scheint auf die Nichthaem-Eisenkomponente der Atmungskette einzuwirken.

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Finite Growth Span of Mouse Mammary Gland Serially Propagated in vivo

In several recent reports[1-4] the assertion was made that normal mouse mammary gland has a limited capacity for growth when repeatedly isografted into young hosts. This finding has attracted interest because it appears to furnish a useful in vivo comparison to the cell culture findings of HAYFLICK[5,6] and others[7], who report that non-transformed fibroblasts also display a limited number of potential cell doublings. The mammary transplant technique has thus become a useful model system for the study of cellular aging, when aging is defined in terms of a time-related decline in growth rate.

This reported inability of mammary tissue to grow without limit has been disputed[8], most recently in a 1970 report by HOSHINO[9]: In these studies HOSHINO measured the survival rate of grafts, and scored as successes any transplants that were able to reconstruct identifiable mammary outgrowths. Using this criterion of regeneration rather than growth, as used by DANIEL et al.[1-4], HOSHINO found that 2 of his transplant lines survived for nearly 4 years, a period well beyond the ordinary life span of the mice used by him. He concluded on the basis of these and similar experiments that the life span of mouse mammary gland is potentially indefinite[8,9]. By extension it could be argued that the use of the mammary transplant system as a model of cellular aging is inappropriate.

In the present paper I submit results indicating that this contradiction is more apparent than real, and that the data of both investigators are mutually consistent when differences in terminology and experimental design are taken into account.

Materials and methods. Mice of the Balb/cCrgl strain were used. Female hosts 3 weeks of age were prepared for transplantation by excising the small mammary rudiment from each inguinal (No. 4) mammary fat pad[10]. This operation makes available 2 mammary gland-free fat pads which provide a natural site for the growth of mammary transplants. Primary implants (consisting of 0.5 mm pieces of either primary or secondary duct) were taken from the No. 3 mammary fat pad of a single 10 week virgin. In the primary transplant, 48 grafts were made into 24 hosts; these animals were then randomly distributed into 2 experimental groups.

At each passage all hosts from the previous transplant received a single i.p. injection of 0.5 ml of a 0.5% suspension of trypan blue, which aids in visualizing the mammary outgrowths[11]. All previously transplanted mice were anesthetized, their inguinal fat pads exposed, and the extent of mammary outgrowth in each fat pad estimated. The most vigorously growing gland was always selected for subsequent propagation, and 24 pieces 0.5 mm in size were removed and transplanted into the cleared fat pads of hosts for the next transplant generation. At the end of each passage, all transplanted glands and a sample of host gland were removed, fixed, extracted in acetone, stained with hematoxylin, dehydrated in alcohols to xylene, and stored in methyl salicylate. In each successful transplant the extent of mammary outgrowth was measured and for each generation the mean percent fat pad filled was calculated; this mean was used as an indicator of growth. These methods have been described in detail elsewhere[3].

Results. The experiment was designed to permit study of the potential growth span of mammary tissue by choosing conditions of serial passage which either did or did not allow unrestricted growth to take place. This was accomplished by making use of the following facts regarding mammary morphogenesis. A transplant of young mammary gland placed in the center of the cleared fat pad regenerates recognizable gland within about 2 weeks, and grows to fill the available fat within 2 to 3 months[4]. Growth ceases when the elongating ducts reach the limits of the fat pads, and unless the gland is again transplanted and more vacant fat is made available for growth, the gland will, in the absence of pregnancy, remain mitotically inactive for the life of the host.

It is therefore possible to control the extent of cell proliferation in each transplant line by varying the duration of the transplant interval. In the present experiment two intervals were used. In one subline transplants were made at 3 month intervals, so that when the outgrowth filled the fat pad it was again transplanted and vacant fat was again available for continued growth. In the second subline a 12 month transplant interval was selected, in which growth took place only at the beginning of each generation. For most of the 12 month period the gland in this subline was mitotically quiescent, although the cells remained metabolically

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