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Sesquiterpene juvenile hormones: novel uncouplers of oxidative phosphorylation

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SUMMARY: Several natural acyclic sesquiterpenes with capacity for insect growth regulation have been shown to uncouple oxidative phosphorylation in mouse-liver mitochondria. These agents stimulate succinate oxidation, reverse oligomycin-inhibited state 3 respiration, activate ATP-hydrolysis, induce loss of respiratory control and abolish ADP/O ratio. Permeability of the inner membrane to potassium, sodium, ammonium and chloride ions as well as to protons is also enhanced. Since the structure of these agents precludes protonophoric activity, the possible mechanism of uncoupling by these juvenile hormones is discussed.

INTRODUCTION: Sesquiterpenes constitute the largest class of terpenoid natural products. Many members of this class are biologically active in plants [1,2] and in lower animals, particularly in insects, where they constitute two important groups of chemical messengers for growth regulation (e.g. juvenile hormones) and for communication (e.g. pheromones) in defence, alarm, aggregation, feeding and sex [3,4].

In spite of the tremendous effort that has been invested in synthesis of sesquiterpenes with juvenile hormone activity [5] aimed mainly at producing "third generation" insecticides [6] very little is known about the site and mode of action of either the natural or synthetic sesquiterpenes. While some progress has been made in relating their effects to protein and nucleic acid synthesis [7] , the possibility of their use as probes of cellular metabolism has been virtually disregarded. That this aspect is worthy of further pursuit is evident from reports that certain di- and sesquiterpene lactones inhibit glycolytic enzymes [8] as well as state 4 and state 3 respiration of Ehrlich ascites cells [9] . To our knowledge, no studies are available on the effect of sesquiterpenes on mitochondrial function. The present study reports on the uncoupling properties of several acyclic natural and synthetic sesquiterpenes

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with significant capacity for insect growth regulation. Such data may also provide a basis for new strategies for insect control.

METHODS AND MATERIALS: Mouse-liver mitochondria were isolated according to Hogeboom [10] as described by Myers and Slater [11]. Protein was determined by the method of Lowry et al [12] using bovine serum album as standard.

Oxygen consumption was measured polarographically using an oxygen electrode as described elsewhere [13] at 23° in a 3 ml-medium consisting of 200 mM mannitol, 83 mM sucrose, 2 mM KCl, 3 mM $MgCl_2$, 15 mM phosphate pH 7.4 and 20 mM Tris-HCl buffer pH 7.4.

ATPase activity was determined by measuring the pH change of the reaction medium at 23° using a Beckman combination electrode (No. GK 2321C) connected to a Radiometer pH meter (model 22) and a 10 mV Varian recorder. The 6 ml - reaction mixture consisted of 5 mM glycylglycine buffer pH 7.2, 1 mM ATP pH 7.2 and 156 mM KCl.

Swelling of mitochondria in iso-osmolar salt solutions was determined by monitoring optical density changes at 520 nm according to Chappell and Crofts [14] using a Gilford 2400 spectrophotometer. The 3 ml-reaction mixture consisted of 10 mM triethanolamine-HCl buffer pH 7.4, 150 mM ammonium, sodium or potassium chloride, 3 µg rotenone and 0.065 µg antimycin.

The permeability to protons was measured using the $H^{\pm}$ pulse technique [15]. Anaerobiosis of the reaction mixture was achieved by flushing with argon at 23° for 15 - 30 min. All measurements were made under a stream of argon. Mitochondria were rendered anaerobic by preincubation for 10 min at 23° in the reaction mixture consisting of 125 mM KCl, 33 mM sucrose and 1.5 mM Tris-HCl buffer pH 7.2 and the juvenile hormone. The pH change of the reaction medium was initiated by a pulse of HCl.

The respiratory control ratio (RCR) and ADP/O ratio were determined according to Estabrook [16].

3,11-dimethyl-10-epoxy-7-ethyl-2,6-tridecadienoic acid methyl ester (JH-I), 10-epoxy-3,7,11-trimethyl-trans, trans-2,6-tridecadienoic acid methyl ester (JH-II) and 10-epoxy-3,7,11-trimethyl-2,6-trans trans-dodecadienoic acid methyl ester (JH-III), were obtained from Calbiochem Corp. Isopropyl (2E, 4E)-11-methoxy-2,4-dodecadienoate (methoprene) was obtained from Dr. D. A. Schooley, Zoecon Corp., Palo Alto, Calif. 6,7-epoxy-3-ethyl-1-(p-ethylphenoxy)-7-methyl-nonane (epofenonane) was obtained from Hoffmann-La Roche & Co. Switzerland. Carbonyl cyanide m-chlorophenylhydrazone (m-Cl-CCP) was provided by Dr. R. G. Heytler, Central Research Department, E. I. DuPont de Nemours and Co., Wilmington, Delaware. All other reagents including 2,4-dinitrophenol (DNP) were of highest purity from commerical sources. Only fresh ethanolic solutions of sesquiterpenes were used and were protected from light to avoid photo-decomposition.

RESULTS: Figure 1 shows the effect of JH-I on respiration of mouse-liver mitochondria using succinate as substrate. JH-I released the oligomycin-inhibited state 3 respiration completely at about 400 nmoles/mg protein (trace A) and stimulated state 4 respiration (trace B) but not to the maximum extent as that obtained with ADP (state 3) or DNP. Release of respiration by JH-I at suboptimal concentrations occurred after a lag period (trace B), the length of which was dependent on the concentration. High concentrations of JH-I caused a transient stimulation of state 4 respiration followed by an inhibition

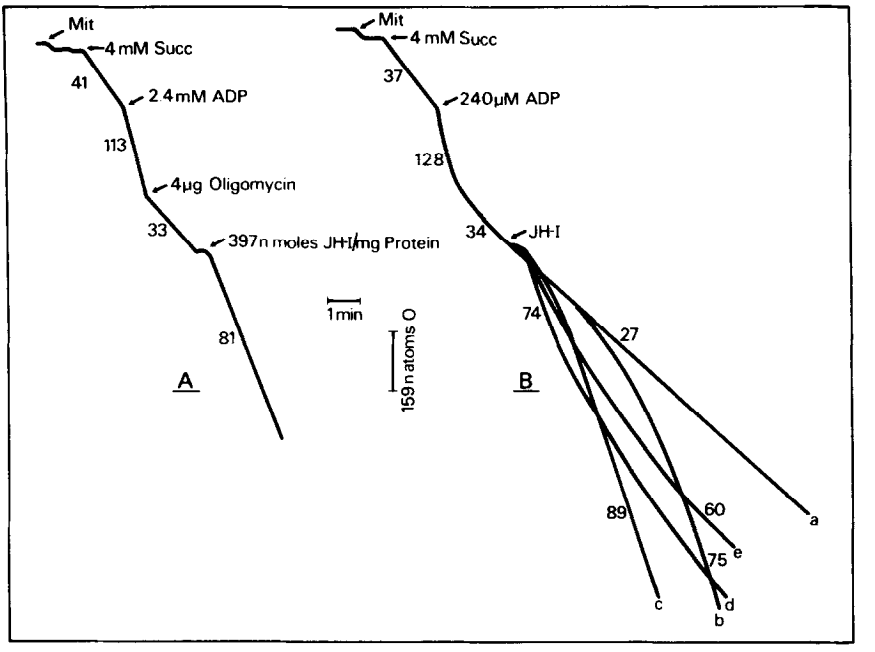

Figure 1 The effect of juvenile hormones on mitochondrial respiration using succinate as substrate. Trace A - reversal of oligomycin-inhibited state 3 respiration by JH-I. Trace B - stimulation of state 4 respiration by JH-I; a - 99 nmoles/mg protein; b - 199 nmoles/mg protein; c- 298 nmoles/mg protein; d - 596 nmoles/mg protein; e - 794 nmoles/mg protein. The numbers under the lines are respiration rates in natoms oxygen/min/mg protein. Rotenone (0.5 µg) and mitochondria (about 3.3 mg protein) were present in total volume of 3.0 ml.

(trace B).

As can be seen in Figure 2 the maximum stimulation of state 4 respiration occurred in the presence of 400, 450 and about 500 nmoles/mg protein of JH-I, -II and -III respectively. Like other uncouplers [17] higher concentrations of juvenile hormones inhibited the respiration. The synthetic sesquiterpenes with juvenile hormone activity (methoprene and epofenonane) were strikingly less effective by at least one order of magnitude (Figure 2).

The effect of these sesquiterpenes was dependent on the protein concentration (not shown) suggesting that they bind to the inner mitochondrial membrane. By contrast, the effect of DNP, used as a standard was independent of the protein concentration (see however [18]).

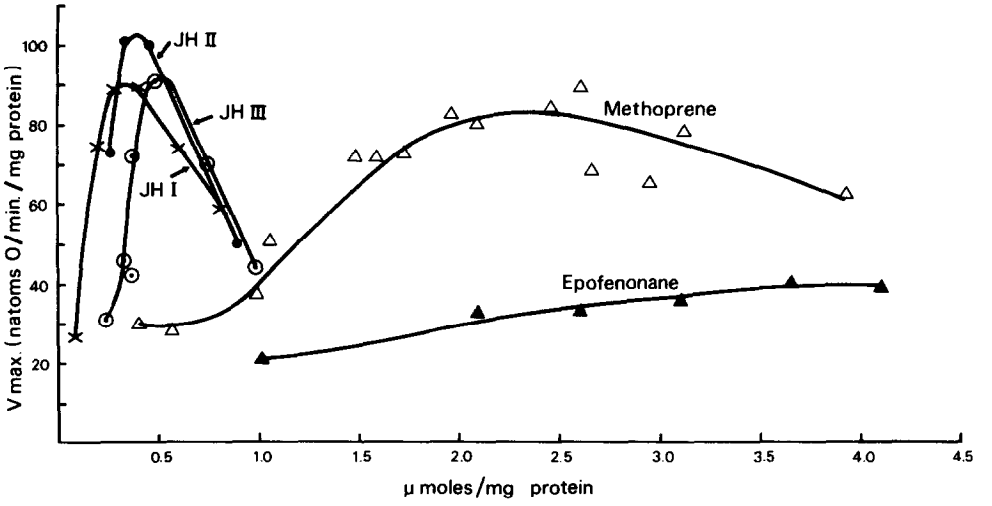

Figure 2 Titration of state 4 respiration with natural and synthetic juvenile hormones. Conditions were exactly as in Figure 1, trace B.

Since this pattern of activity resembled that observed with classical uncouplers [19-22] , the effect of JH-I on the ADP/O and RCR was measured. The ADP/O ratio for succinate oxidation was reduced from 1.24 to 0.38 and the RCR was reduced from 5.0 to 1.4 by 300 nmoles JH-I/mg protein. JH-II and JH-III produced similar effects in this concentration range.

The data in Figure 3 provide further evidence for the uncoupling activity of JH-I, -II and -III. Maximum activation of ATP'ase by the juvenile hormones was obtained at concentrations similar to those enhancing state 4 respiration. The activation at suboptimal concentrations was also preceded by a lag period (not shown) which disappeared as the concentration was increased. The juvenile hormone-activated hydrolysis of ATP was inhibited by oligomycin as has been observed for other uncouplers [23] .

Cunarro and Weiner [24] have reported a correlation between the ability of compounds to uncouple and their ability to promote swelling of mitochondria in iso-osmolar sodium chloride. They have suggested that this is evidence for the protonophoric nature of uncouplers. Table I compares the ability of JH-III, DNP and m-Cl-CCP to induce mitochondrial swelling in iso-osmolar ammonium chloride, sodium chloride and potassium chloride

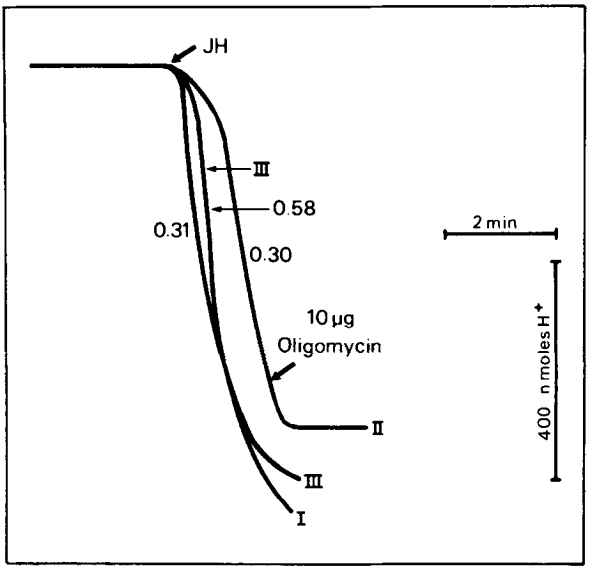

Figure 3 Effect of JH-I, -II and -III on the mitochondrial ATP'ase activity. ATP'ase activity was measured by monitoring pH changes (see methods and materials) of the reaction medium. Total volume was 6.0 ml and mitochondrial protein was about 13 mg. The numbers are concentration of juvenile hormones in nmoles/mg protein. The concentration of H^+ in the medium was standardized with HCl.

Table I Mitochondrial swelling in ammonium chloride, sodium chloride and potassium chloride.

Additions	Rate of swelling (OD units/min) in		
	NH_4Cl	NaCl	KCl
none	0.008	0.002	0.007
DNP (500 µM)	0.160	0.047	0.007
m-Cl-CCP (1.5 µM)	0.225	0.047	0.010
JH-III (1.7 µmoles/mg protein)	0.176	0.182	0.065

Mitochondria (about 0.70 mg protein) were suspended in 3.0 ml of reaction mixture containing the appropriate iso-osmolar salt solution (see methods and materials). Light scattering changes were begun immediately after addition of appropriate swelling agent.

solutions in the absence of energy. Both classical uncouplers induce swelling in ammonium chloride but not potassium chloride solutions suggesting that even though the protonated uncouplers induce an electrogenic movement of Cl^- the inner membrane of mouse-liver mitochondria remains relatively imper-

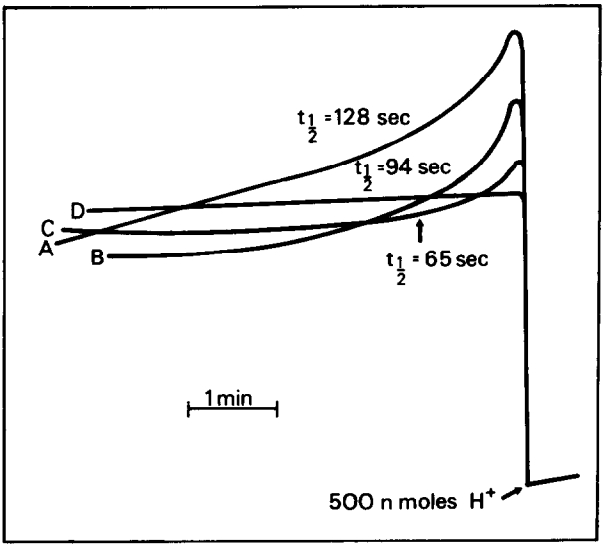

Figure 4 Effect of JH-III on proton permeability of mitochondria. The pH of the reaction medium containing untreated (A) or JH-III treated mitochondria (B, C, D) was monitored (see methods and materials) after addition of 500 nmoles HCl (arrow). The $t_{1/2}$ was calculated according to [15]; (B) - 125 nmoles/mg protein; (C) - 172 nmoles/mg protein; (D) - 188 nmoles/mg protein.

meable to K^+. By contrast JH-III induced swelling in the three salt solutions suggesting the mechanism of swelling is basically different from that of DNP or m-Cl-CCP.

JH-III also enhanced the permeability of the inner membrane to protons. The data in Figure 4 show that addition of HCl to an anaerobic suspension of mitochondria results in an acidification of the external medium followed by a decay of the external pH suggesting titration of the matrix buffer. The half-equilibration of protons ($t_{1/2}$) across the inner membrane of untreated mitochondria was 128 sec. This was decreased to 94 sec and to 65 sec following treatment with 125 and 172 nmoles JH-III per mg. protein respectively. Virtually instantaneous equilibration was achieved by treatment with 188 nmoles/mg protein.

DISCUSSION: The loss of respiratory control, the aboliton of the ADP/O ratio, the stimulation of succinate oxidation, the reversal of oligomycin-inhibited state 3 respiration and the oligomycin-sensitive stimulation of ATP hydrolysis

by the natural insect growth regulators indicates that these sesquiterpene compounds are uncouplers of oxidative phosphorylation.

It is generally believed that uncouplers act as protonophoric agents promoting the transfer of protons across the inner membrane thereby collapsing the proton gradient [25]. However the structure of these acyclic sesquiterpenes precludes the possibility of their being protonophoric agents. To our knowledge this is the first report that sesquiterpenes are a novel class of compounds sharing two apparently incompatible properties of being uncouplers and non-protonophores.

It is well known however that sesquiterpenes are potent alkylating agents. Their hydrophobic nature would increase their ability to penetrate the membrane and hence alkylation within the membrane. It is tempting to speculate that possible candidates for alkylation could be the membrane thiols which have been implicated in oxidative phosphorylation [26-29]. Lee *et al* [9] have shown the possibility of interaction between sesquiterpene lactones and thiols in *in vitro* studies employing cysteine and glutathione. Scott *et al* [30] have shown that mercurials, reacting with thiols, enhance the chloride permeability of the inner membrane which leads to swelling in potassium chloride and ammonium chloride as do the sesquiterpenes reported here. However by contrast with the uncoupling properties of these acyclic sesquiterpenes, the mercurials act as inhibitors of oxidative phosphorylation [31-34] . If the membrane thiols are involved in controlling the ionic traffic across the inner membrane, then their role in maintaining an ionic gradient, particularly the proton gradient, and hence coupling becomes of great interest. This will be the subject of another communication.

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