domethacin-treated artery. In other words, EC_{50} values were 3.3 mM and 3.9 mM, respectively. The results indicate that prostanoids are not involved in NaF-induced relaxation of the rabbit ear artery.

EDRF has been shown to behave in the same way as nitric oxide (NO), and methylene blue, L-NMMA and other agents have been used to inhibit the synthesis of EDRF (NO). Indeed, in the present experiment 10 μM methylene blue abolished the NaF-induced relaxation of the rabbit ear artery. L-NMMA also significantly depressed the NaF-induced relaxation, i.e., relaxation as a percentage of the maximum relaxation induced by 0.1 mM papaverine $90.4 \pm 5.2\%$ in the absence and $23.6 \pm 5.4\%$ in the presence of 0.1 mM L-NMMA ($N = 5$).

Data to date show that NaF affects not only stimulatory and inhibitory GTP-binding regulatory proteins (G-protein)[2] but also various other G-proteins, i.e., a G-protein coupling receptor activation to the breakdown of phosphatidylinositol 4,5-bisphosphate by a phosphodiesterase[14] and transducin[15]. Thus, Cushing et al.[11] have suggested that NaF-induced relaxation and contraction in the coronary artery may be G-protein-mediated, based on sensitivity to G-protein modulators, i.e., NaF can interact with a G-protein(s) and cause an increase in intracellular calcium concentration which leads to release of EDRFs. The suggestion is in accordance with the observation that the production of NO in the rat cerebellum is calcium-calmodulin-dependent[16]. Furthermore, Flavahan and Vanhoutte[17] have shown that NaF-induced endothelium-dependent relaxation of the canine coronary artery is mediated in part by activation of a pertussis toxin-sensitive G-protein.

It is concluded that NaF produces endothelium-dependent relaxation of the rabbit ear artery by releasing some relaxant substance, possibly an EDRF, from the endothelium. The process appears to be different from that in the coronary artery[11], and may not involve prostanoid.

1 Kramer, G. L., and Hardman, J. G., in: Handbook of Physiology, Section 2: The Cardiovascular System, vol. 2. Vascular Smooth Muscle, p. 179. Eds D. F. Bohr, A. P. Somlyo and H. V. Sparks Jr. American Physiological Society, Bethesda 1980.
2 Birnbaumer, L., Codina, J., Mattera, R., Cerione, R. A., Hildebrandt, J. D., Sunyer, T., Rojas, F. J., Caron, M. G., Lefkowitz, R. J., and Iyengar, R., in: Molecular Mechanisms of Transmembrane Signalling. Molecular Aspects of Cellular Regulation, vol. 4, p. 131. Eds P. Cohen and M. D. Houslay. Elsevier, Amsterdam 1985.
3 Tsuru, H., and Negita, S., Jap. J. Pharmac. *51* (1989) 385.
4 Furchgott, R. F., and Zawadzki, J. V., Nature *288* (1980) 373.
5 Moncada, S., Radomski, M. W., and Palmer, R. M., Biochem. Pharmac. *37* (1988) 2495.
6 Ignarro, L. J., and Kadowitz, P. J., A. Rev. Pharmac. Toxic. *25* (1985) 171.
7 Rall, T. W., and Sutherland, E. W., J. biol. Chem. *232* (1958) 1065.
8 Zeng, Y. Y., Benishin, C. G., and Pang, P. K. T., J. Pharmac. exp. Ther. *250* (1989) 343.
9 Jeremy, J. Y., and Dandona, P., Eur. J. Pharmac. *146 (1988)* 279.
10 Pirotton, S., Erneux, C., and Boeynaems, J. M., Biochem. biophys. Res. Commun. *147* (1987) 1113.
11 Cushing, D. J., Sabouni, M. H., Brown, G. L., and Mustafa, S. J., J. Pharmac. exp. Ther. *254* (1990) 28.
12 Griffith, S. G., Crowe, R., Lincoln, J., Haven, A. J., and Burnstock, G., Blood Vessels *19* (1982) 41.
13 Tsuru, H., Kohno, S., Iwata, M., and Shigei, T., J. Pharmac. exp. Ther. *243* (1987) 696.
14 Cockcroft, S., and Taylor, J. A., Biochem. J. *241* (1987) 409.
15 Bigay, J., Deterre, P., Pfister, C., and Chabre, M., FEBS Lett. *191* (1985) 181.
16 Bredt, D. S., and Snyder, S. H., Proc. natl Acad. Sci. USA *87* (1990) 682.
17 Flavahan, N. A., and Vanhoutte, P. M., Eur. J. Pharmac. *178* (1990) 121.

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Effects of methoprene and juvenile hormone on the oxidative metabolism of isolated mitochondria from flight muscle of *Locusta migratoria* L.

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Summary. In vitro applications of juvenile hormone III and a juvenile hormone analogue, methoprene, were made to mitochondria isolated from dorsal longitudinal flight muscles of adult *Locusta migratoria* L. Both compounds completely inhibited oxygen consumption at the highest concentrations used. At lower concentrations, state 3 respiration and respiratory control were reduced but the ADP/O ratio was largely unaffected.
Key words. Locusta migratoria; methoprene; juvenile hormone; juvenile hormone analogue; mitochondria; oxidative metabolism.

In recent years, there has been considerable interest shown in the use of juvenile hormone analogues (JHAs) as novel agents for pest insect control. However, the majority of studies have investigated the morphological and pesticidal effects of the JHAs, so that relatively little is known about the effects of JHAs at the subcellular

level. In a previous paper, we reported that topical application of a juvenile hormone analogue, methoprene, to newly moulted fifth instar *Locusta migratoria* disrupted the subsequent development of the insects' dorsal longitudinal flight muscles[1]. Mitochondria extracted from the flight muscles of adult locusts which had previously been treated with methoprene showed impaired metabolic activity compared to flight muscle mitochondria from normal adults; both respiratory control and oxidative phosphorylation being reduced. It was suggested that methoprene might have induced this effect by acting directly on the flight muscle mitochondria. Several studies have shown that in vitro applications of juvenile hormone (JH) to isolated insect mitochondria can alter their oxidative metabolism but the effects varied widely depending on the substrate used in the mitochondrial incubations (see review by Steele[2]). Chefurka[3] investigated the effects of juvenile hormones and JH analogues (methoprene and epofenonane) upon isolated mouse liver mitochondria. Using succinate as the respiratory substrate, both JHs and JHAs were found to cause uncoupling of oxidative phosphorylation so that all respiratory control was lost, apparently due to an increased permeability of the mitochondria to various ions.

The present study was carried out to investigate the in vitro effects of methoprene upon mitochondria isolated from locust flight muscles and to compare these with the effects of juvenile hormone and with the in vivo effects of methoprene reported previously[1].

Materials and methods

The insects used in the present study were 10–15-day-old adult *Locusta migratoria migratorioides* (R&F) reared under standard conditions described previously[1]. The method of mitochondrial extraction from locust dorsal longitudinal flight muscles was similar to that described by Chance and Hagihara[4], and Chappell and Hansford[5]. Flight muscles were dissected out from equal numbers of male and female insects, and pooled in 10 ml of ice-cold (0–4 °C) isolation medium (0.32 M sucrose, 1 mM EDTA and 5 mM Tris/HCl buffer, pH 7.3) in a glass homogenisation tube. Following the addition of proteinase (5 mg of Nagarse in 5 ml of isolation medium), the tissue was allowed to digest for 6 min at 0–4 °C, in which time 5–6 gentle passes of a teflon pestle were made by hand through the suspension. The suspension was filtered through 4 layers of muslin (previously boiled in distilled water and soaked in ice-cold isolation medium). The residue was washed with 5 ml of isolation medium and the muslin gently squeezed to expel the filtrate. The pooled filtrate was centrifuged at 4000 × g for 8 min at 0–4 °C and the supernatant discarded. The pellet was resuspended in 10 ml of ice-cold isolation medium, centrifuged as before and the supernatant again discarded. The final pellet was gently washed with 1–2 ml of fresh isolation medium to remove the 'fluffy' layer on top of it. This was discarded and the residual pellet was resuspended in a suitable volume of isolation medium to give a mitochondrial protein suspension of ca 10–30 mg/ml.

Mitochondrial oxygen consumption was measured polarographically at 30 °C using a Rank oxygen electrode. Following the introduction of 2 ml of reaction medium (154 mM KCl, 0.4 mg/ml bovine serum albumin, 10 mM Tris and 30 mM phosphate buffer, pH 7.3), 100–150 µl of mitochondrial suspension and 10 µl of substrate solution (1 M pyruvate + 1 M proline) into the reaction chamber of the oxygen electrode, various concentrations of methoprene or juvenile hormone III were added to the chamber in 5 µl of ethanol. In control determinations, 5 µl of absolute ethanol only was added instead. Proline was provided in addition to pyruvate since it has been suggested that proline may supply citric acid intermediates to isolated insect mitochondria for maximal oxidation of pyruvate[6]. Precisely 1 min after the addition of JH or JHA, 10 µl of 50 mM ADP solution (made up in 30 mM phosphate buffer, pH 6.8) was injected into the chamber and oxygen consumption was monitored with a chart recorder. The initial oxygen content of the reaction mixture was 0.43 µg atoms oxygen · ml^{-1} at 30 °C[7]. Determinations of oxygen uptake (state 3 and state 4 rates), respiratory control ratio (RCR) and ADP/O ratio were made according to the method of Chance and Williams[8,9]. Mitochondrial respiration was deemed not to be measurable when the mitochondria were incapable of displaying a steady rate of oxygen consumption. Mitochondrial protein was determined by the Folin's method of Lowry et al.[10].

Results

Four separate experiments, using independent mitochondrial extractions, were carried out and the results obtained are shown in table 1. Preliminary studies showed that the addition of up to 20 µl of absolute ethanol to the reaction mixture had no significant effect on mitochondrial oxygen consumption nor RCR and ADP/O ratios when compared with untreated mitochondria. Thus, under control conditions (i.e. in the presence of 5 µl of ethanol), mitochondria exhibited good respiratory control and coupling of oxygen consumption to ADP phosphorylation; the results comparing favourably with those from other studies[11,12]. However, some of the ADP/O values calculated in the present study were greater than the theoretical maximum of 3.0 (table 1). Similar results have been recorded elsewhere but no explanation for this phenomenon has yet been provided[12–14].

In contrast, both methoprene and JH had a disruptive effect on mitochondrial respiration which became more marked with increasing concentrations of JH or JHA per mg of mitochondrial protein. The results of the experiments are shown in table 1 and table 2, and the figure shows some typical oxygen electrode recordings from one experiment. At the highest concentrations of JH and JHA (ca 182 nmoles · mg $protein^{-1}$), respiration was rapidly inhibited; ADP stimulating the metabolic rate in

Table 1. Effects of methoprene and JH III on the oxidative metabolism of isolated flight muscle mitochondria

Expt.	Treatment	n	State 3 rate	State 4 rate	RCR	ADP/O
1.	Untreated	3	5.7 ± 0.5	0.8 ± 0.1	7.2 ± 0.4	3.0 ± 0.0
1.	20 µl ethanol	4	5.7 ± 0.7	0.8 ± 0.2	7.5 ± 0.7	3.2 ± 0.2
2.	Control	3	9.6 ± 0.3	1.1 ± 0.0	8.7 ± 0.3	2.7 ± 0.1
2.	60.2 nmoles JHA	2	9.2	1.1	8.8	2.8
2.	77.9 nmoles JHA	4	8.2 ± 0.1	1.8 ± 0.1	4.7 ± 0.4	2.8 ± 0.1
2.	97.3 nmoles JHA	4	8.5 ± 0.5	2.0 ± 0.2	4.4 ± 0.6	2.7 ± 0.1
2.	108.2 nmoles JHA	4	6.5 ± 1.2	2.4*[2]	3.5*[2]	2.4*[2]
2.	116.6 nmoles JHA	1	7.0	2.8	2.5	3.3
3.	Control	6	14.3 ± 0.8	2.3 ± 0.1	6.2 ± 0.3	2.3 ± 0.3
3.	182.0 nmoles JHA	3	4.9*[1]	**	**	**
3.	182.1 nmoles JH	3	**	**	**	**
4.	Control	3	8.1 ± 0.9	1.7 ± 0.2	4.7 ± 0.6	2.9 ± 0.3
4.	74.7 nmoles JHA	2	9.1	3.1	3.2	2.7
4.	44.6 nmoles JH	2	5.5	1.7	3.3	2.3
4.	59.7 nmoles JH	2	4.1	1.8	2.3	2.1
4.	74.7 nmoles JH	2	3.1	1.7	1.9	1.9

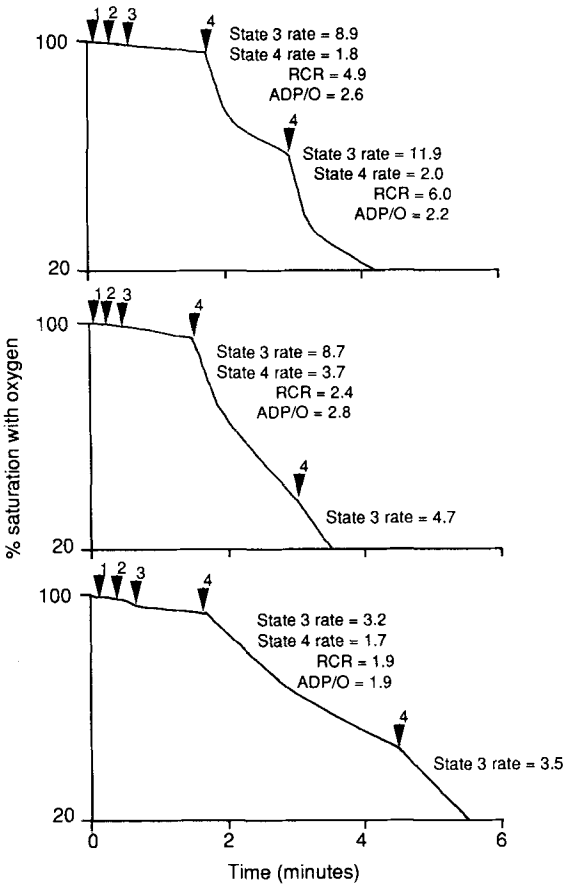
Measurements of the respiratory metabolism of isolated mitochondria are given as Means ± SEM. Means were calculated from the number (n) of determinations. Treatments are given as nmoles of JH or JHA · mg of mitochondrial protein^{-1}; state 3 and state 4 rates are in units of µg atoms of oxygen · mg of mitochondrial protein^{-1} h^{-1}; RCR values represent the mean ratios of state 3 to state 4 rates; ADP/O ratios are the numbers of µmoles of ADP esterified to ATP for each µg atom of oxygen consumed. *[n]Only n number of determinations gave measurable results. **None of the determinations gave a measurable result.

Table 2. Effects of methoprene and JH III on the oxidative metabolism of isolated flight muscle mitochondria (normalised results)

Treatment	State 3	State 4	RCR	ADP/O
Control	1.00	1.00	1.00	1.00
60.2 nmoles JHA	0.96	1.00	1.01	1.04
74.7 nmoles JHA	1.12	1.82	0.68	0.93
77.9 nmoles JHA	0.85	1.64	0.54	1.04
97.3 nmoles JHA	0.89	1.82	0.51	1.00
108.2 nmoles JHA	0.68	2.18	0.40	0.89
116.6 nmoles JHA	0.73	2.55	0.29	1.22
182.0 nmoles JHA	0.34	**	**	**
44.6 nmoles JH	0.68	1.00	0.70	0.79
59.7 nmoles JH	0.51	1.06	0.49	0.72
74.7 nmoles JH	0.38	1.00	0.40	0.66
182.1 nmoles JH	**	**	**	**

Measurements of the respiratory metabolism of isolated mitochondria are given as normalised results to allow easier comparison of results from separate experiments. State 3 and state 4 rates, RCR values and ADP/O ratios of experimental determinations were calculated as proportions of the corresponding control values. Treatments are given as nmoles of JH or JHA · mg of mitochondrial protein^{-1}. **None of the determinations gave a measurable result.

only one determination involving methoprene. At lower concentrations (ca 75–117 nmoles · mg protein^{-1}), methoprene tended to increase the state 4 rate, whereas it was either unaffected or slightly inhibited by JH III (ca 45–75 nmoles · mg protein^{-1}). The ADP/O ratio was unaffected by methoprene whereas it was slightly reduced by JH III, but in other respects the two treatments had similar effects, reducing state 3 rates and RCR values. In terms of molar activity, JH III was more potent than methoprene. However, it should be noted that the average values quoted in tables 1 and 2 are derived only from the determinations that gave measurable results and therefore underestimate the effects of the higher concentrations of the compounds, which caused a complete inhibition of respiration in some determinations.



Oxygen electrode recordings from Experiment 4 showing differences in mitochondrial respiration following the addition of (a) 5 µl of absolute ethanol, (b) 74.7 nmoles of methoprene · mg mitochondrial protein^{-1} and (c) 74.7 nmoles of JH III · mg mitochondrial protein^{-1}. State 3 and state 4 rates are in units of µg atoms of oxygen · mg mitochondrial protein^{-1} h^{-1}; RCR values represent the ratio of state 3 rate to state 4 rate; ADP/O ratios represent µmoles of ADP esterified to ATP for each µg atom of oxygen consumed. ↓[1] addition of 120 µl mitochondrial suspension; ↓[2]addition of pyruvate + proline; ↓[3] addition of JH or JHA in ethanol, or of ethanol alone; ↓[4] addition of ADP.

Discussion

Direct additions of methoprene and JH III to isolated mitochondria had very damaging effects on respiratory metabolism, with the higher concentrations of JH and JHA causing a rapid loss of mitochondrial respiration. The results are similar to effects of JH on mitochondrial activity reported by Firstenberg and Silhacek[15] using mitochondria extracted from whole body homogenates of larval *Plodia interpunctella*. They found that in vitro addition of JH only prevented the oxidation of substrate systems which entered the electron transport chain at NADH (e.g. pyruvate). Substrate oxidations which entered the chain at later points (e.g. succinate and α-glycerophosphate) were not inhibited by JH and succinate oxidation was actually stimulated by hormone treatment. They suggested that JH inhibited the oxidation of NAD-linked substrates by preventing the transfer of electrons between NADH and ubiquinone.

Where respiratory rates were measurable following the addition of methoprene in the present study (JHA concentrations of 75–117 nmoles · mg mitochondrial protein^{-1}), state 3 rates were reduced but state 4 rates were increased and this reduction of respiratory control suggests that methoprene may also have an uncoupling effect. This would be in agreement with Steele's[2] proposal that JH affected mitochondrial oxidative metabolism by causing disruption of mitochondrial membranes. This is supported by earlier studies which have shown that in vitro applications of JH and some JHAs to isolated insect cells[16] and insect cell membranes[17] increased membrane permeability and caused the appearance of lesions in the plasma membranes, apparently by interacting directly with membrane lipids[18]. An increase in mitochondrial membrane permeability could explain why JH stimulated succinate oxidation since insect mitochondria are normally impermeable to such intermediates of the citric acid cycle[13,19]. Disruption of the mitochondrial membranes could also explain the JH-mediated inhibition of oxidation of NAD-linked substrates since the components of the electron transport chain are embedded in the inner membrane of the mitochondrion[20] and any damage to the mitochondrial membranes could affect the passage of electrons between the carrier molecules. However, uncoupling of mitochondrial respiration would be expected to be largely masked in the present study by inhibition of NADH-ubiquinone electron transport and this might explain why JH seemed to have no stimulatory effect on state 4 rates.

Chefurka's[3] finding that methoprene caused uncoupling of oxidative phosphorylation in mouse liver mitochondria is compatible with Steele's[2] proposals since vertebrate mitochondria are readily permeable to succinate and succinate oxidation enters the electron transport after NADH[17]. Chefurka also found that juvenile hormones were far more effective than methoprene in disrupting mitochondrial respiration, similar to the present results. However, the concentrations that he required for maximal stimulation of state 4 respiration (ca 400–500 nmoles · mg mitochondrial protein^{-1} for the JHs and ca 2500 nmoles · mg protein^{-1} for methoprene), were much higher than those used in the present study, suggesting that insect mitochondria may be considerably more sensitive to such compounds than mammalian mitochondria.

The effects of in vitro treatments with methoprene upon mitochondrial oxidative metabolism were very similar to those we reported previously following in vivo applications of the JHA to fifth instar locusts[1], in which flight muscle mitochondria showed impaired state 3 respiration and respiratory control but with no significant change in ADP/O ratios or state 4 respiration. This raises the possibility that the mode of action of methoprene was similar in both cases, with the JHA acting directly on the flight muscle mitochondria after its topical application to locusts and presumably causing a loss of structural integrity of the inner mitochondrial membrane by penetrating the lipid bilayer.

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1 Cotton, G., and Anstee, J. H., J. Insect Physiol. *36* (1990) 959.
2 Steele, J. E., Adv. Insect Physiol. *12* (1976) 239.
3 Chefurka, W., Biochem. biophys. Res. Commun. *83* (1978) 571.
4 Chance, B., and Hagihara, B., in: Proceedings of the Fifth International Congress of Biochemistry, vol. 5, p. 3, Moscow 1961. Ed. E. C. Slater. Pergamon Press, Oxford 1963.
5 Chappell, J. B., and Hansford, R. G., in: Subcellular Components, Preparation and Fractionation, p. 77. Ed. G. D. Birnie. Butterworth & Co., London 1972.
6 Beenakkers, A. M. Th., Van der Horst, D. J., and Van Marrewijk, W. J., in: Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 10, p. 451. Eds G. A. Kerkut and L. I. Gilbert. Pergamon Press, Oxford 1985.
7 Davison, T. F., An investigation into the factors involved in acclimatization to temperature and death at high temperature in *Calliphora erythrocephala* (Meig.). Ph. D. Thesis, University of Durham 1970.
8 Chance, B., and Williams, G. R., Nature *175* (1955) 1120.
9 Chance, B., and Williams, G. R., Adv. Enzymol. *17* (1956) 65.
10 Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. biol. Chem. *193* (1951) 265.
11 Minks, A. K., Archs, Néerl. Zool. *17* (1967) 175.
12 Al-Robai, A. A. S., Studies on developmental changes in fine structure and metabolism in flight muscle of *Locusta migratoria* L. Ph. D. Thesis, University of Durham 1981.
13 Van den Bergh, S. G., and Slater, E. C., Biochem. J. *82* (1962) 362.
14 Tribe, M. A., and Ashurst, D. E., J. Cell Sci. *10* (1972) 443.
15 Firstenberg, D. E., and Silhacek, D. L., Experientia *29* (1973) 1420.
16 Cohen, E., and Gilbert, L. I., J. Insect Physiol. *18* (1972) 1061.
17 Baumann, G., J. Insect Physiol. *14* (1968) 1459.
18 Baumann, G., Nature *223* (1969) 316.
19 Van den Bergh, S. G., Biochem. J. *93* (1964) 128.
20 Lehninger, A. L., Principles of Biochemistry. Worth Publishers, New York 1982.

0014-4754/91/070705-04$1.50 + 0.20/0