that JH acts on the follicle cells through the activation of protein kinase C. Incubation of follicles with PDBU, which is known to activate protein kinase C[11,12], increases the patency index ($p > 0.001$). This effect was inhibited by the inclusion of ouabain in the medium ($p > 0.001$). Thus, PDBU, like JH, appears to stimulate patency via Na^+, K^+-ATPase. These facts taken together suggest the following scheme for the action of JH on the follicle cells of *Rhodnius*. JH binds to the receptor on the membrane and initiates a series of events which stimulate protein kinase C. This leads to the phosphorylation of one or more membrane proteins, including, possibly, the JH specific Na^+, K^+-ATPase, thereby increasing the activity of that enzyme, leading to a reduction in volume of the follicle cells and the appearance of spaces between them. Experiments are currently under way to test this hypothesis. A similar membrane protein mediated effect of JH involving protein kinase C has been reported in the male accessory gland of Drosophila[13].

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* Author for correspondence.

1 Engelmann, F., in: Endocrinology of insects, p. 259. Eds R. G. H. Downer and H. Laufer. Alan R. Liss Inc., New York 1983.
2 Pratt, G. E., and Davey, K. G., J. exp. Biol. *56* (1972) 201.
3 Davey, K. G., and Huebner, E., Can. J. Zool. *52* (1974) 1407.
4 Abu-Hakima, R., and Davey, K. G., J. Insect Physiol. *23* (1977) 913.
5 Abu-Hakima, R., and Davey, K. G., J. exp. Biol. *69* (1977) 33.
6 Abu-Hakima, R., and Davey, K. G., Insect Biochem. *9* (1979) 195.
7 Ilenchuk, T. T., and Davey, K. G., Insect Biochem. *12* (1982) 675.
8 Ilenchuk, T. T., and Davey, K. G., Can. J. Biochem. Cell Biol. *61* (1985) 286.
9 Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y., Biochemistry *23* (1984) 5036.
10 Kawamoto, S., and Hidaka, H., Biochem. biophys. Res. Commun. *125* (1984) 258.
11 Nishizuka, Y., Nature (London) *308* (1984) 693.
12 Berridge, M. J., Biochem. J. *220* (1984) 345.
13 Yamamoto, K., Chadarevian, A., and Pellegrini, M., Science *239* (1988) 916.

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Relationship between the absolute configuration and the biological activity of juvenile hormone III

H. Kindle, M. Winistörfer, B. Lanzrein and K. Mori[a]

University of Berne, Zoological Institute, Division of Animal Physiology, Erlachstrasse 9a, CH-3012 Berne (Switzerland), and [a]University of Tokyo, Department of Agricultural Chemistry, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113 (Japan)
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Summary. The activity of the pure 10R (= natural) and 10S enantiomers of juvenile hormone III (JH III) was determined in 3 different bioassays, and the relative binding affinity of the 2 enantiomers to the haemolymph JH-binding protein of the cockroach *Nauphoeta cinerea* was measured. In the *Galleria* wax test, a local morphogenetic assay, the 10R enantiomer was 5240 times more active than the 10S enantiomer, 1 *Galleria* unit corresponding to 0.42 pg of 10R-JH III as compared to 2.2 ng for 10S-JH III. In a systemic morphogenetic assay with the cockroach *Nauphoeta cinerea* 380 times less 10R enantiomer was necessary in order to induce detectable juvenilisation (58 ng 10R and 22 µg 10S) and in a systemic gonadotropic assay with *Nauphoeta cinerea* 255 times less 10R was needed to induce vitellogenin synthesis in 50% of the insects (6.7 ng 10R and 1710 ng 10S). In the JH-binding protein assay 10R-JH III had an affinity for the JH-binding protein (lipophorin) which was approximately 46 times higher than that of 10S-JH III.
Keywords. Juvenile hormone; natural enantiomer; juvenilising activity; vitellogenin induction; lipophorin; cockroach; *Galleria*.

Juvenile hormone III [methyl (2*E*,6*E*)-10,11-epoxy-3,7,11-trimethyl 2,6-dodecadienoate, JH III] plays a major role in regulating development and reproduction in many insect species. The absolute configuration of the natural hormone has been established as 10R[1]. Owing to difficulties in preparing the 10R and 10S enantiomers in a state of very high purity, their biological activity has not yet been determined. Nevertheless, it has been shown that the 10R enantiomer of JH III binds to haemolymph binding proteins with higher affinity than the racemate in *Manduca sexta*[2], *Locusta migratoria*[3] and *Leptinotarsa decemlineata*[4], and to cytosolic and nuclear receptors of the fat body in *Leucophaea maderae*[5]. In addition, in the *Galleria* wax test a 12-fold higher activity was observed for 10R-JH III than for 10S-JH III (both 92% pure)[2]. A method of synthesising the pure ($\approx$ 100% enantiomeric excess) enantiomers of JH III has recently been developed[6] and we report here on their biological activity. Using the waxmoth *Galleria mellonella* we carried out a local morphogenetic assay (*Galleria* wax test), and, using the cockroach *Nauphoeta cinerea*, a systemic morphogenetic (induction of juvenile characteristics) and a gonadotropic (induction of vitellogenin synthesis) assay. Furthermore we present data on the affinity of the two

enantiomers to the haemolymph JH binding protein of *Nauphoeta cinerea*.

Materials and methods. Nauphoeta cinerea was reared at 26 °C and *Galleria mellonella* at 30 °C. The *Galleria* wax test was carried out according to the method described [7]; 25 or 50 pupae were treated with each dilution and 1 *Galleria* unit (G.U.) is defined as the amount of JH per test insect which induces a positive response in 50% of the pupae treated. In order to measure the juvenilising activity of 10R-JH III and 10S-JH III in *Nauphoeta cinerea* the hormone was injected in 10 µl olive oil into the abdomen of 14-day-old female last stadium larvae. After the moult the juvenilising effect was quantified according to a score system [8] which we modified by including the evaluation of the abdominal pigmentation (adult-like = 0 points; pale, spots visible = 1 point; dark, spots barely visible = 2 points; larva-like = 3 points). With this modified score system a perfect extra larva is awarded 21 points: 6 points each are awarded for forewings and hindwings and 3 points each for pronotum, head and abdomen.

In order to measure the activity of 10R-JH III and 10S-JH III in the induction of vitellogenin synthesis in *Nauphoeta cinerea*, females were decapitated one day after adult ecdysis, i.e. before vitellogenesis sets in [9] and injected with 10R-JH III or 10S-JH III dissolved in 10 µl olive oil. Three days later haemolymph was collected as described [9] and analysed for the presence of vitellogenin by using an Ouchterlony agar diffusion test and a vitellogenin-specific antibody [10].

Binding studies were carried out with a slightly modified version of the polyethylene glycol (PEG) precipitation assay described by Kovalick and Koeppe [11]. When analysing haemolymph of adult females fractionated on a KBr gradient [12] we found that only the fraction containing lipophorin bound racemic JH III specifically (Lanzrein, unpublished results). In the present study we measured the competition for binding [^{3}H]-10R-JH III (purity $\geq$ 95%, specific activity $\approx$ 12 Ci/mmol, a generous gift from Dr G. Prestwich) to lipophorin by 10R-JH III and 10S-JH III. The lipophorin was purified from warm-bled haemolymph (bleeding method described in reference 13) by KBr gradient centrifugation [12]. The assay was carried out as follows: 200 µl TMK-buffer (10 mM Tris, 5 mM $MgCl_2 \cdot 6\ H_2O$, 150 mM KCl, pH 7.4) containing 14650 dpm [^{3}H]-10R-JH III ($\approx 3 \times 10^{-9}$ M), 3×10^{-4} M PMSF (Fluka), 0.5 mg IgG (Sigma, Cohn fraction II), 1.42 µg lipophorin and various concentrations of unlabelled 10R-JH III or 10S-JH III were incubated for 30 min at room temperature. The JHs were dissolved in ethanol for addition to the incubation vials; the final ethanol concentration did not exceed 2% (v/v). The JH-lipophorin complex was precipitated with PEG (Fluka) as described [11], and after centrifugation the supernatant was discarded and the pellet dissolved in 100 µl NCS tissue solubiliser (Amersham). The radioactivity was determined after 500 µl PPO/toluene had been added.

Results. The biological activity of locally applied 10R-JH III and 10S-JH III in the *Galleria* wax test is shown in figure 1 and reveals a much higher level of activity for 10R-JH III (1 G.U. = 0.42 pg) than for 10S-JH III (1 G.U. = 2.2 ng). In a systemic morphogenetic assay with *Nauphoeta cinerea* the 10R-JH III was also much more effective in inducing juvenilisation than the 10S-JH III (fig. 2). For 1 larva point approximately 58 ng of 10R were sufficient as compared with approximately 22 µg for the 10S enantiomer. Detailed analysis (table) showed that the degree of juvenilisation of pronotum, head and abdomen increased steadily with increasing concentrations of injected 10R-JH III up to almost 100% (2.7 to 2.9 out of 3 points), whereas for fore- and

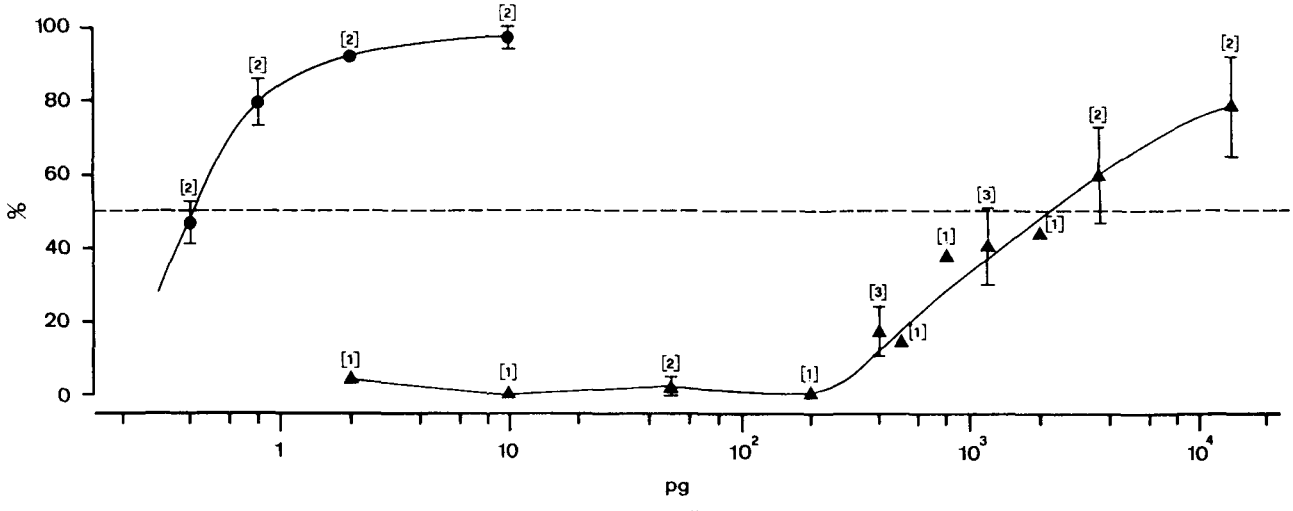

Figure 1. Dose-response curves for 10R-JH III (dots) and 10S-JH III (triangles) in the *Galleria* wax test. Abscissa: dose applied per test pupa; ordinate: percentage with positive response. Number of determinations given in parentheses.

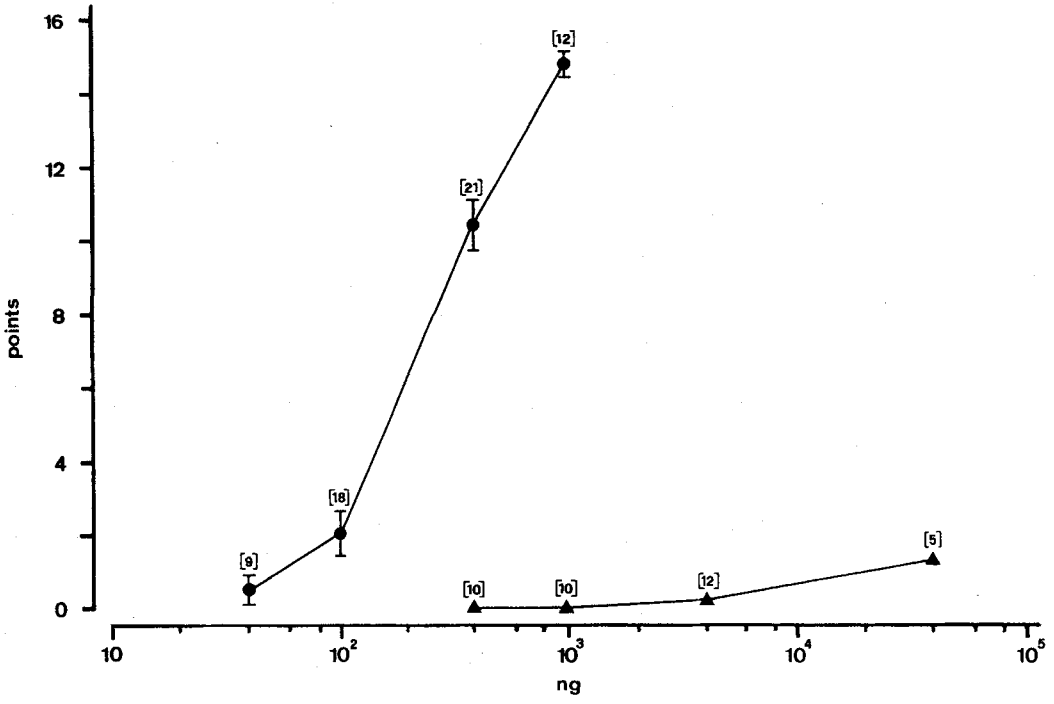

Figure 2. Dose-response curves for induction of juvenilisation in last stadium larvae of *Nauphoeta cinerea* by 10R-JH III (dots) and 10S-JH III (triangles). Abscissa: dose injected per larva; ordinate: degree of juvenilisation in larva points. Data are means ± SEM, number of larvae treated is given in parentheses.

Juvenilising activity* of 10R and 10S-JH III injected into last stadium larvae of the cockroach *Nauphoeta cinerea*

Dose and enantiomer of injected JH III	Forewings	Hindwings	Pronotum	Head	Abdomen	Total	n
Control (oil)	0	0	0	0	0	0	19
40 ng 10R	0.1 ± 0.1	0.1 ± 0.1	0	0.2 ± 0.1	0.1 ± 0.1	0.5 ± 0.4	9
100 ng 10R	0.9 ± 0.3	0.7 ± 0.2	0	0.3 ± 0.1	0.3 ± 0.1	2.1 ± 0.6	18
400 ng 10S	0	0	0	0	0	0	10
400 ng 10R	3.4 ± 0.3	3.1 ± 0.3	1.3 ± 0.2	1.0 ± 0.1	1.7 ± 0.2	10.4 ± 0.7	21
1 µg 10S	0	0	0	0	0	0	10
1 µg 10R	3.1 ± 0.2	3.3 ± 0.1	2.8 ± 0.1	2.7 ± 0.1	2.9 ± 0.1	14.8 ± 0.4	12
4 µg 10S	0	0	0	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	12
40 µg 10S	0.5 ± 0.2	0.5 ± 0.2	0	0.2 ± 0.1	0.1 ± 0.1	1.3 ± 0.5	5

* The degree of juvenilisation is given in larva points (see Materials and Methods); data are mean values ± SEM.

hindwings the degree of juvenilisation remained constant (approximately 55%, 3.1 to 3.4 points out of 6) for 400 ng and 1 µg 10R-JH III.

The 10S enantiomer had only a very weak juvenilising activity even when 40 µg were injected. The dose of 10S-JH III necessary to induce 50% juvenilisation (10.5 points) could not be determined because we did not have a sufficient quantity of this enantiomer available. In inducing vitellogenin synthesis the 10R enantiomer was also much more active than the 10S enantiomer (fig. 3). In this assay a 50% positive reaction was seen with approximately 6.7 ng 10R-JH III as compared with 1.7 µg 10S-JH III. A typical example of competitive displacement of binding of [^{3}H]-10R-JH III to lipophorin of *Nauphoeta* by unlabelled 10R-JH III and 10S-JH III is shown in figure 4. The curves show that 10S-JH III can compete for binding of [^{3}H]-10R-JH III, although less efficiently than 10R-JH III; to reduce binding of [^{3}H]-10R-JH III (3×10^{-9} M) to 50%, approximately 46 times more 10S-JH III (1.6×10^{-6} M) than 10R-JH III (3.5×10^{-8} M) was necessary.

Discussion. The results in figures 1–3 show that the 10R (= natural) enantiomer of JH III was much more active than the 10S enantiomer in all three bioassays; this indicates enantioselectivity of the JH III receptors in the target tissues. In the *Galleria* wax test (fig. 1), where the JH was applied locally, the difference in level of activity of the two enantiomers was most pronounced: 5240 times more 10S-JH III than 10R-JH III was needed to induce a 50% positive response. We also observed a qualitative difference in the effect of the two enantiomers: 10R-JH III induced the formation of a typical

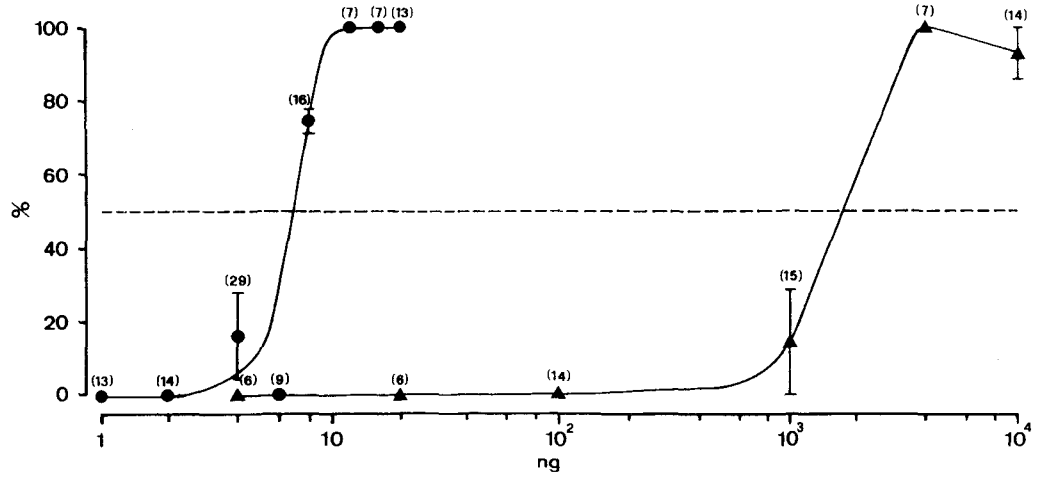

Figure 3. Dose-response curves for induction of vitellogenin synthesis in decapitated adult females of *Nauphoeta cinerea* by 10R-JH III (dots) and 10S-JH III (triangles). Abscissa: dose injected per female; ordinate: percentage of vitellogenin-positive females. Data are means ± SEM, number of females treated is given in parentheses.

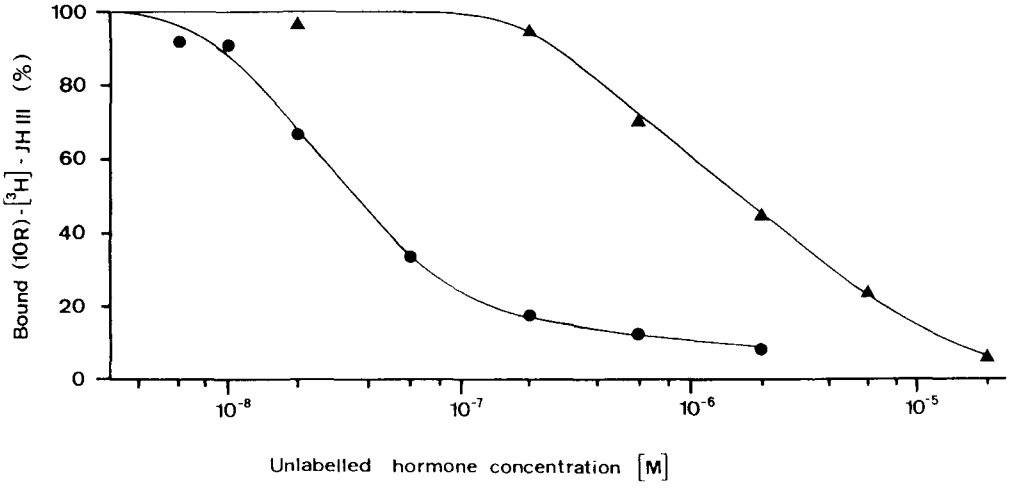

Figure 4. Competitive displacement of [^{3}H]-10R-JH III (3×10^{-9} M) from the lipophorin of *Nauphoeta cinerea* by 10R-JH III (dots) and 10S-JH III (triangles). 100%-bound represents binding of [^{3}H]-10R-JH III in the absence of a competitor.

rough, bumpy pupal cuticle, whereas the application of 10S-JH III gave rise to smoother, scar-like markings. In comparison Schooley et al.[2] reported only a 12-fold higher level of activity for the 10R enantiomer of JH III in the *Galleria* wax test, but the 10S-JH III they used was contaminated with 8% 10R-JH III. Likewise, using the 10R and 10S enantiomers of JH I, Meyer et al.[14] found only a 10-fold higher level of activity for 10R-JH I, and in their case the substance tested was contaminated with 4-10% 10R. In *Galleria* JH I, II and III have been found, JH II being predominant[15].

In the two systemic bioassays with *Nauphoeta cinerea* (figs 2 and 3; table), an insect in which only JH III has been found[16], the difference in the level of activity between the 2 enantiomers was less pronounced than in the *Galleria* wax test. We suppose that the difference in activity levels between the 10R and 10S enantiomers is smaller in the systemic assays than in the local *Galleria* assay because in the former the injected hormones are subject to degradation and the 10R enantiomer of JH III was seen to be degraded more rapidly than racemic JH III in *Nauphoeta cinerea* (Meyer and Lanzrein, submitted). In order to induce slight juvenilisation (1 point) 380 times less 10R-JH III than 10S-JH III (58 ng and 22 µg, respectively) had to be injected (fig. 2), and in order to induce vitellogenin synthesis in 50% of the injected females 255 times less 10R-JH III than 10S-JH III (6.7 ng and 1710 ng, respectively) had to be injected (fig. 3). With regard to the absolute quantities necessary to induce juvenilisation and vitellogenin synthesis, a dose of 400 ng 10R-JH III was necessary to obtain a degree of 50% juvenilisation, whereas a dose of only 6.7 ng was sufficient to induce vitellogenin synthesis in 50% of the injected females. A similar difference was observed in earlier studies when racemic JH III was injected, namely 3 µg for 50% juvenilisation and 18 ng for induction of vitel-

logenin synthesis in 50% of the injected females[8]. We attribute this to the fact that induction of juvenilisation is a long-term effect, in which the different larval target tissues have their own stage-specific sensitivity to JH (table, and Lanzrein[8]) and respond to different threshold doses of JH[8], whereas induction of vitellogenin synthesis is rather a short-term effect.

The endogenous titre of JH III is similar in penultimate stadium larvae and in young adult females at the time when the vitellogenin titre increases, namely 2–10 ng/ml[18] and 0.2–4.0 ng/ml[17], respectively. As JH injected in olive oil diffuses only slowly into the haemolymph, and as it is also subject to degradation, the concentration of injected JH in the haemolymph is difficult to estimate. Nevertheless, from the observations that around 1% of injected JH circulates (Lanzrein[8], and unpublished observations) and that the haemolymph volume in adult females is approximately 200 µl (Meyer, unpublished observations), it can be calculated that injection of 6.7 ng 10R-JH III (dose necessary to induce vitellogenin synthesis in 50% of the insects, fig. 3) leads to a titre of around 0.3 ng/ml exogenous JH III, corresponding to that of endogenous JH III in young adult females[17, 18].

In the binding assay with lipophorin we observed that the 10S enantiomer competed for binding of [^{3}H]-10R-JH III but that 46 times more unlabelled 10S-JH III than 10R-JH III was necessary to reduce binding of [^{3}H]-10R-JH III to 50% (fig. 4). In comparison Schooley et al.[2], using a 10S-JH III preparation contaminated with 8% 10R, reported a 14-fold higher affinity of 10R-JH III than of 10S-JH III to the haemolymph JH binding protein of *Manduca sexta*. Prestwich et al.[19], using enantiomerically enriched ($\geq$ 95% e.e.) 10R-JH I and II and 10S-JH I and JH II, reported up to 10 times higher affinity of the haemolymph JH-binding protein in 4 lepidopterous species for the natural enantiomer. A lower affinity of the 10S-JH III than the 10R-JH III enantiomer for the haemolymph JH-binding protein has also been reported for *Leptinotarsa decemlineata* and *Locusta migratoria*[4]; in this study a 10S preparation containing 8% 10R was used. It should be mentioned that in all these binding studies except ours racemic [^{3}H]-JH was used as the radiolabelled ligand.

In conclusion, our data show that 10S-JH III, which for the first time could be tested at 100% purity, binds to the haemolymph JH-binding protein, although with lower affinity than 10R-JH III, and that 10S-JH III can induce juvenilisation and vitellogenin synthesis when applied in high quantities. These observations, together with the finding that the cytosol and nuclear JH receptor of the fat body in *Leucophaea maderae* bound 10R-JH III with about 1.5 times higher affinity than the racemate[5], indicate that haemolymph JH-binding proteins and receptors in target tissues both display a degree of enantioselectivity.

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1 Judy, K. J., Schooley, D. A., Dunham, L. L., Hall, M. S., Bergot, B. J., and Siddall, J. B., Proc. natl Acad. Sci. USA *70* (1973) 1509.
2 Schooley, D. A., Bergot, B. J., Goodman, W., and Gilbert, L. I., Biochem. biophys. Res. Comm. *81* (1978) 743.
3 Peter, M. G., Gunawan, S., Gellissen, G., and Emmerich, H., Z. Naturforsch. *34C* (1979) 588.
4 De Kort, C. A. D., and Koopmanschap, A. B., Experientia *43* (1987) 904.
5 Engelmann, F., Mala, J., and Tobe, S. S., Insect Biochem. *17* (1987) 1045.
6 Mori, K., and Mori, H., Tetrahedron *43* (1987) 4097.
7 De Wilde, J., Staal, G. B., De Kort, C. A. D., De Loof, A., and Baard, G., Proc. Kon. Ned. Akad. Wetensch. *71* (1968) 321.
8 Lanzrein, B., Gen. comp. Endocr. *39* (1979) 69.
9 Buschor, J., and Lanzrein, B., Comp. Biochem. Physiol. *76B* (1983) 65.
10 Bühlmann, G., Rev. Suisse Zool. *81* (1974) 642.
11 Kovalick, G. E., and Koeppe, J. K., Molec. Cell Endocr. *31* (1983) 271.
12 Shapiro, J., and Law, J. H., Biochem. Biophys. Res. Comm. *115* (1983) 924.
13 Wilhelm, R., Riechsteiner, R., and Lanzrein, B., Insect Biochem. *17* (1987) 971.
14 In: Meyer, D. R., Habilitationsschrift Universität Freiburg, Stettler Diss. Point 1974.
15 Rembold, H., and Sehnal, F., Insect Biochem. *17* (1987) 997.
16 Baker, F. C., Lanzrein, B., Miller, C. A., Tsai, L. W., Jamieson, G. C., and Schooley, D. A., Life Sci. *35* (1984) 1553.
17 Lanzrein, B., Gentinetta, V., Fehr, R., and Lüscher, M., Gen. comp. Endocr. *36* (1978) 339.
18 Lanzrein, B., Gentinetta, V., Abegglen, H., Baker, F. C., Miller, C. A., and Schooley, D. A., Experientia *41* (1985) 913.
19 Prestwich, G. D., Robles, S., Wawrzenczyk, C., and Bühler, A., Insect Biochem. *17* (1987) 551.

0014-4754/89/040356-05$1.50 + 0.20/0