placed in ice-cold Krebs-Ringer buffer ph 7.4 (gassed with 95% O_2 + 5% CO_2), containing 1 mg/ml glucose and 1 mg/ml BSA (Fr. V. Armour Pharmaceutical Co.). Homogenization of the pituitaries was performed with 10 strokes, using glass homogenizers and teflon pestlers in 1.0 ml solution containing: 0.3 m*M* sucrose, 1 m*M* mercaptoethanol, 1 m*M* disodium EDTA and 1 m*M* $MgCl_2$ in 3 m*M* *Tris* HCl (ph 7.5)[6]. The homogenate was centrifuged at 800 rpm for 10 min. The resulting supernatant was centrifuged at 11,000 rpm in a Beckman L-2 ultracentrifuge for 30 min. The pellet was suspended in 41% sucrose solution, which was then overlaid with 63, 45, 37, 32, and 28% sucrose solutions. Centrifugation was then carried out at 22,000 rpm in the Beckman L-2 ultra-centrifuge (rotor SW 25) for 2 h[10]. The interface from the 33% sucrose was collected and washed with 1 m*M* $NaHCO_3$ (1:1) and centrifuged again for 30 min at 20,000 rpm.

The pellet was removed and resuspended in 1 m*M* $NaHCO_3$ and 50 µl aliquots used for the protein determination[11], the 5-nucleotidase[12] assay and the adenyl cyclase assay[13]. Adenyl cyclase activity was measured by conversion of ^{32}P-ATP to ^{32}P cyclic AMP (Krishna et al.[13]; RAMACHANDRAN[14].

3′,5′ cyclic AMP level. Anterior pituitary glands from 'Sabra' strain rats (4 groups of 6 rats) weighing 150–200 g were used for measurement of cyclic AMP levels, using the GILLMAN assay[15].

Cyclic AMP formation by anterior pituitary membrane fractions and cyclic AMP content of the pituitary glands of rats exposed to different environmental temperatures

Temperature (°C)	c-AMP Formation[a] (% over control)	c-AMP Level (pmole/mg protein)	No. of rats
22	54–60	5.0±0.6	12
34	78–86[b]	9.8±3.4[b]	12
37	144–160[b]	16.8±0.52[b]	12
4	0[b]	2.7±0.2	12

± SEM. [a] Stimulation by 50 ng TRH. [b] p 0.001.

The TRH was a generous gift from Farbwerke Hoechst AG., Frankfurt, phospho-enol-pyruvate, pyruvate kinase and cyclic AMP were purchased from Sigma Co., myokinase from Boeringer Co., and ^{32}P-ATP from The Radiochemical Co., Amersham.

Results and discussion. The Table shows that there is a difference between the adenyl cyclase activity in the membrane of the pituitary gland from rats which had been exposed to different temperatures. Adenyl cyclase activity at 34 °C was 22% higher, and at 37 °C 56% higher than the control (22 °C). At 4 °C the adenyl cyclase activity was 98% lower than in the control group. The level of cyclic AMP in the pituitary shows a significant increase at 34 °C and at 37 °C, and a decrease at 4 °C. At 34 °C the pituitary cyclic AMP level was 150% higher, and at 37 °C 210% higher than the control, but at 4 °C there was a decrease of 50%.

In a previous study[14], it was observed that at 37 °C and 0 °C there was a significant decrease in the level of TSH in the blood, but there was an increase in the level of TSH content of pituitary 37 °C, and at 0 °C no difference in TSH content of the pituitary. It was also observed that the level of cyclic AMP in the pituitary gland was increased at 37 °C, and that there was a significant decrease at 0 °C. From this study it has become apparent that adenyl cyclase activity in rats exposed to 37 °C for 4 days is very strong.

These results allow postulation that at 37 °C perhaps the involvement of cyclic AMP is only essential for the biosynthesis of TSH and has no direct bearing on the secretion process. One thing that is clear from this study is that the action of the heat is not on the phosphodiesterase system.

However the possibility is not ruled out that, at 37 °C, the membrane has a higher sensitivity to TRH.

[11] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, J. biol. Chem. *193*, 265 (1959).
[12] R. H. MITCHELL and J. M. HAWTHORNE, Biochem. biophys. Res. Commun. *21*, 333 (1965).
[10] O. M. NEVILLE, Biochim. biophys. Acta *154*, 540 (1968).
[13] C. KRISHNA and L. BIRNBAUBER, Analyt. Biochem. *35*, 393 (1970).
[14] J. RAMACHANDRAN, Analyt. Biochem. *43*, 227 (1971).
[15] A. G. GILMAN, Proc. natn. Acad. Sci., USA *67*, 305 (1970).

Occurrence of Ecdysone in the Blood of the Chelicerate Arthropod, *Limulus polyphemus*[1]

R. R. WINGET and W. S. HERMAN

Department of Zoology, University of Minnesota, 108 Zoology Building, Minneapolis (Minnesota 55455, USA), 5 April 1976.

Summary. A molt promoting substance, assumed to be ecdysone, was discovered in *Limulus* haemolymph. Preliminary bioassay suggests a titre of 9 ng ecdysone per ml of haemolymph.

Seven species of polyhydroxy steroid molting hormones (ecdysones) have been isolated from whole arthropod bodies or eggs[2,3]. Few studies have reported ecdysone from the blood of arthropods[4–6] and none have concerned the blood of a chelicerate, although JEGLA[7] has reported β-ecdysone in whole *Limulus* larvae. We have therefore estimated the ecdysone blood titre in intermolt, juvenile *Limulus*.

Materials and methods. Juvenile *Limulus* (66–235 g fresh weight) were obtained in June and August 1975 from the Marine Biological Laboratory, Woods Hole, Massa-

[1] Supported by the University of Minnesota Graduate School, NSF Grant No. GB 16607 and USPHS Grant No. HO 07336.
[2] J. N. KAPLANIS, W. E. ROBBINS, M. J. THOMPSON and S. R. DUTKY, Science *180*, 307 (1973).
[3] M. J. THOMPSON, J. N. KAPLANIS, W. E. ROBBINS and J. A. SVOBODA, Adv. Lipid Res. *11*, 219 (1973).
[4] E. SHAAYA, Am. Zool. *7*, 756 (1967).
[5] T. OHTAKI, R. D. MILKMAN and C. M. WILLIAMS, Biol. Bull. *135*, 322 (1968).
[6] W. E. BOLLENBACHER, W. V. VEDECKIS and L. I. GILBERT, Devl. Biol. *44*, 46 (1975).
[7] T. C. JEGLA, Am. Zool. *14*, 1288 (1974).

chusetts, and maintained in artifical sea water at 13–14 °C without feeding. Blood was removed by syringe within a few days after the animals arrived in the laboratory and placed into at least two volumes of MeOH.

Isolation of ecdysones followed the general procedure of Kaplanis et al.[8] and details are given in WINGET[9]. Blood was extracted in MeOH, washed with hexane, partitioned into *n*-butanol, dried in vacuo and extracted in 20% MeOH in benzene. The supernatant was purified by silicic acid chromatography (5% MeOH: Benzene followed by 25% MeOH: Benzene) and thin layer chromatography (TLC) (Brinkman silica gel HF 254, $ChCl_3$: MeOH 4:1, UV visualization at 254 nm). The silica gel was eluted with tetrahydrofuran and the extract solubilized in 10% ETOH for bioassay and 100% ETOH for high pressure liquid chromatography (HPLC) (Corasil II, $ChCl_3$: 95% ETOH 9:1, UV visualization at 254 nm). Based on analysis of 100 ml blood aliquots injected with $^3H\alpha$- or β-ecdysone, the extraction procedure through the silicic acid stage was nearly quantitative (95%). However, a 15% loss of radioactivity occurred during TLC, primarily due to lack of quantitative elution by tetrahydrofuran. Elution with hot ETOH did not improve recovery.

Quantification of ecdysones was determined by the *Limulus* bioassay. This assay, developed by JEGLA et al.[10] consisted of injecting first instar *Limulus*, 14–16 days after hatching, with 0.5 µl of purified extract or known amount of β-ecdysone. Precocious ecdysis within 12 days compared to controls injected with an equal volume of 10% ETOH indicated the presence of a molecule with molting activity.

Results. 220 ml of blood were removed from 42 animals (totaling 5285 g fresh weight) collected in early June. After extraction and development of 4 successive thin layer chromatographs, 3 UV absorbing bands appeared in co-migration with authentic α- and β-ecdysone and inokosterone. Several other less polar bands also appeared. Although authentic α-ecdysone was clearly separated from β-ecdysone in this system, inokosterone and β-ecdysone overlapped, as did the corresponding material from *Limulus* blood.

Bioassay of the fractions co-migrating with α- and β-ecdysone and inokosterone produced the results given in the Table. It is clear that these fractions contained molting activity and thus presumably one or more ecdysones. Although quantification of the *Limulus* assay is still in the initial stages in this laboratory, preliminary results indicate that the concentration of ecdysone from all 3 fractions considered together is approximately 9 ng/ml blood, based on an overall extraction efficiency of 50%.

Effect of *Limulus* blood extracts on molt cycle duration in first instar *Limulus*

Treatment	No. of animals injected	Molt within 12 days after injection (%)
10% ETOH	29	0
β-ecdysone (ng/animal)		
5	28	54
25	9	100
50	11	100
blood extracts		
α	7	71
β	10	70
I	8	75
Sum	25	72

α, β and I refer to material that co-migrated with authentic α-ecdysone, β-ecdysone and inokosterone on TLC. Sum equals consideration of these fractions in total.

Considerably more than this amount of material appeared on the thin layer plates. In order to test the assumption that the extra material represented impurities with chromatographic behavior similar to ecdysones, the fractions from TLC were analyzed with HPLC. In each case, peaks appeared with retention volumes that indicated less polarity than the corresponding authentic ecdysone. No peaks appeared at the retention volume of β-ecdysone. UV spectral analysis of HPLC fractions in ETOH, that represented the observed peaks or the retention volume of β-ecdysone, did not indicate the presence of ecdysone. Based on a sensitivity of approximately 500 ng of β-ecdysone for the particular liquid chromatograph used, the HPLC data indicate that considerable quantities of UV absorbing impurities from *Limulus* blood co-migrate with various ecdysones on TLC and that the amount of ecdysone present should be less than 37 ng/ml, as confirmed by the bioassay.

In an earlier attempt to measure *Limulus* ecdysone titres, blood extracts from juveniles collected in August were analyzed by the *Musca* assay, as developed by KAPLANIS et al.[11]. Some cuticle tanning was observed in larval *Musca* abdomens (the criterion for ecdysone presence) compared to no tanning in larvae injected with 10% ETOH. However, the results were not statistically meaningful. Many larvae injected with blood extracts became necrotic, while necrosis was not a serious problem with controls. It therefore appears that *Limulus* blood contains material which is toxic to *Musca* larvae, even after TLC and HPLC purification. Toxicity is not an uncommon problem with this assay[12].

Discussion. We have demonstrated that material obtained from TLC fractionation of juvenile *Limulus* blood extract produced precocious molting in *Limulus* larvae and assume that these observations indicate the presence of ecdysone. We do not have sufficient evidence to identify the ecdysone (s) involved, but their thin layer chromatographic behavior is similar to α- and β-ecdysone and inokosterone. The existence of ecdysone in the blood provides further support for the contention that this steroid is a *Limulus* hormone under the classic definition that hormones represent a blood borne communication system.

A plethora of studies[9,13] have measured ecdysone concentration in whole animal extracts (which may contain gut contents), but few have examined isolated blood. OHTAKI et al.[5] reported ecdysone concentrations of 10–50 ng/ml in larval *Sarcophaga* blood, depending on age. The lower end of this range compares favorably with 9 ng/ml estimated for *Limulus* juveniles in the intermolt stage, which presumably represents the lowest molt hormone levels. However, *Limulus* molt in late July and August in the Woods Hole area. The individuals assayed appeared to be intermolts but were collected about 6–8 weeks before late premolts normally appear and may have been in the earliest, undetectable stages of premolt, when ecdysone titres are increasing in other arthropods[13,14].

[8] J. N. KAPLANIS, M. J. THOMPSON, R. T. YAMAMOTO, W. E. ROBBINS and S. J. LOULOUDES, Steroids *8*, 605 (1966).
[9] R. R. WINGET, Ph. D. thesis, University of Minnesota (1975).
[10] T. C. JEGLA, J. D. COSTLOW and J. ALSPAUGH, Gen. comp. Endocr. *19*, 159 (1972).
[11] J. N. KAPLANIS, L. A. TABOR, M. J. THOMPSON, W. E. ROBBINS and T. J. SHORTINO, Steroids *8*, 625 (1966).
[12] J. A. THOMSON, *Invertebrate Endocrinology and Hormonal Heterophylly* (Ed. W. J. BURDETTE; Springer-Verlag, New York 1974), p. 121.
[13] L. I. GILBERT and D. S. KING, *Physiology of Insecta* (Ed. M. ROCKSTEIN; Academic Press, New York 1973), vol. 1, p. 249.
[14] A. WILLIG and R. KELLER, J. comp. Physiol. *86*, 377 (1973).