Proc. Nat. Acad. Sci. USA
Vol. 69, No. 4, pp. 812–815, April 1972

Binding Proteins for an Ecdysone Metabolite in the Crustacean Hepatopancreas

(molting hormone/steroid/sucrose gradient centrifugation)

THOMAS A. GORELL*, LAWRENCE I. GILBERT†, AND JOHN B. SIDDALL

Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201; and
Zoecon Research Corporation, Palo Alto, California 94304

Communicated by William S. Johnson, January 20, 1972

ABSTRACT **When crustacean hepatopancreas is incubated in the presence of α-[^{3}H]ecdysone of high specific activity and is then homogenized and centrifuged, a peak of protein-radioactivity is recovered after gel filtration of the 105,000 × *g* supernatant. Analysis of this peak by sucrose gradient centrifugation revealed the presence of two complexes of protein and labeled material (~11.5 S and 6.35 S). The same results were obtained *in vivo*. On standing at low ionic strength, the lighter component disappeared, suggesting that the heavier component is an aggregate of the lighter one. Chemical analysis of radioactive material in the complex revealed that it is not α- or β-ecdysone nor any previously described metabolite of the ecdysones. This new metabolite of α-ecdysone is found mainly in the incubated hepatopancreas. Partial structures consistent with the analytical data are inferred for this metabolite. It is suggested that the metabolite may be active in the action of molting hormone.**

Although highly significant advances regarding the chemical nature of arthropod growth hormones have occurred in recent years (1, 2), we do not yet fully understand how these molecules elicit their effects. This lack of understanding is due in part to a paucity of information regarding the primary step in hormone action, i.e., the means by which the hormone recognizes the target tissue (or the converse) and is "bound" to cell constituents. The existence of proteins that bind steroid hormones is an established fact in several mammalian systems (3, 4), and progress in that field is due in great part to the synthesis of labeled hormones of high specific activity. The synthesis of tritiated α-ecdysone of high specific activity (5) provided the critical tool for studies on the binding of molting hormone to arthropod target systems. We now know that α-ecdysone is rapidly converted to β-ecdysone (ecdysterone, 20-hydroxyecdysone, crustecdysone) by insects and crustaceans (6, 7), and it is believed that β-ecdysone is the molting hormone of crustaceans (8–11). The present study examines the possibility of hormone-binding macromolecules in the crayfish hepatopancreas, an organ analogous to the vertebrate liver and the insect fat body.

MATERIALS AND METHODS

The α-[23,24-^{3}H]ecdysone was synthesized by catalytic tritiation of 2β,3β,14α,22β,25-pentahydroxy-5β-cholest-7-en-23-yn-6-one (5). Male crayfish (*Orconectes virilis*) were cooled on ice for 30 min, and the hepatopancreas was removed. The hepatopancreas was rinsed in crayfish saline (12) and incubated at 25° in 3 ml of saline containing [^{3}H]ecdysone (see figure legends for quantities). After incubation, the tissue was rinsed twice in cold 0.1 M phosphate buffer (pH 7.3) and homogenized in buffer in a ground glass homogenizer at 4°. After centrifugation of the homogenate at 105,000 × *g* for 1.5 hr at 4°, the supernatant was concentrated with lyphogel and aliquots of the concentrated supernatant were assayed for protein (13) and radioactivity (Packard Tri-carb scintillation spectrometer, model 3375) in ethyl alcohol–toluene [2,5 diphenyloxazole (PPO), 5 g/l; 1,4-bis-2-(4-methyl-5-phenoxazolyl)-benzene (POPOP), 0.1 g/l] scintillation mixture.

The hepatopancreas is rich in lipid (14, 15), which necessitated partial purification of the 105,000 × *g* supernatant by gel filtration. Sephadex G-200 was swollen in water at room temperature, and the column (24 × 2 cm) was packed and equilibrated with 0.1 M phosphate buffer (pH 7.3) at 4°. The concentrated supernatant (1 ml) was layered on the column, eluted with buffer (flow rate, 10 ml/hr), and 2.5-ml fractions were collected. After aliquots of individual fractions were assayed for protein content and radioactivity, individual fractions and pooled fractions were subjected to sucrose density gradient centrifugation.

Column fractions were concentrated with lyphogel and layered on 5-ml discontinuous linear gradients of 5–20% sucrose in 0.1 M phosphate buffer (pH 7.3) and 0.0015 M EDTA, and then centrifuged for 5 or 6 hr at 258,000 × *g* (4°) in a Spinco L2-65B preparative ultracentrifuge with an SW-65 K rotor. The centrifuge tubes were then punctured, and 10-drop (0.27 ml) fractions were collected and radioassayed. Approximate sedimentation coefficients were determined (16) with beef liver catalase (EC 1.11.1.6; molecular weight, 250,000; 11.3 S) and yeast alcohol dehydrogenase (EC 1.1.1.1; molecular weight, 150,000; 7.4 S) as standards.

RESULTS

Characterization of binding proteins

Fig. 1 reveals that when hepatopancreas is incubated in the presence of [^{3}H]ecdysone, homogenized, centrifuged, and the supernatant subjected to gel filtration, a peak of radioactivity (fractions 5–7) accompanies the protein initially eluted from the column although the bulk of radioactivity is not associated with protein. When fractions 5–7 are individually analyzed by sucrose gradient centrifugation, each fraction possesses radioactivity (Fig. 2). The peak of radioactivity from fraction 5 (Fig. 2*A*) corresponds to the heavier sedimenting peak of fraction 6 (Fig. 2*B*) and persists as a slight shoulder in

* Present address: The Ben May Laboratory for Cancer Research, University of Chicago, Chicago, Ill. 60637.
† To whom reprint requests should be sent.

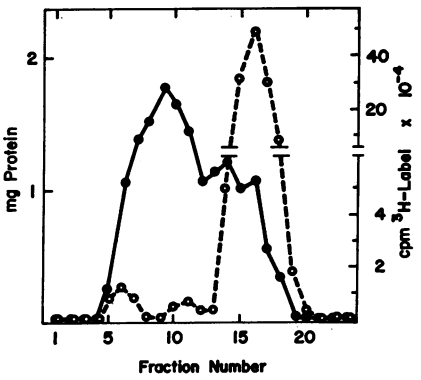

FIG. 1. Elution pattern from Sephadex G-200 of 1 ml concentrated 105,000 × *g* supernatant from three hepatopancreases after 5-hr incubation in crayfish saline containing [³H]ecdysone (30 μCi/ml). ●, mg protein; O, cpm.

fraction 7 (Fig. 2*C*). These data show that the ³H-label is complexed to two macromolecules (or aggregates of one macromolecule) in the hepatopancreas and that these macromolecules are separable by gel filtration and sucrose gradient centrifugation.

Although the elution pattern (Fig. 1) demonstrates a relatively large quantity of protein beyond the initial radioactivity peak and a slight rise in radioactivity at fractions 10–12, sucrose gradient analysis of these fractions showed no evidence for a label–macromolecular complex.

For determination of the size of the complexes, Sephadex column chromatography was again conducted on the 105,000 × *g* supernatant from hepatopancreas previously incubated with [³H]ecdysone, and fractions 5–7 were subjected to sucrose gradient centrifugation along with the catalase and alcohol dehydrogenase markers. The results (Fig. 3) show the heavier radioactive peak to be about 11.3 S (molecular weight about 250,000) while the lighter peak is about 6.35 S (molecular weight about 130,000). Since the protein estrogen receptor of the rat uterus appears to be composed of lighter subunits that tend to aggregate on standing at low ionic strengths (17), we investigated the same possibility in the crayfish system. Fig. 4 reveals that when the 105,000 × *g* supernatant is allowed to stand for several days, the lighter component disappears, suggesting that the heavier component is an aggregate of the lighter one.

Thus far, the evidence suggests the presence of an ecdysone receptor(s) in the crayfish hepatopancreas, but it is

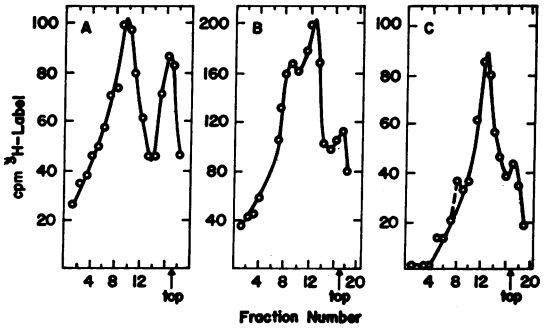

FIG. 2. Sucrose gradient analysis of 0.5-ml aliquots from Sephadex fractions 5(*A*), 6(*B*), and 7(*C*) obtained in Fig. 1 after centrifugation for 5 hr.

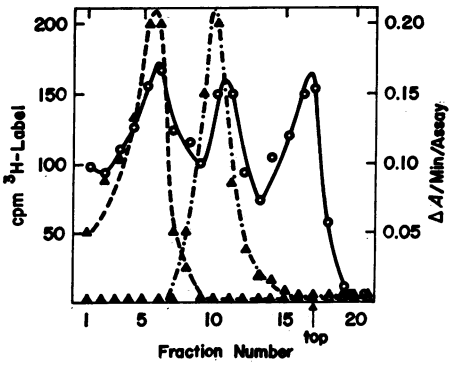

FIG. 3. Sucrose gradient analysis of 0.4-ml aliquots from Sephadex fractions 5, 6, and 7 (combined and concentrated) containing catalase and alcohol dehydrogenase markers assayed as described (16). Samples were centrifuged for 6 hr. O, cpm; △, catalase; ▲, alcohol dehydrogenase.

based on the assumption that the radioactivity associated with the protein fractions represents unaltered ecdysone. To determine the validity of this assumption, we investigated the chemical nature of the radioactive material bound to the hepatopancreas proteins.

Preliminary characterization of radioactive material

We next examined the nature of the radioactivity from fractions 5, 6, and 7 (Fig. 1) to verify that both macromolecular fractions contained the same label and to analyze the chemical nature of the bound label. An aliquot (10,000 cpm) of pooled fractions 5–7 from a similar Sephadex G-200 elution was added to a solution of 2.5 ml of *n*-butyl alcohol containing unlabeled α-ecdysone (1.67 mg), β-ecdysone (2.00 mg), and ponasterone A (1.23 mg). After thorough shaking to break the protein complex, the *n*-butyl alcohol phase was removed and evaporated (at 10° in high vacuum) and its content was applied to a preparative silica gel GF (Merck) chromatoplate. Development in 8% methanol–chloroform cleanly resolved the unlabeled carriers (seen under UV irradiation), and radioscanning located the radioactivity in one narrow band (R_f =

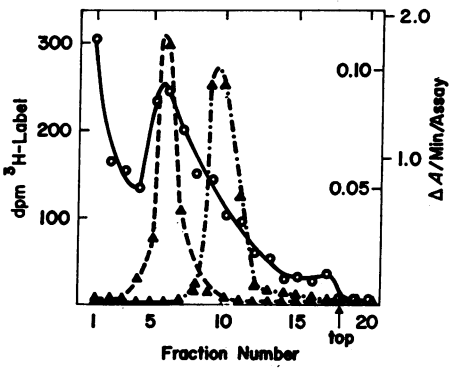

FIG. 4. Sucrose gradient analysis of radioactive protein fractions (cpm were corrected to dpm) from Sephadex G-200 elution of 1 ml concentrated 105,000 × *g* supernatant. Four hepatopancreases were incubated for 5 hr in 3 ml of crayfish saline with [³H]ecdysone (1 μCi/ml) and kept at 4° for 8 days before column chromatography. The samples were centrifuged with catalase and alcohol dehydrogenase as markers for 6 hr. O, dpm; △, catalase; ▲, alcohol dehydrogenase.

FIG. 5. Radiochromatogram of *n*-butyl alcohol extracts of homogenates prepared from crayfish hepatopancreas after each hepatopancreas was incubated for 5 hr in 2 ml crayfish saline containing 0.5 μCi [^{3}H]ecdysone. Extraction and thin-layer chromatography are as described in the *text*. *O*, origin; *1*, polar metabolite; *2*, probably β-ecdysone; *3*, α-ecdysone; *4*, metabolite that binds to hepatopancreatic proteins; *S*, solvent front.

1.65 relative to α-ecdysone in 25% ethanol–chloroform on 0.25-mm silica HF plates) moving slightly ahead of ponasterone A. Clearly, the radioactivity is not associated with α- or β-ecdysone, nor is the labeled compound identical with ponasterone A, 25-deoxyecdysone, 14-deoxyecdysone, 22-deoxyecdysone, inokosterone, or 7,8-dihydro-α-ecdysone, but appears to be a previously unidentified, less-polar metabolite. Several incubations of crayfish hepatopancreas tissues with fresh highly-purified α-[^{3}H]ecdysone gave a consistent picture of clean conversion (Fig. 5) into the same less-polar metabolite, which was always found *predominantly* in the tissue (46–58%) with some highly polar material (6–8%), the remaining label being unchanged α-ecdysone (20–40%) found both in the tissue and the medium. Verification of this conversion to the same metabolite (56% in 5 hr) by the hepatopancreas of another crustacean species (*Procambarus clarki*) was made with the same incubation procedure (King, D. S., personal communication).

The radioactive metabolite showed several properties that clearly distinguish it from the common ecdysones and the phytoecdysones (2), which for the most part possess the acid-labile 14-α-hydroxy 7,8-dehydro-6-ketone system. With the minute quantities of metabolite available, only limited chemical analysis could be conducted, and the results are summarized below.

(*A*) Treatment with 0.05 N aqueous methanolic hydrochloric acid leaves the metabolite unchanged, but unlabeled α-ecdysone is simultaneously dehydrated in the same reaction solution.

(*B*) 0.01 M Aqueous sodium metaperiodate cleanly and quickly cleaves the metabolite to a single radioactive spot (thin-layer chromatography), indicating a vicinal glycol or α-hydroxy-ketone system. The product is neither 4-hydroxy-4-methyl pentanal nor α,α-dimethyl butyrolactone, which would have arisen from β-[^{3}H]ecdysone or from a 20-hydroxy-22-ketone system. The cleavage product undergoes facile acetylation (acetic anhydride/pyridine at 20°), indicating a primary or secondary hydroxyl in addition to the vicinal glycol or α-hydroxyketone above.

(*C*) The metabolite forms an acetonide (acetone/phosphomolybdic acid catalysis), which, on further reaction with cold acetic anhydride/pyridine, forms a single acetate. The acetonide is not cleaved by 0.01 M sodium metaperiodate, indicating that the metabolite probably has only one vicinal glycol grouping.

(*D*) The metabolite fails to react with selenium dioxide in warm (65°) dioxane and, hence, is neither 14-deoxyecdysone nor a $\Delta^{8(14)}$-6-keto steroid.

(*E*) The metabolite reacts readily with lithium aluminum hydride or tri-*t*-butoxy lithium aluminum hydride in cold (20°) tetrahydrofuran to produce new, more polar products, indicating at least one reducible (carbonyl) group.

(*F*) The metabolite is neither acidic nor basic and contains no highly polar group(s), such as a sulfate linkage.

On the basis of these observations and in the absence of any classical spectra that would require considerably larger quantities of material, we conclude that the metabolite very probably retains the skeletal carbon atoms and the hydroxylated side chain of α-ecdysone but possesses a radically different pattern of nuclear functionalization. Two possible partial structures consistent with these data require either a 13,14-*seco*-14-keto system or an 18-nor-14β-methyl steroid.

DISCUSSION

The data clearly indicate that when crayfish hepatopancreas is incubated in the presence of α-[^{3}H]ecdysone, the 105,000 $\times$ *g* supernatant yields two macromolecules (or aggregates of a single macromolecule) that show affinity for the labeled metabolite of ecdysone not demonstrated by the remainder of the hepatopancreatic protein fractions eluted from a Sephadex column. The results suggest that both macromolecules are protein and that a possible subunit–aggregate relationship exists between the two. It should be noted that the same results were obtained when intact animals were injected with [^{3}H]ecdysone and the hepatopancreas subsequently removed, fractionated, and analyzed. This strongly suggests that neither the binding nor metabolite conversion is an artifact of the *in vitro* procedures used in the majority of experiments.

Recent data suggest that α-ecdysone binds to one or more hemolymph (blood) proteins in the bug *Pyrrhocoris apterus* (18), although this does not appear to take place in silkworm hemolymph (19). It was reported that α-ecdysone is reversibly bound to three hemolymph proteins in *Drosophila hydei* and that it forms a macromolecular complex of molecular weight about 65,000 in the cytoplasm of the salivary glands (20). No chemical studies were reported on the nature of the radioactive material associated with the protein although the R_f of the bound material appeared to be similar to that of α-ecdysone on the basis of thin-layer chromatography.

The existence and characteristics of the crustacean binding proteins are similar to the estrogen-binding proteins of the rat uterus (3, 4, 17) although the crustacean proteins are larger molecules. The major difference is that the radioactivity associated with the proteins of the crustacean hepatopancreas is neither α- nor β-ecdysone but a steroid metabolite not previously reported, while authentic 17-β-estradiol is bound to proteins of the rat uterus. Although the possible significance of this metabolite will be discussed below, a precedent exists in vertebrates for analogous hormone modification, since testosterone is converted to an active metabolite, 5α-dihydrotestosterone, in its target tissues. It has been postulated that

the active androgen may be dihydrotestosterone and that this is the material that binds to target cell constituents (21, 22).

It is of interest that similar binding patterns have been found in crustacean epidermis, which is a target tissue for the molting hormone(s), but not in muscle (unpublished information). The crayfish hepatopancreas responds to exogenous β-ecdysone by increased RNA and protein synthesis (23) and exhibits fluctuations in protein synthesis (24) and lipid metabolism (14, 15) during the normal molt cycle that are presumably due to changing titers of endogenous molting hormone.

One can only speculate on the physiological significance of the metabolite since we do not yet have enough material for bioassay. It may be that the conversion product is an active material responsible for certain phenomena attributed to the molting hormone and that α-ecdysone is a hormone precursor. Ecdysone may enter all tissues (7, 25) but hormonal effects may be elicited in only those tissues that have the enzyme(s) necessary for converting α-ecdysone to "active" metabolites. On the other hand, the metabolite may be a degradation product of the hormone, although it would be unusual for such a product to be specifically bound.

Finally, the binding protein–metabolite complex may act to stimulate RNA polymerase, as has been recently shown for the estradiol-binding protein complex (26).

We thank Mr. B. J. Bergot (Zoecon Research Corp.) for assistance with microchemical reactions. This work was supported in part by grants AM-02818 from the National Institutes of Health and GB-27574 from the National Science Foundation. T. A. G. is a former predoctoral fellow of NIH Training Grant.

1. Gilbert, L. I. (1969) "The chemistry of insect hormones," *Proc. 3rd Int. Cong. Endocrinol.* 340–346.
2. Siddall, J. B. (1970) in *Chemical Ecology*, eds. Sondheimer, E. & Simeone, J. B. (Academic Press, New York), pp. 281–306.
3. Jensen, E. V. & Jacobson, H. I. (1962) "Basic guides to the mechanism of estrogen action," *Rec. Progr. Hormone Res.* **18**, 387–414.
4. Gorski, J., Toft, D., Shyamala, G., Smith, D. & Notides, A. (1968) "Hormone receptors: Studies on the interaction of estrogen with the uterus," *Rec. Progr. Horm. Res.* **24**, 45–80.
5. Hafferl, W., Wren, D. L., Marshall, J. P., Calzada, M. C. & Siddall, J. B. "The synthesis of tritiated α-ecdysone and 25-deoxyecdysone of high specific activity," *J. Label. Compounds*, in press.
6. King, D. S. & Siddall, J. B. (1969) "Conversion of α-ecdysone to β-ecdysone by crustaceans and insects," *Nature* **221**, 955–956.
7. Moriyama, H., Nakanishi, K., King, D. S., Okauchi, T., Siddall, J. B. & Hafferl, W. (1970) "On the origin and metabolic fate of α-ecdysone in insects," *Gen. Comp. Endocrinol.* **15**, 80–87.
8. Horn, D. H. S., Middleton, E. J., Wunderlich, J. A. & Hampshire, F. (1966) "Identity of the moulting hormones of insects and crustaceans," *Chem. Commun.* 339–341.
9. Lowe, M. E., Horn, D. H. S. & Galbraith, M. N. (1968) "The role of crustecdysone in the moulting crayfish," *Experientia* **24**, 518–519.
10. Krishnakumaran, A. & Schneiderman, H. A. (1968) "Chemical control of molting in arthropods," *Nature* **220**, 601–603.
11. Krishnakumaran, A. & Schneiderman, H. A. (1969) "Induction of molting in crustacea by an insect molting hormone," *Gen. Comp. Endocrinol.* **12**, 515–518.
12. Van Harreveld, A. (1936) "A physiological solution for fresh water crustaceans," *Proc. Soc. Exp. Biol. Med.* **34**, 428–432.
13. Lowry, O. H., Rosenbrough, N. J., Farr, A. L. & Randall, R. J. (1951) "Protein measurement with Folin phenol reagent," *J. Biol. Chem.* **193**, 265–275.
14. O'Connor, J. D. & Gilbert, L. I. (1968) "Aspects of lipid metabolism in crustaceans," *Amer. Zool.* **8**, 529–539.
15. O'Connor, J. D. & Gilbert, L. I. (1969) "Alterations in lipid metabolism associated with premolt activity in a land crab and fresh water crayfish," *Comp. Biochem. Physiol.* **29**, 889–904.
16. Martin, R. G. & Ames, B. N. (1961) "A method for determining the sedimentation behavior of enzymes: Application to protein mixtures," *J. Biol. Chem.* **236**, 1372–1379.
17. Jensen, E. V., Suzuki, T., Numata, M., Smith, S. & DeSombre, E. R. (1969) "Estrogen-binding substances of target tissues," *Steroids* **13**, 417–427.
18. Emmerich, H. (1970) "Über die Hämolymphproteine von *Pyrrhocoris apterus* und über die Bindung von Ecdyson durch Hämolymphproteine," *J. Insect. Physiol.* **16**, 725–747.
19. Chino, H., Gilbert, L. I., Siddall, J. B. & Hafferl, W. (1970) "Studies on ecdysone transport in insect haemolymph," *J. Insect Physiol.* **16**, 2033–2040.
20. Emmerich, H. (1970) "Ecdysonbindende Proteinfraktionen in den Speicheldrüsen von *Drosophila hydei*," *Z. Vergl. Physiol.* **68**, 385–402.
21. Wilson, J. D., Bruchovsky, N. & Chatfield, J. N. (1969) "Intranuclear localization of testosterone-1,2-^{3}H in rat prostate," *Proc. 3rd Int. Cong. Endocrinol.* 17–23.
22. Baulieu, E. E., Lasnitzki, I. & Robel, P. (1968) "Testosterone, prostate gland and hormone action," *Biochem. Biophys. Res. Commun.* **32**, 575–577.
23. Gorell, T. A. & Gilbert, L. I. (1969) "Stimulation of protein and RNA synthesis in the crayfish hepatopancreas by crustecdysone," *Gen. Comp. Endocrinol.* **13**, 308–310.
24. Gorell, T. A. & Gilbert, L. I. (1971) "Protein and RNA synthesis in the premolt crayfish," *Z. Vergl. Physiol.* **73**, 345–356.
25. Gorell, T. A., Gilbert, L. I. & Tash, J. "The uptake and conversion of α-ecdysone by the pupal tissues of *Hyalophora cecropia*," Insect Biochem., in press.
26. Mohla, S., DeSombre, E. R. & Jensen, E. V. (1971) "Stimulation of RNA synthesis in uterine nuclei by estradiol *in vitro*," *Fed. Proc.* **30**, 1214.