Ecdysone and 20 hydroxyecdysone: new hormones for the human parasite *Schistosoma mansoni*

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The insect moulting hormones, ecdysone and 20 hydroxyecdysone, were detected by the combined use of radioimmunoassay and high performance liquid chromatography in the human parasite *Schistosoma mansoni*. On day 11 after infection only the ecdysone form is present, but, on day 40 after infection the ratio between ecdysone and 20 hydroxyecdysone changes with anatomic localization of the adult worms in mammalian host. In the eggs, the ratio of these two hormones is identical to the ratio found in sexually mature worms located in mesenteric veins. These data demonstrate for the first time that *S.mansoni* synthesizes the steroid hormones ecdysone and 20 OH ecdysone which are potent molecules in stimulating growth and vitello-genesis of this gonochoric trematode.

Schistosoma mansoni *Fasciola hepatica* *Ecdysone/analysis* *Ecdysterone/analysis*
Radio immunoassay – chromatography, high pressure liquid

1. INTRODUCTION

More than 350 million people are exposed to *Schistosoma* infection throughout the world. No preventive methods are at present developed and vaccination or immunological therapy are still long term goals. A better knowledge of the physiology of the worm would provide an alternative approach to the control of the proliferation of this disease. Little is known about the neurological or endocrinological control of the development of the parasite. At present only neuromediators have been studied, such as biogenic amines or synaptic molecules [1]. We have shown that ecdysteroid material appeared in *S.mansoni* on day 6 after infection [2]. Here, we demonstrate that these ecdysteroids are the insect moulting hormones ecdysone (E) and 20 hydroxyecdysone (20 OH . E). The quantities of these two hormones undergo variations which are related to specific developmental stages in the juvenile forms as well as in adult parasites. A dramatic increase occurs during the first larval stage (day 11 after infection), a critical period in the development of the parasite which has left the lungs and just reached the liver. In addition, ecdysone and 20 OH . ecdysone are present in adult males and females and also in the eggs. Combined use of high performance liquid chromatography [3] and radioimmunoassay [4] allowed the measurement of both hormones in the following critical developmental stages: (i) artificially transformed schistosomula; (ii) day old worms; (iii) 20 day old parasites; (iv) adult males and females collected on day 40 after infection and in the eggs.

2. MATERIALS AND METHODS

2.1. *Schistosomula*

The cercariae of *S.mansoni* is a procaryote formed with a tail and a head. This infective form is shed into conditioned fresh water by the intermediate snail host *Biomphalaria glabrata*. After shearing off the tails of cooled cercariae by 1 min vortex mixing, the heads are named mechanically-

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derived schistosomula or schistosomula [5]. Purification of schistosomula from tails was done using a 54% Percoll gradient (Pharmacia) in minimum essential medium (MEM, Gibco) at $350 \times g$ for 15 min. The pellet of pure schistosomula was washed 3-times in 0.9% NaCl.

2.2. *Infestation of animals*

Hamsters *Mesocricetus auratus)* were infected by exposure to cerariae as in [6].

2.3. *11-Day old worms*

These have left the lungs of infected hamsters and have just reached the liver for further maturation. Infected hamsters were killed 11 days after infection by cervical dislocation without pentobarbital or heparin treatment. The abdominal and thoracic cavities were opened and the liver removed. The worms were then recovered by perfusion of the liver with MEM. Worms were picked up under light microscopy and washed 3-times in 0.9% NaCl prior to storage at −70°C until further experiments.

2.4. *20-Day old worms*

These worms are heterogenous in size and a few already show the first signs of sexual differentiation. They were recovered as above and two batches were made: worms longer than 1.5 mm (L) and less than 1.5 mm (S), in order to collect more homogenous samples.

2.5. *40-Day old worms*

40-Day old parasites were well differentiated and mature. The normal location for these parasites for copulation and egg laying are the portal and mesenteric vessels. They were recovered by total perfusion of the whole blood system of hamsters with NaCl 0.9% or only from portal and mesenteric vessels of hamsters by local puncture and aspiration of the content of these veins. Males and females were separated in MEM with a paint brush, then washed 3-times in 0.9% NaCl prior to storage at −70°C until further experiments.

2.6. *Egg of 40-day* S.Mansoni

Eggs were recovered from the liver of infected hamsters. Livers were homogenized in 17% NaCl, then filtered through medical gauze (1 mm) prior to sieving (0.124 mm). The filtrate was homogenized in EDTA 3.36 mM, NaCl 140 mM, KCl 2.68 mM, $Na_2HPO_4 \cdot 12H_2O$ 8 mM, KH_2PO_4 0.47 mM, solution (pH 7.4) for 30 s and decanted after 2 h at 4°C. The pellets were centrifuged on a 54% Percoll gradient in MEM at $350 \times g$ for 20 min. The supernatant was discarded and the eggs washed 3-times in 17% NaCl.

2.7. *Ecdysteroid assay*

For all the experiments, about 300000 schistosomula, 2.10^6 eggs and 40000 adults were used. Batches of 20–50 mg were homogenized in methanol–water–hexane (5/2.5/1.5, by vol.), the upper phase was discarded and the hypophase was dried under nitrogen flow. The extract was then diluted in 0.1 M citrate buffer (pH 6.1) and this solution used in the RIA method. The blank was run with liver or blood of uninfected animals. The ^{125}I-labelled hormone was a succinyl ecdysterone coupled to [^{125}I]tyrosine-methyl-ester (Immunotech, Marseille). The binding assays were made in a plexiglass apparatus containing 10 dialysis compartments, each divided into two 200-μl chambers by a cellulose membrane [7]. One chamber was filled with 150 μl of antibody and the other with 150 μl of a mixture of equal parts of the unknown ecdysteroid solution and the ^{125}I-labelled hormone. After 24 h shaking at 4°C, equilibrium was achieved and 100 μl of each compartment was counted. The sum of the bound (B) and free (F) lavelled analogue was found on the antibody side and the free (F) alone on the other. The ratio of binding was computed as $r = B/T$ where $T = B + F$. The standard curve was made with pure 20OH.E (Sigma) over 5×10^{-11} M–10^{-8} M. The maximum binding in these conditions was 0.67 ($r_0 = 67\%$). Crude extract bound fractions were then calculated on a Wang 2200 S computer system, with a linear regression of the standard curve. The results were expressed as pmol ecdysone equiv./mg protein (Lowry determination [8]).

2.8. *High pressure liquid chromatography (HPLC)*

A modification of the method in [3] was used. HPLC was performed on a reverse phase column (Whatman, Partisil 10–25/ODS 3) using acetonitrile in a 0.1% solution of trifluoroacetic acid in water as solvent. A linear gradient (1 ml/min for 20 min) was used (Pump, Waters 6000 A). Absorbance was measured at 245 nm, fractions of 30 s

were collected, dried under nitrogen flow then dissolved in 0.1 M citrate buffer (pH 6.1) and analysed by the RIA procedure.

3. RESULTS

Fig.1A shows that newly derived schistosomula contain 4.3 pmol ecdysone equivalents/100 mg protein. On arrival in the liver of the host on day 11 after infection the concentration of the hormone was increased to 25 pmol ecdysone equiv./100 protein (fig.1B). At this stage of infection only the ecdysone form was detected using HPLC – RIA combination ($E/(E + 20\,OH.E) = 1$).

The lowest level of ecdysteroid was detected on day 20 after infection, representing the background levels in this parasite, though not of the method (fig.1C). Both males and females collected on day 40 after infection by total perfusion of hamsters exhibit an ecdysone level about 30 pmol/100 mg protein (fig.1D). However, there were differences in the ratio of ecdysone to 20 hydroxyecdysone: $E/(E + 20\,OH.E) = 0.32$ for the females (fig.2A) and $E/(E + 20\,OH.E) = 0.38$ for the males. On the other hand, adults collected exclusively by local puncture of the portal vein and mesenteric vessels showed significant sexual differences in hormone level. Males contained <1 pmol/100 mg protein whilst females possessed 2.5 pmol/100 mg protein (fig.1E). As shown in fig.2B, parasites in the mesenteric and portal system exhibit an inverse ratio of ecdysone to 20 hydroxyecdysone as compared with those collected by total perfusion: $E/(E + 20\,OH.E) = 0.76$ for the females (fig.2B) and $E/(E + 20\,OH.E) = 0.60$ for the males. The hormonal content of eggs was comparable to that of females. The total activity measured by RIA was 2.6 pmol/100 mg protein (fig.1F) and the $E/(E + 20\,OH.E)$ ratio was 0.76. It should be emphasized that after HPLC separation the RIA activity was detected exclusively in fractions 50, 51, 52, 56 and 57 (arrows in fig.2). The first 3 peaks correspond to the standard migration of 20OH ecdysone and the two others to the standard migration of ecdysone. Controls performed on uninfected hamster tissues gave a concentration of 0.15 pmol/100 mg protein (fig.1G). In all cases, the RIA activity on collected HPLC fractions gave a greater amount of ecdysone and 20OH ecdysone than before HPLC.

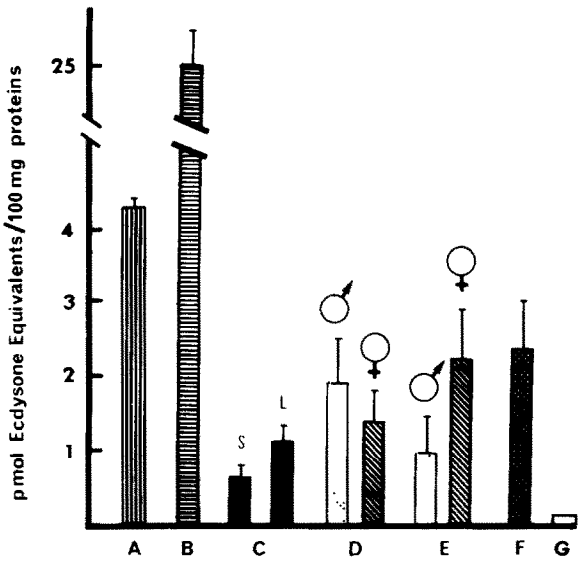

Fig.1. Variation in ecdysone levels during *Schistosoma mansoni* development in vivo using RIA procedure: (A) mechanical schistosomula ($N = 2$); (B) 11-day old worms ($N = 4$); (C) 20-day old worms ($N = 4$). (L) are worms >1.5 mm and (S) are worms <1.5; (D) adult parasites (40-day old) collected by total perfusion of infected hamsters ($N = 6$ for each sex); (E) adult parasites collected only from mesenteric vessels and portal system of animals ($N = 4$); (G) controls performed on uninfected animal tissue ($N > 10$); bars represent SD.

4. DISCUSSION

Combined use of HPLC and RIA has demonstrated for the first time the presence of ecdysone and 20OH ecdysone in a human trematode. In addition, the insect moulting hormones were also formed in *Fasciola hepatica*. The hormonal content of this digenic trematode was comparable to that for *S.masoni* adult females and eggs: 4.4 pmol/100 mg protein (unpublished).

Our work demonstrates that hormonal activity is present at two crucial periods of development in *S.mansoni*. During day 6–day 11 after infection the observed rise in ecdysone levels can be correlated to 3 linked events:

(1) Migration and development of the juvenile worms [9] which have left the lungs and just reached the liver. The process of transformation from the lung to the liver form is charac-

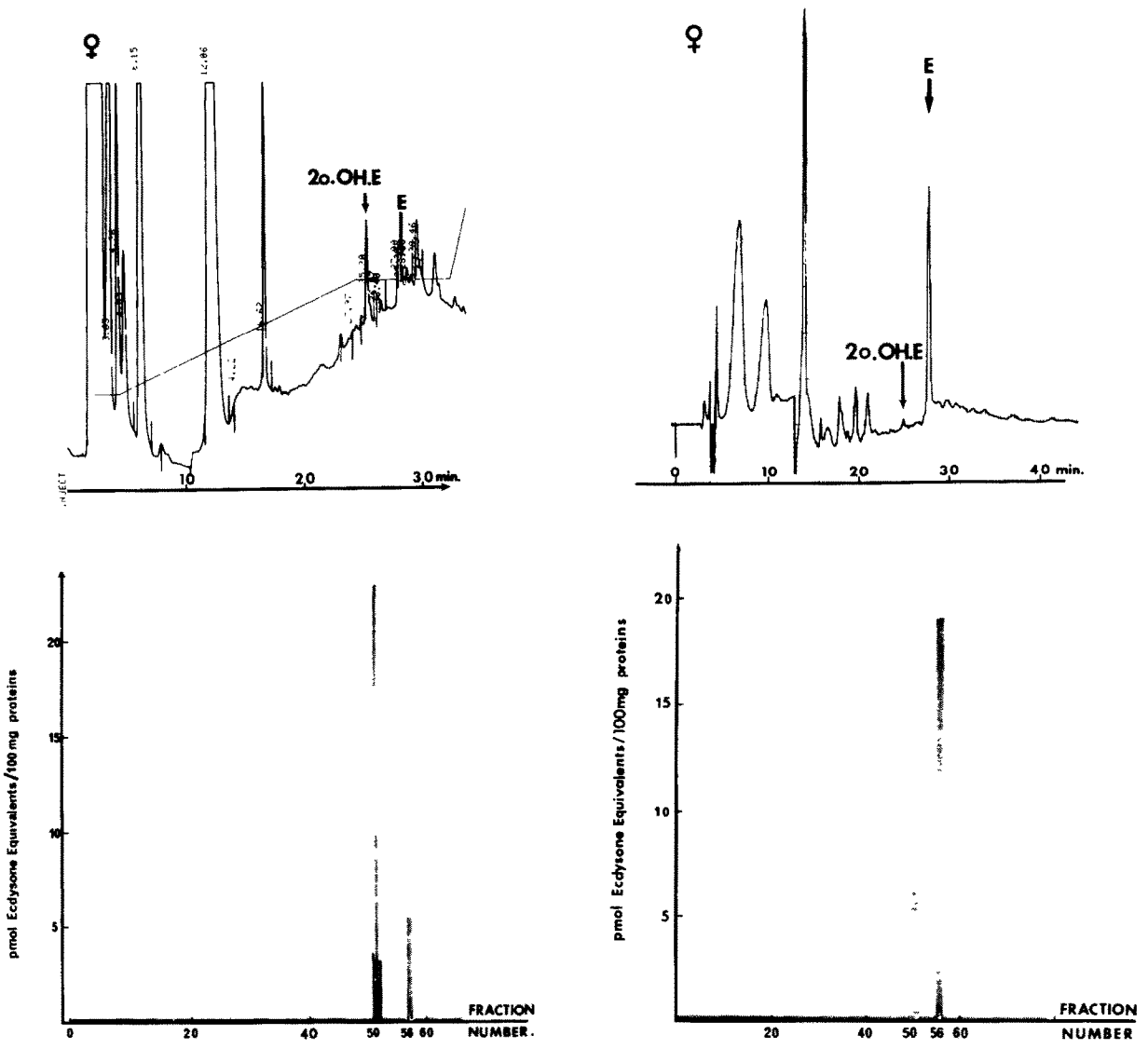

Fig.2. Chromatography of extracts of female schistosomes (top) and radioimmunoassay on collected fraction from high pressure liquid chromatography: (A) female schistosomes from total perfusion of hamsters (Waters data module M 730, system controller M 720); (B) female Schistosomes from mesenteric vessels (Programmator Waters M 650). E = ecdysone; 20E = 20 hydroxyecdysone; Arrows = standard elution of ecdysone (or 20 hydroxyecdysone) from Sigma.

terized by a number of changes in biological functions such as the ability to migrate, length, shape and coiling.

(2) Metabolic changes [10] occur in the parasite which leaves a semi-quiescent metabolic state. An increase in several biological parameters also occurs during this period (density, nitrogen content, growth initiation).

(3) Membrane modifications of *S.mansoni* [2] have been demonstrated by freeze fracture studies. The observation of membrane exuviation on 10–20-day old schistosomes gave rise to the hypothesis of a possible hormonal control of this moulting process. By analogy with the insect model, it is possible that the increase in ecdysone levels at the juvenile period could

participate in the initiation of these phenomena. It is of interest to notice that only the ecdysone form is present at this period.

The results clearly demonstrate that ecdysone is produced by adult schistosomes. Parasites obtained by total perfusion contain ecdysone and 20OH ecdysone. In this case, where all the parasites were collected from infected hamsters, no sexual differences in hormonal levels can be observed by the RIA method. It is commonly accepted that the portal and mesenteric system are the normal location for the parasite for copulation and egg laying. Adult worms, collected only from these locations, exhibit significant sexual differences in hormone levels. These data indirectly suggest an important potential role for these steroids in the induction of the maturation of the female reproductive system. Moreover, the presence of the ecdysone form may be correlated with vitellogenesis [11]. It has been shown that acetone or ether extracts of male schistosomes strongly influence the vitalline cell activity of females [12].

In *Drosophila,* ecdysone stimulates the synthesis and secretion of yolk polypeptides [13] and it is possible that the same could be true for *S.mansoni*. The large amount of the ecdysones found in the eggs suggest an analogous role to that in insects; e.g., in the growth of the ovocyte [14]. In addition, L-dopa decarboxylase, an essential enzyme in the pathway of sclerotinisation, a crucial process for eggshell formation, is known to be under control of ecdysone in insect model [15]. The recent evidence for the presence of L-dopa decarboxylase in adult and larval schistosomes [16] provides the basis for a precise strategy aiming at the correlation of the activity of this essential enzyme with the parasite is production of steroid hormones. Thus, as in insect model, ecdysone and 20OH ecdysone may play a key role in parasite development. Hormonal action on the juvenile form and on adult parasites to brake parasite development or to prevent egg formation could also be considered. An attractive hypothesis would be that ecdysone does not simply accumulate in the eggs as a passive end component, but may be a source of ecdysteroids during embryogenesis.

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REFERENCES

[1] Bennett, J. and Bueding, E. (1971) Comp. Biochem. Physiol. 39A, 857–867.
[2] Torpier, G., Hirn, M.H., Nirde, Ph., De Reggi, M.L. and Capron, A. (1982) Parasitology 84, 123–130.
[3] Lafond, R., Somme-Martin, G., Mauchamp, B., Maume, B.F. and Delbecque, J.P. (1980) Prog. Ecdysone Res. 00, 45–68.
[4] De Reggi, M.L., Hirn, M.H. and Delaage, M.A. (1975) Biochem. Biophys. Res. Commun. 66, 1307–1315.
[5] Ramalho-Pinto, F.F., Gazzinelli, G., Howells, R.E., Motta-Santos, T.A. Figueiredo, E.A. and Pellegrino, J. (1974) Exp. Parasitol. 36, 360–372.
[6] Smithers, S.R. and Terry, R.J. (1965) Parasitology 55, 695–700.
[7] Cailla, H.L., Gros, G.S., Jolu, E.P.T., Delaage, M.A. and Depieds, R.C. (1973) Anal. Biochem. 56, 383–393.
[8] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 165–275.
[9] Wilson, R.A., Draskau, T., Miller, P. and Lawson, J.R. (1978) Parasitology 77, 57–73.
[10] Lawson, J.R. and Wilson, R.A. (1980) Parasitology 81, 325–336.
[11] Hagedorn, H.H., O'Connor, J.D., Fuchs, M.S., Sage, B., Schaeger, D.A. and Bohm, H.K. (1975) Proc. Natl. Acad. Sci. USA 72, 3255–3259.
[12] Shaw, J.R., Marshall, I. and Erasmus, D.A. (1977) Exp. Parasitol. 42, 14–20.
[13] Jowett, T. and Postlethwait, J.H. (1981) Nature 292, 633–635.
[14] Legay, J.M., Calvez, B., Hirn, M.H. and De Reggi, M.L. (1976) Nature 262, 489–490.
[15] Kraminsky, G.P., Clark, W.C., Estelle, M.A., Geitz, R.D., Sage, B.A., O'Connor, J. and Hodgetts, R.B. (1980) Proc. Natl. Acad. Sci. USA 77(7), 4175–4179.
[16] Catto, B.A. (1981) Exp. Parasitol. 51, 152–157.