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THE BINDING PROTEIN AS A MODULATOR OF JUVENILE HORMONE STABILITY  
AND UPTAKE

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INTRODUCTION

Past data on the corpora allata and juvenile hormone (see Gilbert and King, 1973) suggest a highly intermeshed regulatory system which must allow for rapid and dramatic changes in hormone titer as well as fine tuning. An important compartment in this control system is the hemolymph since it serves as the shuttle system between the corpora allata and peripheral tissues. This compartment represents the central mixing point for those factors determining the juvenile hormone (JH) titer, the latter determining the hormone concentration at the target tissues. The processes responsible for the hemolymph pool of JH are: synthesis and release of the hormone by the corpora allata (e.g. Röller and Dahm, 1970; Judy et al., 1973; Pratt and Tobe, 1974); under certain conditions there may be a reflux from tissues; metabolic inactivation; and tissue uptake with subsequent degradation and/or excretion.

Recent studies have demonstrated that JH binds specifically to certain hemolymph proteins (Whitmore and Gilbert, 1972; Emmerich and Hartmann, 1973; Goodman and Gilbert, 1974; Kramer et al., 1974). By interaction with these proteins, JH can exist in both a bound and free state, the ratio of which is a function of the total amount of binding proteins present and their binding constants (see Goodman et al., Kramer et al., this volume). As a consequence, this must affect properties of the pool such as metabolic rate, rate of equilibration with other pools, and pool size. In the present paper we report on the roles of the high affinity binding protein in Manduca sexta in the regulation of JH content in hemolymph and other tissues. Since the fat body plays a crucial role in JH metabolism (e.g. Hammock et al., 1975), we have utilized the fat body in organ culture as our primary test system.

## MATERIALS AND METHODS

Experimental Animals. *Manduca sexta* larvae were reared on an artificial diet as described previously (Sroka and Gilbert, 1971) under a 16L:8D photoperiod.

Juvenile Hormone. [7-Ethyl-1,2-<sup>3</sup>H(N)]-JH I (11.17 Ci/mmol) was purchased from New England Nuclear while [<sup>3</sup>H]methoxy-JH I (isomeric mixture, 4.34 Ci/mmol) was a generous gift from K.H. Trautmann (Dr. Maag AG). Radiolabeled JH was used directly or diluted with cold JH I (95% t,t,c, courtesy of K. Dahm). Stock solutions were prepared in benzene:hexane (4:1) or methanol and concentrations were determined by liquid scintillation spectrometry of [<sup>3</sup>H]-JH or from absorbance values of methanolic solutions (cold JH) using a molar extinction coefficient  $\epsilon_{220} = 13,830 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$  (Trautmann et al., 1974). Highly concentrated stock solutions of JH ( $10^{-3} \text{ M}$ ) were obtained by weighing. [<sup>3</sup>H]-JH acid was prepared from chain labeled JH I by esterase hydrolysis in fifth instar larval hemolymph.

In Vitro Procedures. Fat body was removed from larvae as two coherent organs, rinsed twice in Grace's medium and finally washed in the medium to be used for incubation. Under short-term conditions ( $\leq 2 \text{ hr}$ ), fat body was cultured in Grace's medium at pH 6.6 (Grand Island Biological Co.) or in a Ringer's solution containing 1.5 mM  $\text{Na}_2\text{HPO}_4$ , 54.4 mM KCl, 10.5 mM  $\text{CaCl}_2$  and 60.4 mM  $\text{MgCl}_2 \cdot 6 \text{ H}_2\text{O}$ , adjusted with HCl to pH 6.6. For incubations over longer periods of time a specially devised *Manduca* culture medium (Nowock et al., 1975) was used and all procedures carried out under sterile conditions.

All incubations involving JH were carried out in glass vials coated with carbowax 20 M (polyethylene glycol;  $M_n$  20,000) or silicone to reduce adsorption of the hormone. JH<sub>r</sub> was transferred from stock solutions into the culture vials and after evaporation of the organic solvent under  $\text{N}_2$ , medium was added. The hormone was resolubilized by sonication, or by preincubation for at least 30 min when the medium contained proteins. Incubations were carried out in a shaking water bath at 26°C unless otherwise stated.

Binding Protein Preparation. Hemolymph from day 3 and day 4 fifth instar larvae was used to prepare a partially purified binding protein fraction (BPF). Two fractionation procedures were employed, the first already described in detail by Hammock et al. (1975) and involving treatment of cell-free hemolymph with diisopropyl fluorophosphate (DFP) to inhibit esterases, followed by filtration through Sephadex G-150 (0.1 M phosphate, pH 7.4). Fractions with binding activity were concentrated and passed through Sephadex G-75 (0.1 M phosphate, pH 7.4). Binding activity in the eluted fractions was determined by charcoal assay using [<sup>3</sup>H]-JH I as ligand (Hammock

et al., 1975). The active fractions from the G-75 column were lyophilized and desalted by passage through a Sephadex G-25 column equilibrated with Grace's medium. For *in vitro* studies, the binding activity was adjusted by charcoal assay to the activity of DFP-treated L5D3 hemolymph. (L denotes instar while D indicates day of instar in the previous notation, i.e. L5D3.)

An improved two-step procedure was used to prepare BPF for the studies on JH-uptake by fat body (see Goodman et al., this volume). Cell-free hemolymph was saturated to 70% with  $(\text{NH}_4)_2\text{SO}_4$ . The precipitate was redissolved in 0.05 M phosphate buffer, pH 6.6 containing 0.05 M KCl, dialyzed and passed through Sephadex G-150 (same buffer). The active fractions were concentrated by  $(\text{NH}_4)_2\text{SO}_4$  precipitation, the proteins redissolved and dialyzed against 0.05 M acetate buffer, pH 5.0 with 0.01 M KCl, and applied to a Whatman CM 52 column equilibrated with the acetate buffer. BP is uncharged at pH 5.0 and was therefore not retained by the resin. The active fractions were concentrated and dialyzed against Ringer's solution. In this preparation, BP was contaminated by three or four other proteins as determined by gel electrophoresis and no esterase activity was detected.

The concentration of binding sites in this preparation was determined by a saturation procedure (Westphal, 1967) using charcoal as adsorbent to separate bound from free hormone. Optimal conditions for the charcoal adsorption were found to be the same as described by Goodman et al. (this volume).

Extraction, Separation and Characterization of Metabolites. Extraction procedures, separation and identification of JH and metabolites were described previously (Hammock et al., 1975). Recovery was >90% of the total radioactivity applied.

Determination of Esterase Activity. The hydrolytic activity of hemolymph samples and fat body culture media was assayed with  $\alpha$ -naphthyl acetate ( $\alpha$ -NA) and JH as substrates to distinguish in a crude way between "general" and "JH specific" esterases (Sanburg et al., 1975).  $\alpha$ -NA hydrolytic activity was determined spectrophotometrically (van Asperen, 1962). JH-hydrolytic activity was assayed by one of the following methods. Using chain-labeled JH as substrate, quantitation was achieved by extraction of the reaction mixture with ethyl acetate followed by thin layer chromatographic (TLC) separation and subsequent radioassay of the appropriate zones of the silica gel plates (Weirich et al., 1973). A less laborious method was developed with [ $^3\text{H}$ ]-methoxy-JH I. This assay is based on the partition of the generated methanol in a two-phase system (see also Sanburg et al., 1975). Under standard assay conditions 170  $\mu\text{l}$  of  $10^{-5}$  M JH in 0.05 M phosphate buffer (pH 6.6) were mixed with 50  $\mu\text{l}$  of appropriately diluted enzyme solution in carbowed test tubes (7 x 20 mm) and incubated in a shaking water

bath at 25°C for 30 min. The reaction was terminated by adding 200  $\mu$ l methanol and 500  $\mu$ l chloroform. The mixture was vortexed and the phases separated by centrifugation at 2,000 g for 5 min. The phases separate in the ratio 19:27 (v/v; epi-/hypophase). The methanol (80  $\pm$  2%) appeared in the epiphase. Partition of unreacted JH into this phase was negligible. The assay showed proportionality between hydrolysis rate and enzyme concentration at up to 10% substrate conversion.

Polyacrylamide gel electrophoresis was performed with an Ortec 4200 system as described (Nowock et al., 1975). Protein was determined by a modified Lowry procedure (Low et al., 1969).

Determination of JH Uptake. Fat body from L4D2 larvae (0.90-0.95 g) was used for the uptake studies. Tissues were preincubated for 30 min at room temperature with two changes of medium to allow for degradation of endogenous JH. Since endogenous titers were not measured, all calculations assume that residual hormone levels after preincubation are negligible. Fat body was finally rinsed for 5 min in Ringer's solution at incubation temperature. One fat body strand was placed in a culture vial containing 2 ml incubation mixture from which 20  $\mu$ l aliquots were taken at various time intervals. Uptake into tissue was calculated from the depletion of radiolabel from the medium. Incubation of fat body with inulin-[carboxyl-<sup>14</sup>C] demonstrated the absence of extracellular spaces in which label could distribute and be retained. Initial uptake rates were estimated by the 1 min uptake data.

Reversibility of JH-uptake was determined by pre-incubating fat body for 30 min in medium containing JH and reincubating the tissue in JH-free medium after an intermittent rinse in ice cold Ringer's for 20 sec. In several cases media and tissues were extracted with ethyl acetate at the end of the incubation period and the extent of JH degradation was determined (Hammock et al., 1975).

## RESULTS

### Effect of BP on JH Metabolism

Distribution of JH and Metabolites in Fat Body and Medium. To glean preliminary information on the uptake and metabolism of JH and the possible role of binding protein in these processes, fat body from fourth and fifth instar larvae were incubated with [<sup>3</sup>H]-JH in the presence or absence of BPF. BPF was replaced by an equal quantity of bovine serum albumin (crystalline BSA; Miles, Inc.) in the control samples since BSA binds JH only weakly and

unspecifically. The distribution of total label as well as JH and metabolites was monitored as a function of time. The data reveal that in the absence of BPF, label is rapidly taken up by the tissues (Fig. 1). Differences exhibited between the two developmental stages presumably reflect differences in the amount of tissue. The cultured fat body metabolized JH very efficiently with fifth instar tissue exhibiting the higher rate of degradation. However, at both developmental stages a fairly constant level of JH was not degraded during the incubation period indicating that some JH is retained in compartments not accessible to degradative enzymes. In the presence of BPF both the uptake of JH by the tissues and its subsequent metabolism were remarkably reduced.

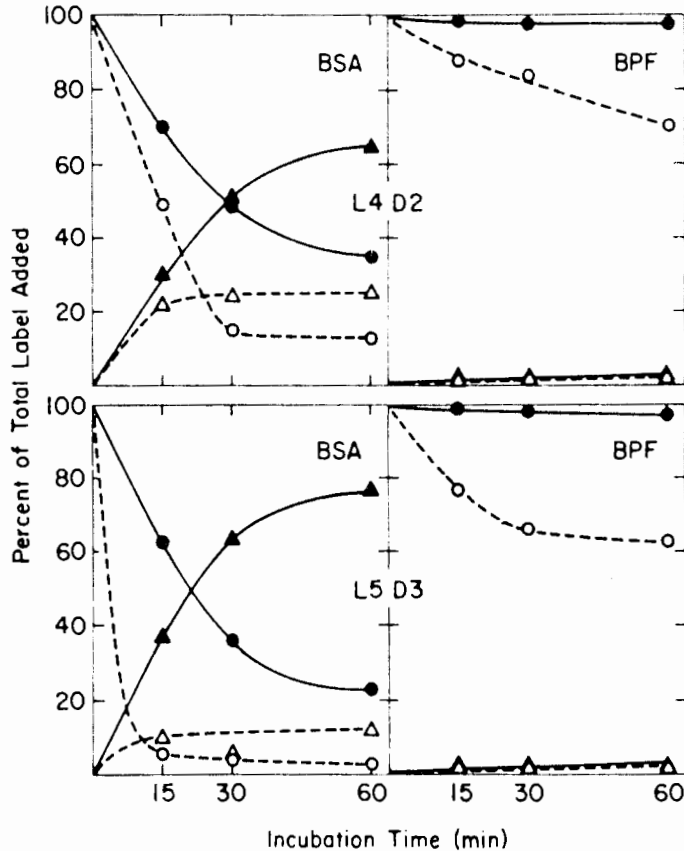


Fig. 1. Time course analysis of the distribution of JH and metabolites between fat body and culture medium. Fat body from L4D2 or L5D3 larvae was incubated in Grace's medium, pH 6.6 containing  $10^{-8}$  M [ $^3$ H]-JH and BPF or BSA. Media and tissue homogenates were extracted and analyzed by TLC for JH and metabolites. Curves represent content of total [ $^3$ H] in medium (●-●) or fat body (▲-▲) and [ $^3$ H] as JH in medium (○--○) or fat body (△--△) expressed as percent of total label added.

To determine the nature of the JH metabolites, extracts of media and tissue homogenates were analyzed by TLC both before and after derivatization. The major metabolites noted were the acid and diol acid while diol was only found in small amounts. Trace amounts of polar metabolites remained at the origin under the TLC conditions employed (Fig. 2) and no oxidative products were detected. To generally localize the catabolic enzymes of the tissue, fat body homogenates were subjected to differential centrifugation. JH degradative activity resided primarily in the soluble fraction and was a consequence of esterase activity, whereas epoxide hydratase activity was associated with the microsomal fraction (Hammock et al., 1975).

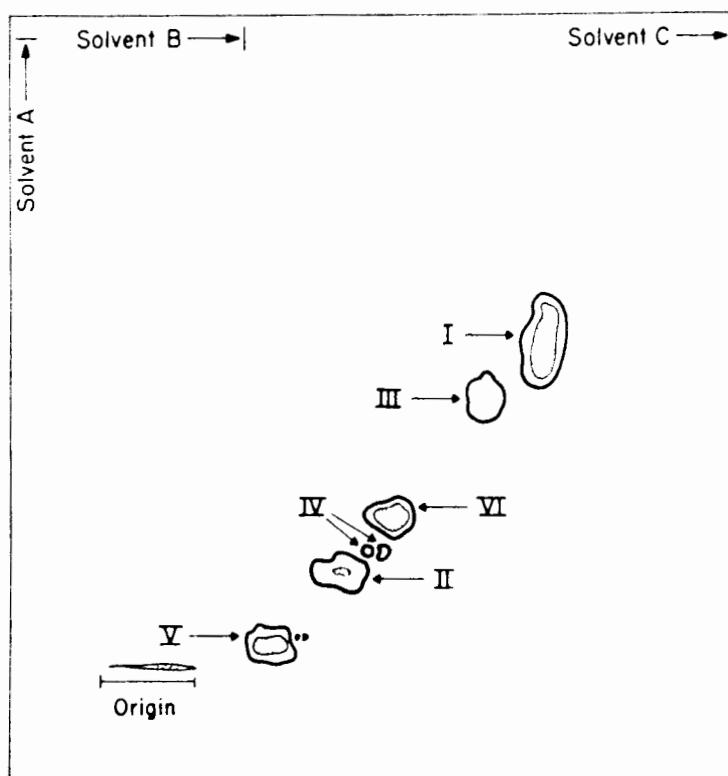


Fig. 2. Scheme of two-dimensional thin-layer chromatography of JH metabolites (I) JH, (II) diol, (III) diepoxide, (IV) tetrahydrofuran diols, (V) acid diol and (VI) acid. Solvent A = hexane:ethyl acetate (2:1); solvent B = benzene:n-propanol (4:1); solvent C = benzene:n-propanol (10:1). Open spots designate metabolite standards while stippled areas represent the result of autoradiographic determination of metabolites from [ $^3\text{H}$ ]-JH (from Hammock et al., 1975).

Although the metabolic pattern was qualitatively identical in all cases, quantitative differences were noted. After 1 hr of incubation the fat body yielded approximately equal amounts of acid and diol acid as did the media devoid of exogenous BPF. With increasing time of incubation a shift towards the diol acid was observed and the diol acid appears to be the final, primary metabolite in this system. However, when BPF is present in the medium, JH acid was the predominant metabolite. The reduction in the quantity of diol and diol acid in the medium in the presence of BPF suggests that BPF inhibits the uptake of JH by the fat body since epoxide hydratases are microsomal enzymes. The occurrence of JH acid in the medium can be a result of either selective transport out of the tissue or the hydrolysis of JH in the medium by esterases released from the fat body. The former alternative can be excluded because a substantial amount of acid was present in the fat body after incubation in a BPF-free medium.

Release of Esterases by the Fat Body. To test the possibility that the fat body actively secretes esterases into the medium, tissues were preincubated for 1 hr, removed, and the preconditioned media were divided into aliquots and combined with an equal amount of fresh medium containing JH and BPF or BSA. When the metabolic activity of these media was assayed, it was found that they degraded the JH to the acid with essentially no other identifiable metabolites being present (Fig. 3). Enzyme activity was greatest in preconditioned medium derived from L5D3 fat body and the presence of BPF dramatically reduced the rate of ester hydrolysis. In media derived from L4D2 fat body, acid formation was nearly completely suppressed whereas some residual activity remained in the L5D3 cultures. JH was not protected by BP from this residual esterase activity. Analysis of the preconditioned media with  $\alpha$ -naphthyl acetate as substrate revealed a higher activity in the L4D2 medium. Since BP appears to protect JH from the attack of "general" esterases but offers only minimal protection from "JH specific" esterases (Sanburg *et al.*, 1975; Hammock *et al.*, 1975) we conclude that L4D2 fat body secreted a predominance of "general" esterases into the medium whereas L5D3 fat body also released a substantial quantity of "JH specific" esterases. We are using the term "general" esterase as defined by Sanburg *et al.* (1975) as those esterases which are capable of degrading free JH, but show little preference for JH over  $\alpha$ -NA as a substrate. It should be kept in mind that the juvenile hormones are enoate esters and thus are quite stable to base hydrolysis (Sanburg *et al.*, 1975), and esterases from many sources are not capable of hydrolyzing JH (unpublished information). Esterases from several insects show very different elution profiles upon Sephadex chromatography when *p*-nitrophenyl acetate,  $\alpha$ -NA and JH are used as substrates and their relative inhibition by many compounds is quite different. Thus, these "general" esterases show specificity for JH but are unable to attack JH when it is complexed to BP.

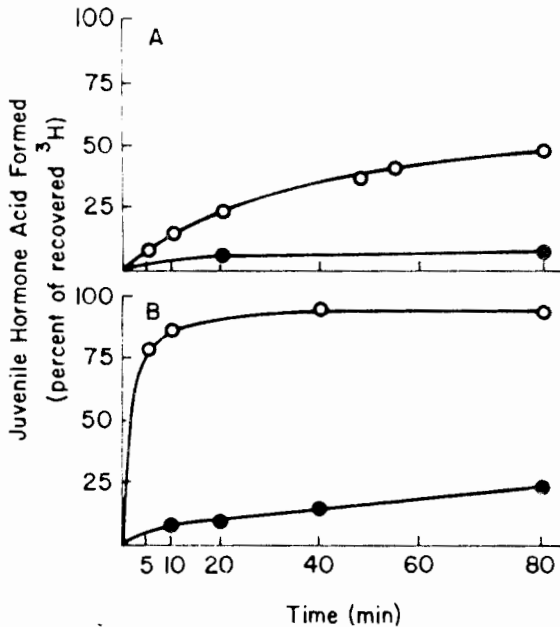


Fig. 3. Hydrolytic activity of esterases released from the fat body into the culture medium. Tissue of approximately the same weight from L4D2 (A) and L5D3 larvae (B) was incubated for 1 hr, after which the tissue was removed. Aliquots of the preconditioned media were combined with equal volumes of medium containing [ $^3\text{H}$ ]-JH in the presence (●-●) or absence (o-o) of BPF, and JH acid formation was monitored (from Hammock *et al.*, 1975).

The activity patterns in the preconditioned media were in accord with those found in hemolymph from the same developmental stage (Weirich *et al.*, 1973; Sanburg *et al.*, 1975). This suggests that hemolymph esterases originate in the fat body. To test this possibility a developmental approach was utilized in which we compared the hydrolytic activity of hemolymph and media preconditioned with fat body, both derived from the same stage of the last larval instar. The developmental pattern of hemolymph esterases exhibited a maximum at days 3 and 4, irrespective of whether  $\alpha$ -naphthyl acetate or JH served as substrate (Fig. 4). In the preconditioned media, total and DFP-resistant (i.e. "JH specific" according to Sanburg *et al.*, 1975) hydrolytic activity increased during the first 3 days of the instar and decreased thereafter.  $\alpha$ -NA hydrolytic activity peaked at day 4 and seems to be separately controlled. Slab gel electrophoresis and subsequent staining with  $\alpha$ -NA and fast blue revealed the same pattern of "general" esterases in hemolymph and incubation medium from the same stage. Indeed, the  $R_f$  values for particular esterase bands were identical. In



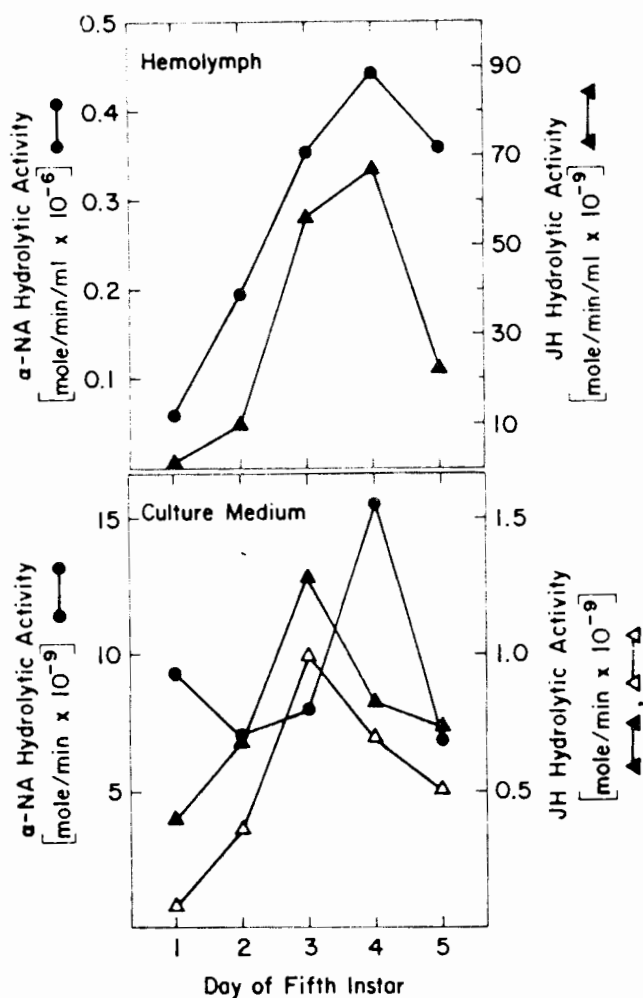


Fig. 4. Developmental analysis of esterase activity in hemolymph and culture medium preconditioned with fat body. Hydrolytic activity was determined with  $\alpha$ -NA and chain-labeled [ $^3$ H]-JH (hemolymph) or [ $^3$ H]-methoxy-JH (culture media) as substrates as described in Materials and Methods. Fat body of various stages was cultured *in vitro* for 24 hr and the media analyzed for esterase activity. Closed triangles indicate total JH hydrolytic activity. A 15 min treatment with DFP ( $10^{-4}$  M) revealed DFP resistant (i.e. "JH specific") esterase activity. Hydrolytic activity in culture media is expressed per mg fat body protein.

contrast, analysis of fat body homogenates yielded a more complex esterase pattern indicating that the esterases found in the medium are specifically released from the fat body and are not a result of leakage or cell breakdown.

Both the developmental pattern of hydrolytic activity and the electrophoretic mobility of the "general" esterases support the assumption that hemolymph esterases originate in the fat body. However, additional information is required to definitively prove that the esterases released in vitro are identical to those occurring in the hemolymph.

#### Effect of BP on JH Uptake Into Tissues

Effect of JH Concentration and Temperature on Uptake. The ideal experimental situation for the study of JH transport between hemolymph and fat body precludes the utilization of heterogeneous tissue, the existence of extracellular spaces and degradation of the hormone. Unfortunately, this ideal model was not found in Nanduca, especially in regard to JH metabolism. Theoretically, one might circumvent the latter problem with the use of juvenoids possessing high metabolic stability (see Hammock and Quistad, this volume). However, these juvenoids do not bind to the hemolymph BP or show such weak affinity (see Goodman et al., this volume) that they would be useless for studying BP involvement on hormone uptake. As a compromise, we therefore selected fourth instar fat body despite its metabolic activity and the fact that it releases both esterases and BP into the medium (Nowock et al., 1975). It does however have the appropriate morphological properties for such studies and can be obtained easily in sufficient quantities.

Fat body was incubated in Ringer's solution containing either  $8 \times 10^{-9}$ ,  $10^{-7}$ ,  $10^{-6}$  or  $10^{-5}$  M [ $^3\text{H}$ ]-JH, and changes in the radiolabel of the medium were monitored over time at both  $4^\circ$  and  $26^\circ\text{C}$ . When the fractional uptake of radiolabel was plotted against time, no differences between the various JH concentrations were noted within a particular temperature series. Therefore, the data of the  $4^\circ$  and  $26^\circ\text{C}$  regimens respectively, were pooled (Fig. 5). At  $26^\circ\text{C}$  label was taken up maximally after 10 min, but subsequently there tended to be a net back flow into the medium. At the lower incubation temperature radiolabel was taken up at a slightly decreased rate, although the difference is not statistically significant, and equilibrium was achieved after about 30 min. By extending the incubation time, a net reflux at a low rate was noted here as well. Analysis of extracts of the media revealed that up to 70% of the initial JH was metabolized at  $26^\circ\text{C}$ , mainly to the acid and acid diol after 30 min. Incubation at  $4^\circ\text{C}$  did not inhibit degradation completely as 25% of the JH was still metabolized, predominantly to the acid. The net reflux of radiolabel from the fat body into

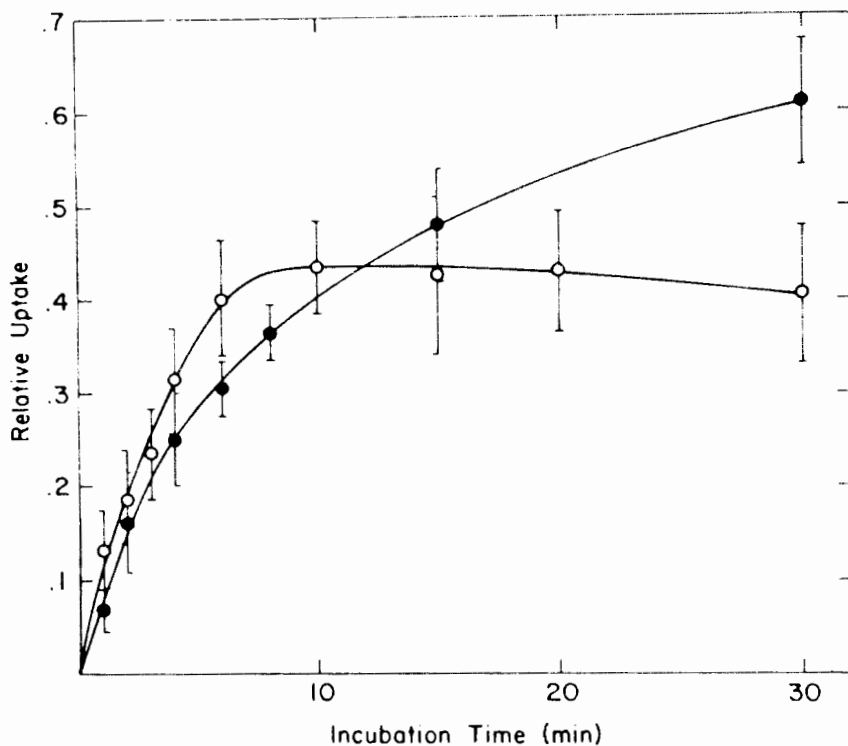


Fig. 5. Time course of radiolabel uptake by fat body incubated in [ $^3\text{H}$ ]-JH. Tissue of comparable weight was incubated in Ringer's solution containing various concentrations of JH ( $8 \times 10^{-9}$ ,  $10^{-7}$ ,  $10^{-6}$ , and  $10^{-5}$  M) and uptake was assayed as described in Materials and Methods. Each concentration series was run at  $4^\circ\text{C}$  ( $\bullet$ — $\bullet$ ) and  $26^\circ\text{C}$  ( $\circ$ — $\circ$ ). As data for fractional uptake showed no significant differences between the JH concentrations tested at a certain temperature, they were pooled and expressed as means  $\pm$  S.D.  $n = 8$  ( $4^\circ\text{C}$ ) and 12 ( $26^\circ\text{C}$ ), respectively.

the medium seems to be principally a function of JH degradation since it was not observed with isopropyl-11-methoxy-3,7,11-trimethyl-trans-2,trans-4 dodecadienoate (Altosid, Zoecon Corp.) which was extremely stable under our in vitro conditions. Interaction with BP which might arise from the fat body during incubation cannot be excluded as a possibility, but it should not have a significant effect, especially at higher JH concentrations.

The similarity of fractional uptake within a concentration series indicated that uptake was not saturable over the tested range, but rather that the rate of uptake was directly proportional to the external concentration of JH. This corrects an earlier

preliminary observation (Hammock et al., 1975). When the initial uptake rate, for which the 1 min data were used as approximations, was plotted against the initial JH concentration, a linear relationship was obtained having the first-order rate constants of  $0.77 \times 10^{-4} \text{ min}^{-1}$  ( $4^\circ\text{C}$ ) and  $1.20 \times 10^{-4} \text{ min}^{-1}$  ( $26^\circ\text{C}$ ) (Fig. 6).

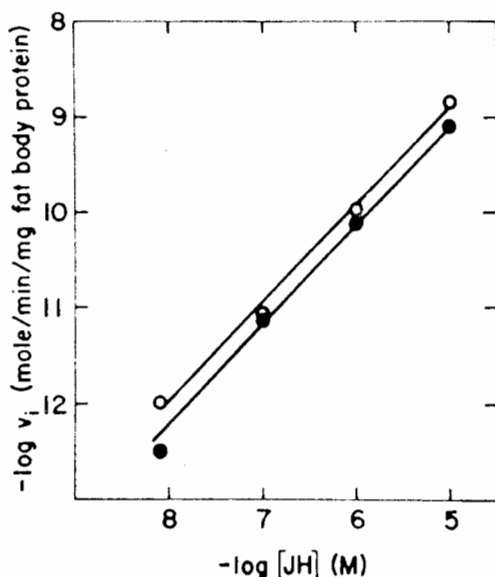


Fig. 6. Initial uptake rate as a function of external JH concentration. Data are from the same experiment as presented in Fig. 5. Determinations were performed at  $4^\circ\text{C}$  (●—●) or  $26^\circ\text{C}$  (○—○).

However, the difference in rate constants at the two temperatures is not statistically significant. Since uptake was not greatly influenced by temperature, further experiments were performed at  $4^\circ\text{C}$  to minimize interference due to JH degradation and the possible release of BP into the medium.

When fat body was incubated in  $10^{-8} \text{ M}$  [ $^3\text{H}$ ]-JH acid, both the initial uptake rate ( $0.38 \times 10^{-12} \text{ mol/min/mg}$ ) and total uptake (20%) were significantly reduced when compared to JH. After 30 min of incubation, about 26% of the acid was further metabolized to the acid diol.

Effect of BP. When free JH rather than JH-BP complex is taken up by the fat body, or when the rate of uptake of the complex is low when compared to free JH, the uptake rate in the presence of BP should be proportional to the concentration of unbound hormone. To

test this assumption,  $10^{-8}$  M JH was incubated with increasing amounts of BP. The controls received equal amounts of BSA. After equilibration, fat body was added and the initial uptake rates determined. From the dissociation constant of the JH-BP complex,  $K_D = 3 \times 10^{-7}$  M (Kramer et al., 1974), the concentration of free hormone can be calculated for a particular  $[JH]/[BP]$  ratio. Using these concentrations and the estimate of the first order rate constant of the uptake process ( $0.77 \times 10^{-4} \text{ min}^{-1}$ ), the initial uptake rates can be obtained and compared with the experimental data. Figure 7 shows that a decrease in the  $[JH]/[\text{binding sites}]$  ratio, i.e. a decrease in the concentration of free hormone, resulted in a lower uptake rate although somewhat greater than expected from theoretical considerations. This may be a consequence of experimental variability, deviation from the linear relationship between JH concentration and uptake rate at lower  $[JH]/[BP]$  ratios, or to the interference of the uptake process by non-binding proteins present in the BPF-preparation. Relatively high concentrations of BSA also slightly reduced the rate of uptake.

Reversibility of Uptake. Fat body was preincubated in  $10^{-8}$  M  $[^3\text{H}]\text{-JH}$  for 30 min, briefly rinsed and transferred to fresh Ringer's solution or medium containing BPF or BSA. Similar amounts of radiolabel were taken up by the tissues (47-56%) during preincubation and the wash fluid contained  $\leq 1.5\%$  of the total label initially applied. Under all conditions tested a reflux of radiolabel occurred (Fig. 8) and was most prominent in the media containing BPF. After 90 min, 75% and 84% of the label taken up was released again depending on the number of binding sites present ( $10^{-8}$ ;  $10^{-7}$  M, respectively). This suggests that only a minute amount of JH is tightly bound to the cells. Testing media with two concentrations of BSA yielded a similar rate and amount of reflux. (It should be noted that up to 5% of the total label released into Ringer's solution was adsorbed to the vial wall compared to  $\leq 1\%$  when proteins are present in the medium.) As JH metabolism still occurred during incubation at  $4^\circ\text{C}$ , it was necessary to ascertain whether BPF favored the preferential reflux of JH or its metabolites. Fat body was incubated in the presence of  $10^{-8}$  M  $[^3\text{H}]\text{-JH}$  for 30 min and reincubated as noted above for another 30 min. Tissue homogenates and media from both incubation periods were extracted with ethyl acetate and analyzed by TLC. In all cases the dominant metabolite was the acid. The data pertaining to the distribution between JH and total metabolites in both compartments (Table 1) are difficult to interpret since degradation occurs both within the tissue and in the medium, creating a complex pattern of fluxes of the various compounds. However, it is evident that under all experimental conditions there was consistently more JH than metabolites in the fat body. This differs from the situation in the incubation media and confirms our earlier observation that JH tends to partition into compartments within the fat body where it is not easily accessible to degradative

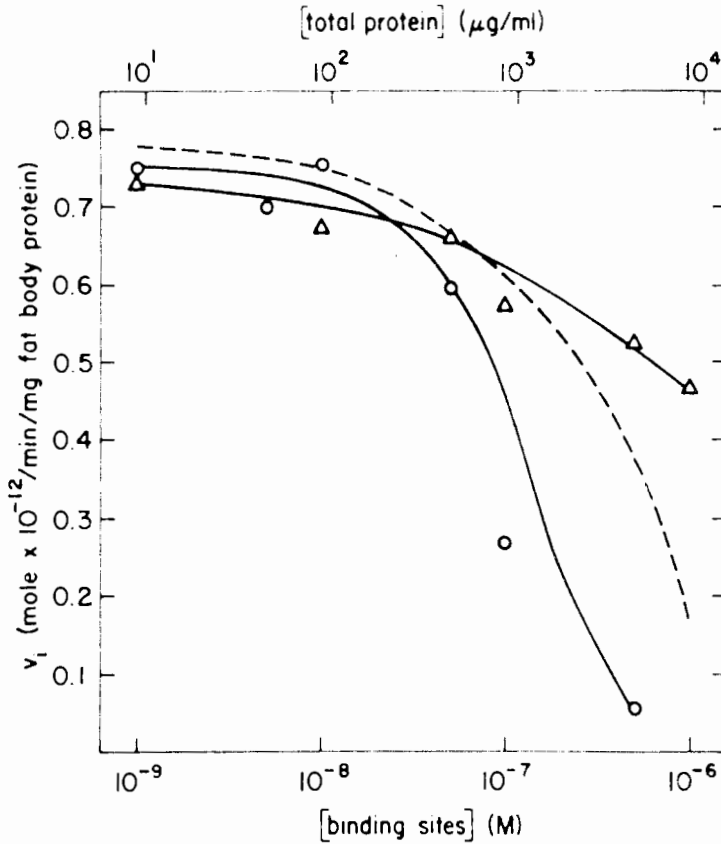


Fig. 7. Effect of BP on the rate of uptake of JH by the fat body. [<sup>3</sup>H]-JH (10<sup>-8</sup> M) was equilibrated with increasing concentrations of BPF, fat body was added and the initial uptake rate determined (o—o). In the controls, BPF was replaced by BSA (Δ—Δ). Assuming that the uptake rate is proportional to the concentration of unbound JH, initial uptake rates were calculated for various [JH]/[binding sites] ratios using a dissociation constant of  $K_D = 3 \times 10^{-7}$  M and a rate constant for the uptake of  $0.77 \times 10^{-4} \text{ min}^{-1}$  (dashed line).

enzymes. When protein-free Ringer's solution was utilized as the second incubation medium, a substantial amount of metabolites was retained in the tissue. Reincubation in either protein-containing medium resulted again in a low level of metabolites being present in the fat body. This observation was also true of the BPF medium, whereas the BSA medium contained slightly more metabolites (mainly acid) than JH. The accumulation of acid could be due to: (1) generation from JH in the medium mediated by esterases originating

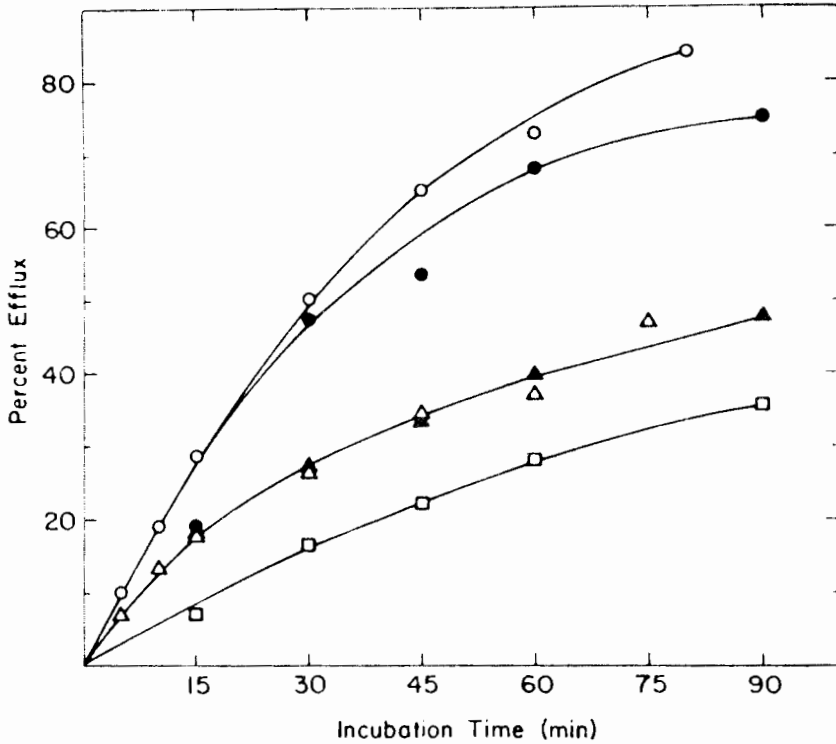


Fig. 8. Reversibility of uptake. Fat body was incubated for 30 min in  $10^{-8}$  M [ $^3\text{H}$ ]-JH, briefly rinsed and re-incubated in either protein-free Ringer's solution (squares) or in Ringer's containing BPF or BSA. Concentration of binding sites:  $10^{-8}$  M (●-●) and  $10^{-7}$  M (○-○); concentration of BSA: 85.5  $\mu\text{g}/\text{ml}$  (▲-▲) and 855  $\mu\text{g}/\text{ml}$  ( $\Delta$ - $\Delta$ ).

from the fat body; (2) release from the tissue by an enhanced efflux in the presence of BSA in the medium; (3) a combination of both processes.

Upon analyzing the reversibility of uptake after pre-incubating fat body in the presence of [ $^3\text{H}$ ]-JH acid, we found that of the radiolabel initially taken up, 60% was released into Ringer's solution, 79% into BSA-medium and 86% into BPF medium after 30 min. Thus, as with the case of JH, the presence of proteins in the medium enhances the reflux of the acid and acid diol formed during incubation. Under all conditions utilized here the rate of radiolabel release was greater when fat body was pre-incubated with [ $^3\text{H}$ ]-JH acid as compared to [ $^3\text{H}$ ]-JH. In this context, it is of interest that after pre-incubation with JH only a small amount of metabolites was released when the fat body was transferred to fresh Ringer's solution devoid of proteins (Table 1). However, a rapid reflux was noted after pre-incubation with JH acid. This suggests

TABLE 1. DISTRIBUTION OF JH AND METABOLITES AFTER PRE-INCUBATION OF FAT BODY WITH JH AND RE-INCUBATION IN DIFFERENT MEDIA.

Incubation medium	Percent of extractable label							
	1st Incubation				2nd Incubation			
	Medium	Met.	Fat body	Incubation medium	Medium	Met.	Fat body	Met.
	33	12	47	8				
[ <sup>3</sup> H]-JH in	39	16	(45)	Ringer's	6	15	45	34
Ringer's	30	5	(65)	Ringer's + BSA	21	26	41	13
	37	15	(48)	Ringer's + BPF	47	11	29	13

Tissues were incubated in  $10^{-8}$  M [<sup>3</sup>H]-JH in Ringer's solution for 30 min and transferred to protein-free medium or medium containing BPF ( $10^{-8}$  M binding sites) or the same amount of BSA for an additional 30 min. Media and fat body homogenates were extracted and analyzed by TLC. The distribution of JH and metabolites (Met.) between medium and tissue is expressed as percent of total label extractable from each incubation (medium + fat body). Data are the means of two determinations.



that metabolites generated internally from JH and those taken up from an external pool are contained in different cellular compartments within the fat body.

#### DISCUSSION

Fat body inactivates JH in vitro by ester hydrolysis and epoxide hydration, which agrees with the pathways established in a variety of insects (Slade and Zibitt, 1972; White, 1972; Ajami and Riddiford, 1973; Slade and Wilkinson, 1974). Oxidative pathways accounted for negligible metabolism in the fat body, although they may be more important in other tissues such as midgut. Conjugate formation was of no significance, at least under the short-term in vitro conditions employed here. This is not surprising since conjugation occurs with great efficiency in gut and Malpighian tubules and is probably related to excretion (Slade and Wilkinson, 1974; Erley et al., 1975). Since epoxide hydratases are membrane bound (Hammock et al., 1975), diol formation requires the uptake of JH into the tissues. The esterase situation was more complex since ester hydrolysis of JH occurred in the medium due to the release of carboxyl esterases from the fat body as well as in the fat body itself. The data strongly suggest that the esterases normally present in the hemolymph are identical to those detected in the incubation medium and that the fat body is the source of these enzymes.

In our in vitro system the presence of BP in the medium affected JH metabolism in two ways. First, BP decreased the uptake of JH into the fat body and thereby reduced intracellular degradation. Second, it protected JH against extracellular esterases with the exception of the so-called "JH specific" esterases (Sanburg et al., 1975) which could mediate JH hydrolysis even when the hormone was complexed to BP. Since JH is transported from the corpora allata to peripheral tissues via the hemolymph, the concentration of BP and activity of esterases in the hemolymph determine to a great extent the JH titer at, or within, target cells. The presence of BP in fourth and fifth instar larvae is well established (Kramer et al., 1974; Goodman and Gilbert, 1974; Gilbert et al., 1976) as are "general" esterases of moderate or high activity (Weirich et al., 1973; Sanburg et al., 1975). "JH specific" esterases are barely detectable in the hemolymph of fourth instar larvae, but exhibit a remarkable increase in activity at the middle of the fifth instar which is followed by a rapid decline. These data are supportive of accepted views of JH requirements for larval development and metamorphosis (Gilbert and King, 1973). The very low activity of "JH specific" esterases and the protection afforded by BP against general esterases in fourth instar hemolymph favor the maintenance of a relatively high JH titer which is requisite for a larval-larval molt. The dramatic

increase in "JH specific" esterase activity during the fifth instar clears JH from the hemolymph as would be required for a larval-pupal molt. However, the situation is more complex and the above explanation is rather simplistic. When assaying the hemolymph of ligated fourth instar larvae by JH bioassay, Fain and Riddiford (1975) determined the half life of endogenous JH to be the surprisingly low value of 90 min. Although the bioassay data alone do not allow a decision between JH decline due to inactivation or tissue uptake, they do demonstrate that the presence of BP is not in itself sufficient for the extended persistence of JH in the hemolymph. The observation that the half life of JH in fifth instar larvae (when esterase activity is high) is even shorter (<30 min), nevertheless indicates a protective role for BP. Nijhout and Williams (1975) found that the JH titer of fifth instar larvae begins to decline when the animals attain a weight of approximately 5 g. The decline requires slightly greater than 24 hr and coincides exactly with the stage at which we found "JH esterase" activity to be maximal. However, since endogenous JH at this stage does not persist longer than 2 hr (Nijhout, 1975), JH release by the corpora allata must still continue during the time that the hemolymph titer is gradually decreasing. This indicates that the control of JH production and release may be as important a component of the JH titer regulating system as the esterase, BP components. It is obvious that the roles of the "JH specific" esterases must be investigated further.

The uptake of JH by the fat body followed first-order kinetics, i.e. the initial uptake rate was proportional to the hormone concentration in the medium over the range of  $8 \times 10^{-9}$  to  $10^{-5}$  M. Due to both the nature of the experimental regimes and the relatively low specific activity of the [ $^3$ H]-JH, lower JH concentrations could not be tested. When JH was applied together with BP, the uptake rate was dependent on the concentration of unbound hormone. It is not yet known whether BP or the JH-BP complex enters the fat body cells. If they do however, uptake should occur at a much lower rate than with free JH. JH titers in fourth instar larvae can be in the range of  $5 \times 10^{-8}$  M (Fain and Riddiford, 1975; Goodman *et al.*, this volume). Assuming a BP concentration of  $3 \times 10^{-6}$  M (Gilbert *et al.*, 1976) and a dissociation constant of  $3 \times 10^{-7}$  M (Kramer *et al.*, 1974), about 10% of the hemolymph JH may be unbound. (It should be noted that the  $K_D$  was determined at 4°C and that it may be different at the rearing temperature.) Since we utilized slightly higher hormone concentrations, it is still possible that under physiological conditions a saturable uptake mechanism exists for free JH.

JH acid exhibited a lower rate of uptake into the fat body than JH. Whether this is due to a difference in charge or polarity between the two molecules is conjectural at this time although with molecules of similar size the more non-polar (i.e. JH)

molecule can transit the cell membrane more readily. Most of the JH taken up by the fat body was only weakly bound since uptake was to a great extent reversible when fat body was transferred to fresh medium containing BPF. Reflux was even more rapid in the case of metabolites and did not appear to require proteins with high binding affinity and specificity. We therefore assume that hemolymph lipoproteins possessing low affinity and high capacity (see Gilbert et al., 1976) may bind JH-metabolites and thus enhance or elicit their exit from the tissues.

From the above, one can readily see that the maintenance of JH titers requires a most complex regulatory system and that BP, lipoproteins, degradative enzymes and activity of the corpora allata may all be critically involved. Information on most of the above is still preliminary and it is therefore obvious that a great deal of research on the topic remains to be conducted. However, it is likely that BP probably does not play a role in target cell recognition and that unbound JH is probably the form that enters peripheral tissues. This supposition is based on the observation that juvenoids with potent JH morphogenetic activity bind very weakly or not at all to the BP (see Goodman et al., this symposium).

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