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# Photoaffinity Labeling of the Hemolymph Juvenile Hormone Binding Protein of *Manduca sexta*†

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ABSTRACT: A synthetic analogue of the insect juvenile hormone (JH) III, 10,11-epoxy[10-$^3$H]farnesyl diazoacetate ([$^3$H]-EFDA), binds to several proteins in a partially purified preparation of hemolymph protein from fourth instar larvae of *Manduca sexta* when irradiated with UV light. Approximately 80% of this binding could be inhibited by the addition of excess unlabeled JH I. To compare the relative affinity of EFDA for the juvenile hormone binding protein (JHBP) with that of the various JH homologues, the ability of unlabeled EFDA and JH homologues to displace [$^3$H]JH I from binding sites was measured. The relative affinities were EFDA > JH I > JH II > JH III. When Scatchard analysis of the binding of [$^3$H]EFDA or [$^3$H]JH I to the larval JHBP was performed, an estimated apparent $K_D$ of $4.5 \times 10^{-8}$ M was found for EFDA, whereas for JH I a slightly higher $K_D$ of $8.8 \times 10^{-8}$ M was calculated. To determine if [$^3$H]EFDA bound at the JH I binding site, displacement of [$^3$H]JH I from the JHBP complex with unlabeled JH I, JH II, and JH III was compared to the displacement of [$^3$H]EFDA with the same homologues. The results demonstrated that the photoaffinity label bound covalently at the JH I binding site on the hemolymph binding protein of *Manduca sexta*. Fluorescence autoradiography of [$^3$H]EFDA photoaffinity labeled proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that [$^3$H]EFDA bound covalently to two major proteins in the absence of JH I. The presence of JH I prevented the binding of [$^3$H]EFDA to one protein having a molecular weight of approximately 32 000, a molecular weight similar to that of the larval JHBP of *Manduca* hemolymph.

The juvenile hormones (JH) are one of the principal regulators of insect development and differentiation. Therefore, precise control of JH hemolymph titers is essential for normal growth and development (Gilbert & Goodman, 1981), and in the tobacco hornworm *Manduca sexta* a specific hemolymph JH binding protein (JHBP) plays an important role in regulating the JH titer [see Gilbert & Goodman (1981)]. This protein consists of a single 28 000-dalton polypeptide chain (Kramer et al., 1976a; Goodman et al., 1978a), is synthesized in the fat body (Nowock et al., 1975; Nowock & Gilbert, 1976), and functions both to transport the hormone in the hemolymph (Gilbert et al., 1976) and to protect JH from degradation by nonspecific esterases (Hammock et al., 1975; Sanburg et al., 1975).

The molecular interaction of the JHBP with JH has been studied extensively. Each JHBP molecule possess a single high-affinity, JH binding site (Kramer et al., 1976a), which binds JH I with an apparent $K_D$ of approximately $9 \times 10^{-8}$ M (Kramer et al., 1976a). The more nonpolar homologues JH 0 and JH I are bound preferentially by the JHBP, whereas it exhibits decreasing affinities for the increasingly polar homologues JH II and JH III (Goodman et al., 1976; Kramer et al., 1976b). In addition, the JHBP has little or no affinity for either of the breakdown products of JH, the JH acid and the diol (Kramer et al., 1974), indicating that the epoxide and ester functions are essential for hormone recognition and binding. On the basis of these and other findings, the JH binding site of the JHBP is purported to be a sterically defined hydrophobic region with polar sites for the recognition of the epoxide and ester groups (Goodman et al., 1978b).

Details of the molecular interaction between hormones and their binding components can be studied by the use of photoreactive analogues of these hormones. In insects, photoaffinity labeling has been used to study the sex pheromone of the moth *Antheraea polyphemus* (Gonjian et al., 1978) and 20-hydroxyecdysone in *Drosophila* (Schaltmann & Pongs, 1982). In addition, a JH III analogue, 10,11-epoxyfarnesyl diazoacetate (EFDA) has been synthesized as a photoaffinity label for juvenile hormone binding proteins (Reich, 1978). More recently, carbon-labeled (Kraft et al., 1982) and tritium-labeled EFDA have been synthesized (Prestwich et al., 1984b). The tritium-labeled analogue, [$^3$H]EFDA, has been used successfully to label the JH binding site on the hemolymph and ovarian proteins in the cockroach *Leucophaea maderae* (Koeppe et al., 1984). This paper demonstrates that EFDA attaches covalently to the JH binding site of the *Manduca* JHBP with relatively high affinity and specificity. Thus, EFDA may now be used as a photoaffinity label for

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further studies on the structure and function of the JHBP in *Manduca*, as well as a probe for putative JH cellular receptors.

## Materials and Methods

*Chemicals.* Radiolabeled JH I, methyl (2*E*,6*E*)-*cis*-10,11-epoxy-7-ethyl-3,11-dimethyl-2,6-[10-$^3$H]tridecadienoate (racemic), was obtained from New England Nuclear Corp. (sp act. 15.5 Ci/mmol). The tritiated and unlabeled 10,11-epoxyfarnesyl diazoacetate (EFDA) were prepared as described previously (Prestwich et al., 1982, 1984a). Labeled EFDA had a specific activity of 2.4 Ci/mmol. Unlabeled JH I, JH II [methyl (2*E*,6*E*)-*cis*-10,11-epoxy-3,7,11-trimethyl-2,6-tridecadienoate], and JH III [methyl (2*E*,6*E*)-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate] (all racemic) were from Calbiochem. All chemicals were reagent grade unless oterhwise indicated. Hexane and methanol used for solubilizing JH were Fisher HPLC grade. The buffer (TK) used throughout the study contained tris(hydroxymethyl)aminomethane (Tris) (10 mM) and potassium chloride (10 mM) at a pH of 7.5. Preparation and storage of JH and EFDA stock solutions are described elsewhere (Kovalick & Koeppe, 1983; Prestwich et al., 1984b). All glassware used with either the JH homologues or JH analogues was coated with a 1% PEG [poly(ethylene glycol), $M_r$ 20 000] solution (Goodman et al., 1978a). Teflon tubing was used for JH homologue/analogue transfer.

*Animals and Extract Preparation. Manduca sexta* larvae were reared at 25 °C under a 16-h photophase as described elsewhere (Goodman et al., 1978a). Hemolymph was drawn from late fourth instar larvae exhibiting head capsule slippage, and partial purification of JHBP from the hemolymph was performed as described previously (Goodman & Goodman, 1981). The 20 000–40 000-dalton fraction from a calibrated Sephacryl S-200 column was employed for these studies.

*Binding Assays.* To separate free hormone/analogue from bound hormone/analogue, two competitive binding assays were used: dextran-coated charcoal (DCC) and the hydroxylapatite (HTP) assay. The methodology for these assays was similar to that described elsewhere (Goodman et al., 1976, 1978b). When [$^3$H]EFDA was used, trichloroacetic acid (TCA) was used to precipitate the labeled proteins from solution [TCA filter assay (Koeppe et al., 1984)]. In all assays, total protein concentration of the extract was constant for a given experiment and was approximately 0.1 $\mu$g/$\mu$L for each mixture. The volume of the reaction mixtures varied with the assay. When binding affinities were determined, the binding capacity of the extract was adjusted so that 50% of the labeled JH I (approximately $6 \times 10^{-9}$ M) was bound. Binding capacities and binding affinities were calculated according to the method of Scatchard (1949).

(*1*) *Dextran-Coated Charcoal Assay* (*DCC Assay*). When bound hormone/analogue was separated from free hormone/analogue via the DCC assay, the reactions were completed under equilibrium conditions (overnight incubation at 4 °C) in 6 × 50 mm test tubes coated with PEG. The reaction volume was 350 $\mu$L and consisted of unlabeled JH homologue/analogue (dried under nitrogen and resuspended in 5 $\mu$L of ethanol), TK buffer, protein extract, and [$^3$H]JH I/[$^3$H]EFDA. To stop the reaction and to bind the free hormone, 50 $\mu$L of DCC was added to the reaction mixture. After being vortexed, the mixture was treated as described previously (Goodman et al., 1976).

(*2*) *Hydroxylapatite Binding Assay* (*HTP Assay*). Reaction mixtures that were assayed for JH binding via the HTP assay were made up as above, except that the final volume was 200 $\mu$L. To separate the free from the bound hormone/analogue, HTP was added (Goodman et al., 1978a,b).

(*3*) *Trichloroacetic Acid Filter Assay* (*TCA Assay*). To separate covalently bound [$^3$H]EFDA from unbound JH homologue/analogue, proteins were precipitated from solution by the addition of TCA to a final concentration of 10%. Unbound homologue/analogue was removed by washing the precipitate on a glass fiber filter with 3 volumes of 5% TCA and 2 volumes of 60% ethanol. Analysis of the bound radioactivity was performed as described elsewhere (Koeppe et al., 1984).

*Photolysis.* Samples were irradiated in a Rayonet photochemical reactor (RPR-100) equipped with eight UV lamps (RPR-2537A) emitting light at a wavelength of 254 nm. Photolysis was performed in quartz tubes previously treated with a 1% solution of poly(ethylene glycol) ($M_r$ 20000). Tubes containing reaction mixtures were kept at 4 °C during UV exposure by submerging them in an ice–water-filled quartz container. While the volumes of these reaction mixtures varied from 350 $\mu$L to 35 mL, the concentrations of the components in the reaction mixtures remained the same (Koeppe et al., 1984).

*Gel Electrophoresis and Fluorography.* Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out as described elsewhere (Goodman et al., 1978a) and followed the general method of Laemmli (1971). The gels (18%) were stained with Coomassie brilliant blue (0.25% in 30% methanol/20% acetic acid) and destained with 5% methanol/10% acetic acid. To identify the labeled bands, the gels were analyzed by a modification of the fluorography technique of Bonner & Laskey (1974). In these studies, EN$^3$HANCE was utilized according to the instructions of the manufacturer (New England Nuclear). After impregnation of the EN$^3$HANCE (2 h) and precipitation (1 h in water), the gels were dried on a Bio-Rad slab gel dryer, placed against a blue sensitive X-ray film (Kodak X-Omat AR film XAR-2), and exposed for 4–6 weeks at −70 °C.

## Results

*Effect of UV Irradiation.* To determine the effects of UV irradiation on the binding capacity of partially purified JHBP, protein solutions were added to PEG-treated quartz tubes that contained approximately 12 700 dpm of [$^3$H]JH I (approximately $8 \times 10^{-9}$ M). After incubation overnight at 4 °C, the solutions were irradiated for 0, 15, 30, 60, 90, and 100 s at 4 °C. Bound JH was quantified by hydroxylapatite precipitation of proteins. Figure 1 illustrates the effects of UV irradiation on these solutions and demonstrates that exposure to UV light for longer than 60 s resulted in the loss of bound [$^3$H]JH I. For this reason, UV irradiation of all reaction mixtures was kept to less than 60 s.

*Binding Affinity of EFDA.* To establish whether EFDA bound specifically to the JHBP, protein solutions were incubated with $2 \times 10^{-7}$ M [$^3$H]EFDA in the presence or absence of JH I at a concentration of either $1 \times 10^{-7}$ or $1 \times 10^{-5}$ M. After UV irradiation, the amount of covalently bound [$^3$H]-EFDA was estimated by the TCA filter assay (Figure 2). Maximum labeling of poteins with [$^3$H]EFDA occurred within 10–20 s. Addition of $1 \times 10^{-7}$ M JH I to the incubation mixture reduced covalent binding of [$^3$H]EFDA to proteins by approximately 58%, whereas the addition of $1 \times 10^{-5}$ M JH I reduced [$^3$H]EFDA binding by almost 75–80%. The amount of [$^3$H]EFDA bound in the presence of $1 \times 10^{-5}$ M unlabeled JH I was considered to be nonspecific binding. Thus, 75–80% of [$^3$H]EFDA bound to protein in the absence of JH was bound specifically. The ability of EFDA, JH I, JH II, and JH III to competitively displace [$^3$H]JH I from

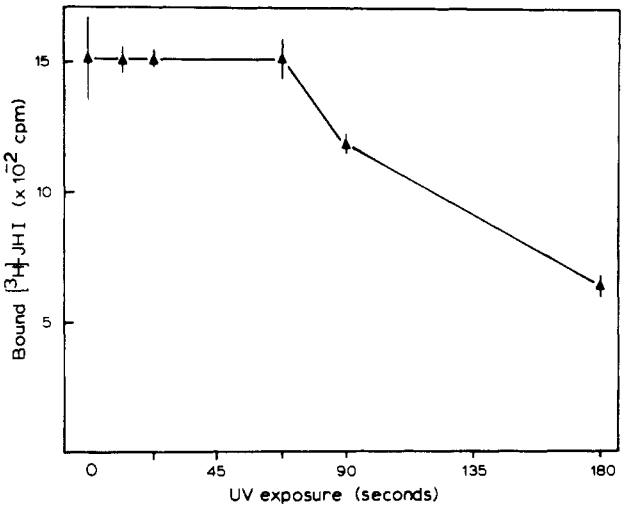


FIGURE 1: JH binding capacity of UV-irradiated partially purified hemolymph protein extract from fourth instar *Manduca sexta* larvae. Reaction mixtures consisted of $6 \times 10^{-9}$ M [$^3$H]JH I and diluted protein extract. UV irradiation was performed under equilibrium conditions (overnight incubation at 4 °C) for the indicated times. Bound [$^3$H]JH I was measured with the HTP assay. The data represent the means of triplicate assays.

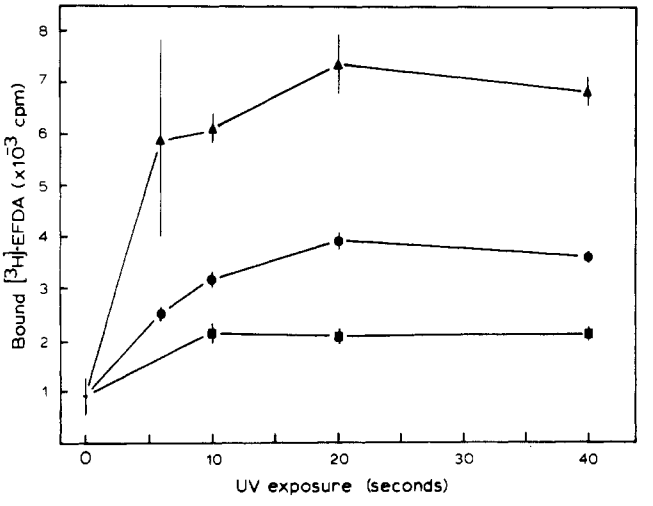


FIGURE 2: Photoaffinity labeling of a partially purified JHBP preparation from fourth instar larval hemolymph. Reaction mixtures of [$^3$H]EFDA and extract were irradiated for the indicated times in the presence or absence of JH I. Bound [$^3$H]EFDA was quantified with the TCA assay. Reaction mixtures were as follows: without JH I (filled triangles); with $1 \times 10^{-7}$ M JH I (filled circles); with $1 \times 10^{-5}$ M JH I (filled squares). Each point represents the mean of triplicate samples.

the JHBP was then examined. Protein solutions were incubated with [$^3$H]JH I and various concentrations of different JH homologues or EFDA. The binding of [$^3$H]JH I was quantified by the DCC assay as well as by the HTP assay. Both techniques yielded similar results. As shown in Figure 3, the competitive displacement of [$^3$H]JH I occurs in the following order: EFDA > JH I > JH II > JH III. Thus, EFDA was a better competitor of [$^3$H]JH I than was unlabeled JH I. This indicates that the relative equilibrium dissociation constant ($K_D$) for the binding of EFDA to the *Manduca* JHBP is lower than that for JH I. The binding affinity of JHBP for EFDA was determined by measuring specific binding over a wide range of concentrations of [$^3$H]EFDA. Nonspecific binding (approximately 20%) was taken as that amount of [$^3$H]EFDA binding that occurred in the presence of excess unlabeled JH I ($2 \times 10^{-5}$ M). Scatchard analysis of these results gave an estimated $K_D$ of $4.5 \times 10^{-8}$

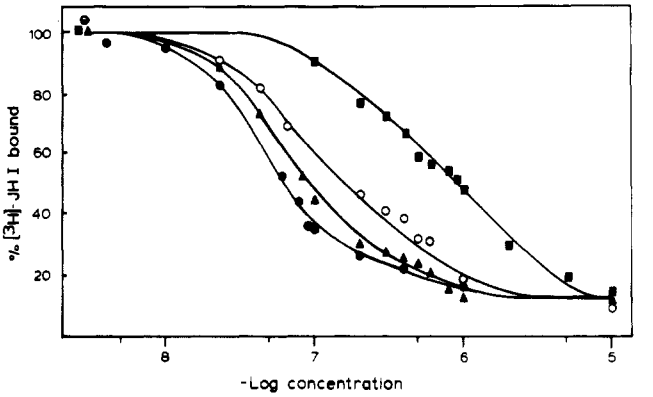


FIGURE 3: Competitive displacement of [$^3$H]JH I from a partially purified JHBP preparation from fourth instar larval hemolymph: by EFDA (filled circles); by JH I (filled triangles); by JH II (open circles); by JH III (filled squares). Free and bound ligand were separated by the DCC assay.

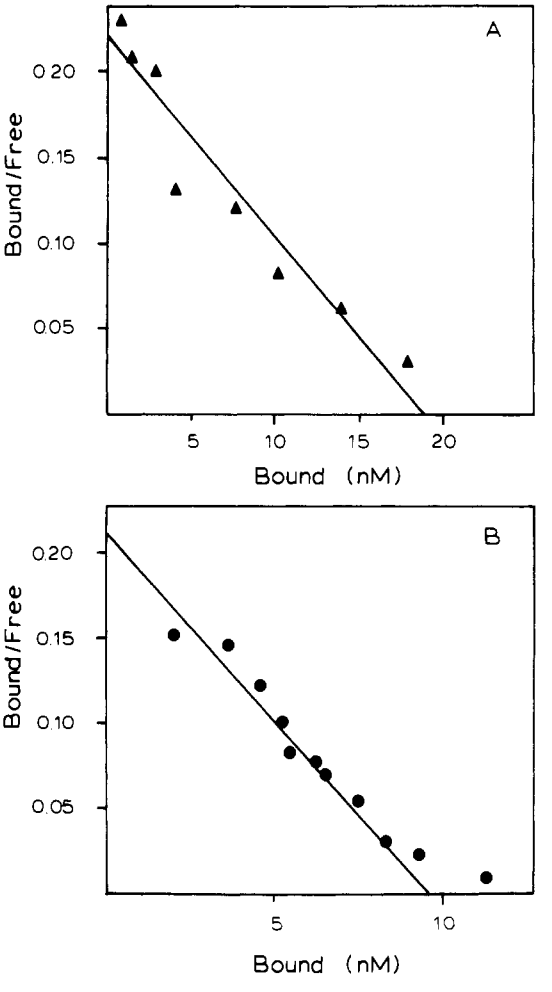


FIGURE 4: Binding affinity of partially purified JHBP from fourth instar larval hemolymph for JH I (A) and EFDA (B). Data were analyzed according to the method of Scatchard (1949). The slope of the lines was determined by linear regression analysis; the data indicate that the apparent $K_D$ for JH I is $8.8 \times 10^{-8}$ M (DCC assay; $r = 0.95$), while the apparent $K_D$ for EFDA is $4.5 \times 10^{-8}$ M (TCA filter assay; $r = 0.91$).

M (Figure 4B). In a control experiment, the $K_D$ of the JHBP with JH I was estimated to be $8.8 \times 10^{-8}$ M (Figure 4A). Nonspecific binding (>10%) was taken as that amount of [$^3$H]JH I binding that occurred in the presence of excess unlabeled JH I ($2 \times 10^{-5}$ M).

*Specificity of EFDA Binding to JH Binding Site of JHBP.* The differential ability of the various JH homologues to dis-

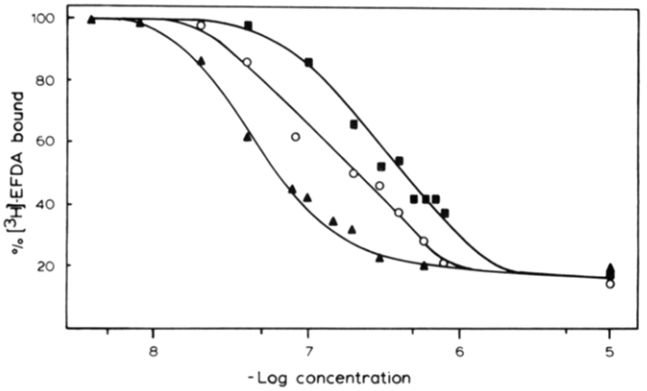


FIGURE 5: Displacement of [$^3$H]EFDA from the JHBP by various concentrations of JH homologues: JH I (filled triangle; JH II (open circles); JH III (filled squares). Reaction mixtures were incubated overnight at 4 °C and then photolyzed for 60 s. Binding was quantified by the TCA filter assay.

place labeled ligand from the JHBP was utilized to determine if [$^3$H]EFDA attached to the JHBP at the JH binding site. [$^3$H]EFDA was photoattached to proteins in the presence of various concentrations of JH I, JH II, and JH III. As shown in Figure 5, the amount of [$^3$H]EFDA bound is dependent on the concentration and the structure of the competitor. The concentration of free JH I needed to displace 50% of the [$^3$H]EFDA from the JHBP was $8.1 \times 10^{-8}$ M. For JH II, this concentration was $2 \times 10^{-7}$ M or 2.5 times the amount of that was required for JH I; for JH III, it was $4.8 \times 10^{-7}$ M or 5.9 times greater. These data were comparable to the results obtained when [$^3$H]JH I was used in place of [$^3$H]-EFDA (Figure 3). The amount of free hormone needed to displace 50% of the [$^3$H]JH I was $9 \times 10^{-8}$ M for JH I, $1.5 \times 10^{-7}$ M for JH II (1.7 times more than JH I), and $8.6 \times 10^{-7}$ M for JH III (9.6 times more). These results suggest that EFDA binds at the JH binding site of the *Manduca* JHBP.

*Identification of [$^3$H]EFDA-Labeled Proteins by SDS–PAGE.* To confirm that [$^3$H]EFDA covalently attached to the JHBP of fourth instar *Manduca* larval hemolymph, partially purified hemolymph protein extracts (30 mL) containing $1 \times 10^{-7}$ M [$^3$H]EFDA were irradiated in the presence or absence of $1 \times 10^{-5}$ M JH I. After irradiation, the proteins were precipitated from solution with TCA, washed 2 times with 95% ethanol, and then resuspended in sample buffer (Laemmli, 1971). After being boiled for 10 min, the proteins were separated electrophoretically on an 18% gel, stained, destained, and then prepared for fluorography. The results in Figure 6 demonstrate that in the absence of JH I [$^3$H]-EFDA bound to two major proteins, one of approximately 32000 daltons. The covalent attachment of [$^3$H]EFDA to this protein was inhibited by the presence of excess unlabeled JH I.

## Discussion

Details of the molecular interaction between hormones and their binding components can be approached by the use of chemically reactive analogues of these hormones. Photoaffinity labeling has been applied in several vertebrate systems for the analysis of receptors and other proteins (Bayley & Knowles, 1977; Chowdhry & Westheimer, 1979). Only recently, however, has this approach been used for binding proteins and receptors of insects (Ganjian et al., 1978; Schaltmann & Pongs, 1982). EFDA has been tested previously in several insect systems as a possible photoaffinity label (Reich, 1978; Krafft et al., 1982; Prestwich et al., 1982, 1984; Koeppe et al., 1984). In this study we examined the potential of EFDA

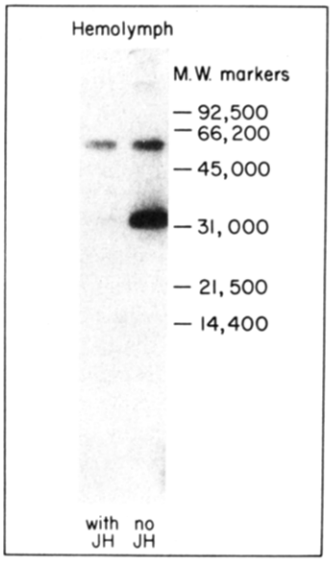


FIGURE 6: Fluorogram of electrophoretically separated proteins labeled with [$^3$H]EFDA. A partially purified JHBP preparation was equilibrated with $1 \times 10^{-7}$ M [$^3$H]EFDA in the presence or absence of JH I. As indicated, two proteins bands bound [$^3$H]EFDA in the absence of JH I, while only one band was labeled when JH was present. As indicated by the position of the molecular weight markers, the protein band that was protected by the JH had an $M_r$ of approximately 32000 while the other protein that is not protected by JH has an approximate $M_r$ of 60000. Molecular weight markers from top to bottom are as follows: Phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

as a photoaffinity label for the hemolymph JHBP of fourth instar *Manduca sexta* larvae.

EFDA is an analogue of JH III, with the carboxymethyl ester function of JH III replaced by a diazocarbonyl group in EFDA (Reich, 1978). The diazo ester has a strong absorbance in the ultraviolet range ($\lambda_{max}$ 244 nm, $\epsilon$ 10090). Absorption of UV light by the diazo ester produces a highly reactive acylcarbene, which, if EFDA is bound to the JHBP, can result in covalent attachment of EFDA to the binding protein. Unfortunately, even brief exposure of proteins and JH to UV light may cause nonspecific degradation of these compounds. Degradation of hormone or binding protein during photolysis would be accompanied by a loss of binding capacity and could seriously affect the results. The effects of various amounts of UV light exposure on binding capacity in JHBP solutions were examined, and a measurable loss of binding capacity occurred after 60 s of UV exposure. In contrast, maximum labeling of proteins with EFDA takes place within 20 s of UV exposure. Thus, the covalent attachment of EFDA to proteins is accomplished prior to significant degradation of the proteins or JH in solution. Since photolysis times of less than 60 s were used, the influence of protein or JH breakdown did not affect the results.

If EFDA is to be used successfully as a photoaffinity label for the JHBP, EFDA must bind specifically and with a relatively high affinity to the JHBP. In addition, EFDA must attach covalently to the JHBP at the JH binding site when exposed to UV light: the data indicate that EFDA fulfills these requirements. The amount of [$^3$H]EFDA attached covalently to proteins was dependent on the concentration of the unlabeled JH I present in the incubation mixture, suggesting that the occupation of JH-specific binding sites by JH I could prevent covalent attachment of EFDA at these sites. Koeppe et al. (1984) demonstrated that the addition of high concentrations ($4 \times 10^{-5}$ M) of a biologically inactive JH analogue to reaction

mixtures did not prevent photolysis. Thus, inhibition of [$^3$H]EFDA attachment by JH I appears to be due to the specific structure of the hormone, rather than to a nonspecific "UV interference" effect.

Further evidence of EFDA binding specificity was provided by competitive binding studies with unlabeled EFDA or various JH homologues as competitors with [$^3$H]JH I for binding sites. Of the three JH homologues used, JH I was the most effective competitor, while JH II was less effective and JH III was the least effective. These results are in agreement with previously published data that showed the least polar homologue to bind to the JHBP with the greatest affinity (Goodman et al., 1976; Kramer et al., 1976b). EFDA also competed well with [$^3$H]JH I for binding sites, proving to be more effective than JH I and suggesting that EFDA was bound by the JHBP with a higher affinity than JH I. This unexpected result was confirmed when data from a competitive binding assay with EFDA was subjected to Scatchard analysis and compared to a similar analysis of data obtained with [$^3$H]JH I. The apparent equilibrium dissociation constant ($K_D$) for EFDA was $4.5 \times 10^{-8}$ M, approximately half the $K_D$ calculated for JH I in these ($8.8 \times 10^{-8}$ M) and prevous studies ($9.35 \times 10^{-8}$ M; Goodman et al., 1976). These results were unexpected since EFDA is more polar than JH III. Perhaps the polarity rule applies to the lipophilicity of the carbon chain and that the carboxymethyl ester end interacts in a different manner with a polar region of the active center. Replacement of this group with the diazo group appears to enhance binding, perhaps because associated amino acids near the active center are now interacting with a more polarizable and electron-rich group.

To determine if [$^3$H]EFDA bound to the JHBP at the JH binding site, a series of comparative displacement studies were performed. In one series of experiments, various concentrations of unlabeled JH I, JH II, or JH III were used to compete with [$^3$H]JH I for binding sites while in the other a similar series of assays was performed with [$^3$H]EFDA as the labeled ligand. If [$^3$H]EFDA and [$^3$H]JH I bind at the same site, then the ratios of the concentrations of JH I to JH II to JH III needed to displace 50% of the labeled ligand from the JHBP should be similar for both labeled ligands. When [$^3$H]JH I was used, this ratio was 1:2.5:5.9; with [$^3$H]EFDA, the ratio was 1:1.7:9.6. The similarity of these ratios suggests strongly that [$^3$H]EFDA and [$^3$H]JH I bind at the same binding site.

Further evidence that [$^3$H]EFDA binds to the JHBP was provided by fluorography of the SDS–PAGE-separated [$^3$H]EFDA-labeled proteins, which revealed that a single protein with a molecular weight of approximately 32000 was specifically labeled by [$^3$H]EFDA. This molecular weight is similar to but not identical with that reported previously for JHBP (Kramer et al., 1976a; Goodman et al., 1978b). More recently, it was noted than when the binding protein was rapidly purified by affinity chromatography, the molecular weight was slightly higher ($M_r$ 32000) (W. Goodman, personal observation). It is possible that in the conventional purification schemes JHBP may cochromatograph with certain proteases that remove a small peptide fragment from the protein without disrupting binding. The rapid labeling method presented by [$^3$H]EFDA and affinity purification procedures may avoid this problem. Other proteins were also labeled, but since increased concentrations of unlabeled JH I did not inhibit covalent attachment of [$^3$H]EFDA to these other proteins, it appeared to be non-JH related and is considered to be nonspecific.

In many insects, such as *Leucophaea maderae*, the rate of dissociation of JH from the binding protein complex is extremely rapid (seconds), making purification and characterization of the protein extremely difficult. The present results demonstrate that [$^3$H]EFDA, a photoaffinity analogue of JH III that covalently binds to the JH active center, has a higher affinity for the hemolymph JHBP from *Manduca* than do the natural hormones, JH I and JH II. This should now permit further characterization of this binding protein, i.e., identification of the amino acids and their sequence in its active center. In addition, [$^3$H]EFDA can be used as a probe for identifying and characterizing putative JH receptors in the target tissues (e.g., epidermis) of *Manduca sexta* and other insects. Such studies are currently under way in our laboratories.

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# Fluorescent Probes as a Measure of Conformational Alterations Induced by Nucleophilic Modification and Proteolysis of Bovine $\alpha_2$-Macroglobulin†

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ABSTRACT: Conformational alterations occurring in bovine $\alpha_2$-macroglobulin ($\alpha_2$M) resulting from proteolysis and nucleophilic modification have been monitored by UV difference spectra, circular dichroism, and changes in the fluorescence of 6-(*p*-toluidino)-2-naphthalenesulfonate (TNS) and bis(8-anilino-1-naphthalenesulfonate) (Bis-ANS). The results of this study indicate that these two dyes appear capable of differentiating between conformational changes induced by proteolysis and those induced by methylamine treatment. It appears that TNS is a sensitive probe for monitoring protease-induced but not methylamine-induced conformational changes in bovine $\alpha_2$M. Bis-ANS, on the other hand, appears suitable for monitoring conformational changes induced by methylamine treatment or proteolysis of the molecule and was used as a probe to monitor the kinetics of the conformational change induced by methylamine treatment. It was found that the conformational change did not occur simultaneously with cleavage of the thiol ester bonds by the nucleophile, measured by titration of free sulfhydryl groups with 5,5′-dithiobis(2-nitrobenzoate). The data are consistent with a model in which initial nucleophilic attack results in exposure of sulfhydryl groups, resulting in a conformational change measured by an increase in fluorescence. This event is followed by a unimolecular step representing a conformational change in the protein that results in a further increase in the fluorescence signal. The second-order rate constant for hydrolysis of the thiol ester bonds was determined to be $3.4 \pm 1.0$ $M^{-1}$ $s^{-1}$, while the rate constant for the conformational change was $(4.4 \pm 0.8) \times 10^{-4}$ $s^{-1}$.

Human $\alpha_2$-macroglobulin ($\alpha_2$M)[1] is a 718 000 molecular weight glycoprotein containing four identical subunits (Swenson & Howard, 1979a,b; Hall & Roberts, 1978). This protein functions as a protease inhibitor and is capable of inhibiting proteases from all four classes (Barrett & Starkey, 1973). Several functionally important regions of the molecule have been identified. Inhibition of a protease has been proposed to occur following a cleavage of the polypeptide chain at a restricted region on $\alpha_2$M (Sottrup-Jensen et al., 1981). This limited proteolysis is followed by a conformational change in $\alpha_2$M, resulting in reduced activity of the protease toward large molecular weight substrates with little change in activity toward lower molecular weight substrates.

A further consequence of the conformational change occurring in $\alpha_2$M is the hydrolysis of thiol ester bonds (Salvesen et al., 1981). Covalent attachment of proteases at this region may occur, via reaction with amino groups on the proteinase (Wu et al., 1981). The function of these thiol ester bonds in $\alpha_2$M is not known at this time. It has been observed that certain nucleophiles, such as methylamine, inactivate $\alpha_2$M, which has been postulated to involve nucleophilic cleavage of the thiol ester bond (Salvesen et al., 1981; Swenson & Howard, 1980). This activity loss is associated with a conformational change occurring in $\alpha_2$M. As a consequence of the conformational change induced in $\alpha_2$M by proteolysis, or by small nucleophiles, the modified molecule interacts with high-affinity receptors present on many fibroblast cell lines and macrophages (Pastan et al., 1977; Van Leuven et al., 1978; Kaplan & Nielsen, 1979; Mosher et al., 1977; Imber & Pizzo, 1981), resulting in a rapid cell uptake and clearance of the modified protein from the circulation.

The conformational changes occurring in $\alpha_2$M have been measured by a variety of techniques, including alterations in circular dichroism spectra (Gonias et al., 1982), ultraviolet difference spectra (Björk & Fish, 1982; Dangott et al., 1983), intrinsic fluorescence (Björk & Fish, 1982; Straight & McKee, 1983), fluorescence of 6-(*p*-toluidino)-2-naphthalenesulfonic acid (TNS) (Strickland & Bhattacharya, 1984), thermal denaturation (Cummings et al., 1984), and altered electro-

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[1] Abbreviations: $\alpha_2$M, $\alpha_2$-macroglobulin; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ANS, 8-anilino-1-naphthalenesulfonate; Bis-ANS, bis(8-anilino-1-naphthalenesulfonate); TNS, 6-(*p*-toluidino)-2-naphthalenesulfonic acid.

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