A Steroid-Binding Protein from Insect Haemolymph Isolated by Photoaffinity Labelling and Immunoadsorption

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Endogenous ecdysteroids as well as radiolabelled exogenous ecdysone were crosslinked to a protein when haemolymph from blowfly larvae was irradiated with UV-light under optimized conditions. This indicates that larval haemolymph of blowflies contains an ecdysteroid-binding protein.

The crosslinked ecdysteroid-protein complex was shown to form a ternary complex when mixed with ecdysteroid-specific antibodies. Formation of the ternary complex was due to a specific interaction of the binding sites of the antibodies with the steroid. This specific interaction was used to isolate the ecdysteroid-protein complex from irradiated crude larval haemolymph by immunoadsorption. The method described here represents a rapid approach for the isolation of steroid-binding proteins by a combination of photoaffinity labelling and immunoadsorption.

Introduction

There is ample evidence for the existence of blood proteins able to bind steroid hormones in mammals (*cf.* [1]). Several types of steroid carriers have been found. Some, like transcortin or the sex hormone-binding globulin, are rather specific for certain steroid hormones. These proteins occur in limited concentrations in the blood and bind the steroids with high affinity. Another mammalian blood protein, albumin, has a low specificity and a low but significant affinity for steroids. Blood proteins able to bind steroids have also been detected in species of the lower vertebrates (in cyclostomes, chondrichtyes, osteichthyes, amphibia, reptiles and birds) (*cf.* [2]). It may thus be speculated that carrier proteins are an integral part of all steroid hormone systems including that of ecdysteroids in invertebrates.

The search for ecdysteroid-binding proteins (EBP) in the blood of arthropod species led so far to conflicting results. No evidence for an ecdysteroid carrier was obtained in two crustacean species (*Pachygrapsus crassipes* [3]; *Orconectes limosus* [4]) or three insect species (the three silkmoths *Philosamia cynthia* [5]; *Antheraea polyphemus* [6]; *Hyalophora cecropia* [7]) although the possibility of a weak binding between haemolymph proteins and ecdysteroids could not be excluded [8]. On the other hand reversible binding of radiolabelled ecdysteroids to blood proteins was concluded from studies with several other insect species. Preliminary examination suggested binding in the bug *Pyrrhocoris apterus* [9], the fruit fly *Drosophila hydei* [10, 11] and the blowfly *Calliphora vicina* [10]. Binding of 20-hydroxyecdysone to a haemolymph protein was definitively shown in the locust *Locusta migratoria* [13−15].

None of the EBP's from arthropods have as yet been isolated and studied biochemically to compare their characteristics to steroid carriers from vertebrates. We therefore attempted to isolate the presumed blood protein which is able to bind ecdysteroids from blowfly larvae. By use of photoaffinity labelling the ecdysteroid carrier was cross-linked to the endogenous ecdysteroid. With an ecdysteroid-specific immunoadsorbent the protein-steroid complex was subsequently isolated. Since the method appears to be applicable to the isolation of steroid-binding proteins in general the method is described here in detail.

Materials and Methods

Reagents

Ecdysone and 20-hydroxyecdysone were purchased from Simes s.p.a. (Milano, Italy). Ponasterone A was

a gift from Dr. D. H. S. Horn (Melbourne, Australia). Radiolabelled [23,24-³H]ecdysone with a specific radioactivity of 68 Ci/mmol was obtained from Zoecon (Palo Alto, California). It was purified by TLC (see below) prior to use. The radiochemical purity of tritiated ecdysone was always better than 97% as judged by TLC. The proteins used in irradiation experiments were obtained from Boehringer (Mannheim): alcohol dehydrogenase (EC 1.1.1.1), isocitrate dehydrogenase (EC 1.1.1.42); Serva (Heidelberg): catalase (EC 1.11.1.6); Sigma (München): albumin (bovine serum albumin), ovalbumin, cytochrome C, alkaline phosphatase (EC 3.1.3.1), aldolase (EC 4.1.2.13); Worthington (München): 3-hydroxysteroid dehydrogenase (EC 1.1.1.50 and 51). Calliphorin was isolated from haemolymph of mature third-instar larvae of the blowfly and purified to homogeneity [16]. Dextran 500 was from Serva (Heidelberg). All other reagents were obtained from Merck in analytical grade.

Insects

Maintenance of the blowfly culture, *Calliphora vicina* R.D., was as described earlier [17].

Photoaffinity labelling

Ecdysteroids dissolved in phosphate-buffered saline (20 mM sodium phosphate, pH 7.3, 150 mM sodium chloride) were irradiated at constant temperature in a cylindrical cell (type 165; Hellma, München) which had an optical length of 20 mm and a stirred volume of 2.0 ml. The high-power light source used (type LX 501 with a 450 Watt Xenon bulb; Zeiss, Oberkochen) was part of a commercial cytophotometer and is also found as a component of Zeiss fluorometers. The light source produced a focused beam which was passed through a 6 cm water filter to absorb the IR light portion. The UV-light of short wavelength was absorbed by the front part of the cuvette (either quartz or special optical glass) and by an additional filter which was either a 1 mm long-pass glass filter (object slide, type Resistance) or an interference filter (λ_{max} 330 nm; width at $T_{50\%}$: 8.5 nm; Spindler and Hoyer, Göttingen). In a normal experiment the special glass cuvette and the long-pass filter was used. The sum of absorbancies of the front part of this cuvette and the glass filter was 0.5 at 316 nm, 1.0 at 307 nm and 2.0 at 296 nm, as compared to air. This type of cuvette plus filter system

had a cutt-off (absorbance = 0.5) at 316 nm. The experiment shown in Table II was performed without glas filter, which resulted in a cut-off at 290 nm.

Irradiation of ecdysteroids in the presence of proteins was normally performed for 30 min at 10° (± 1°) C constant temperature (measured inside the cuvette).

Quantitative analysis of cross-linkage

The ratio of protein linked ecdysteroid to total ecdysteroid was taken as yield of crossreaction. To determine the extent of photoinduced crossreaction of radiolabelled ecdysteroid to protein, aliquots (50 µl) were taken prior to irradiation and at various timed intervals and analyzed by either of the following methods:

Thin-layer chromatography. TLC was performed with silicagel 60 F₂₅₄ plates (Merck). Aliquots of 20 to 50 µl were spotted onto the plate and developed with chloroform-methanol (80/20; v/v). radioactivity on the plates was detected and quantified with a windowless thin-layer scanner attached to an integrator (Berthold, Wildbad). Ecdysone had a R_f of 0.29 [18], whilst protein-bound ecdysteroid remained at the origin and had a R_f less than 0.05.

Gel filtration. Gel filtration of aliquots from the irradiated solution was performed with a column (0.5 cm i.d., 25 cm height) filled with Sephadex G-25 medium (Pharmacia, Freiburg). The column was equilibrated and eluted with phosphate-buffered saline (see above). In separate runs the elution volumes of albumin and ecdysone monitored by measurement of absorbance at 280 and 249 nm, respectively, were estimated. Radioactivity was measured in fractions of 500 µl. Cross-linked steroid was eluted in the void volume.

Precipitation with methanol. Protein in aliquots from the irradiated solution precipitated with methanol (40 volumes) was pelleted by centrifugation and washed twice with methanol. The protein pellet was dissolved in 200 µl tissue solubilizer (TS-1; Koch-Light Laboratories, Colnbrook, England). Following addition of counting cocktail (3.0 ml Quickszint 212; Zinsser, Frankfurt) the vials were stored several hours in the dark to avoid chemiluminescence during counting.

Precipitation with TCA. Aliquots from the irradiated solution were mixed with 3.0 ml aqueous

solution of trichloroacetic acid (5%; w/v). Samples containing less than 1 mg protein were mixed with 50 µl albumin (20 mg/ml water) prior to precipitation. After 2 h at 5 °C the proteins were pelleted and washed with 3.0 ml TCA. The radioactivity in the protein pellet was counted as described above.

Charcoal treatment. The aliquots from the irradiated solution were mixed with one volume of dextran coated charcoal suspension (5% charcoal, 0.5% dextran T 500 in water; w/v) and incubated for 30 min at 0 °C with agitation every 10 min. The charcoal to which the free steroid was adsorbed was removed by centrifugation and discarded. Radioactivity was analyzed in an aliquot of the supernatant.

Assay of alkaline phosphatase

The enzyme activity of alkaline phosphatase (EC 3.1.3.1) was determined according to the protocol of the manufacturer (Sigma) with *p*-nitrophenyl phosphate as substrate.

SDS-PAGE

Polyacrylamide gel electrophoresis under denaturing conditions was performed in the presence of sodium dodecylsulphate and mercaptoethanol [19]. Proteins were stained with Coomassie blue and destained by diffusion. Scans were run at 550 or 625 nm with a spectrophotometer. The following reference proteins (kit LMW, Pharmacia) were used: phosphorylase b (M_r 94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000) and trypsin inhibitor (20 000). The gel was frozen and cut into 2 mm slices which were incubated (12 h at 37 °C) in Ommifluor-Protosol in toluene (NEN, Dreieich) prior to liquid scintillation counting.

Gel chromatography

Gel permeation chromatography was performed at 5 °C on a column (1.5 cm i.d., 100 cm height) filled with Sephacryl S-300 (Pharmacia, Freiburg). Eluant was phosphate-buffered saline (see above). The eluate was fractionated (3.3 ml fractions) and analyzed by liquid scintillation counting. In separate runs the column was calibrated with dextran blue, thyroglobulin (M_r 669 000), ferritin (440 000), catalase (232 000) and aldolase (158 000). The standards were obtained from Pharmacia. For the ex-

periment shown in Fig. 3 a solution (8.0 ml) of purified calliphorin (0.8 mg = 1.6 nmol) was used which was crossreacted to radioactive ecdysone (0.8 nmol, 54.4 nCi) by photoaffinity labelling. One half of the solution in phosphate-buffered saline was submitted to chromatography. The other half was mixed with ecdysone-specific antiserum (undiluted DUL-I, 2 mg protein), incubated at 0 °C for 12 h and analyzed by gel chromatography as above. The antiserum used has a binding capacity of 1.1 µmol ecdysone as determined by RIA [20].

Immunoadsorption of an ecdysteroid-protein complex

Cell-free haemolymph (400 µl with 32 mg protein) was prepared by mass isolation from mature third-instar larvae of the blowfly (day 6 after oviposition). Phenoloxidase was inhibited by addition of 100 µmol/ml phenylthiourea. To crosslink endogenous ecdysteroids (concentration 103 nmol/ml haemolymph; [21]) by photoaffinity labelling with presumed carrier proteins, the haemolymph was diluted with 4 volumes phosphate-buffered saline and irradiated with UV-light (cut-off 316 nm) for 30 min. Ecdysteroids which had not reacted with protein were removed by exhaustive dialysis against saline. An aliquot of the dialysed solution (2.1 mg protein) was submitted to immunoadsorption.

The preparation of the immunoadsorbent (DUL-I)-Sepharose 4 B specific for ecdysteroids and an examination of its binding properties were recently described [20]. The antiserum used was obtained in rabbits with ecdysone coupled at position C-6 in the steroid nucleus to bovine serum albumin with aminooxyacetic acid. A column (0.5 cm i.d. and 12 cm height) filled with 9.0 ml immunoadsorbent bound 9.9 nmol ecdysone. The protein solution containing the irradiated haemolymph proteins was passed through the column and was washed with phosphate-buffered saline until no UV_{280}-absorbing material could be detected in the eluate. The presumed ecdysteroid-protein complex was eluted from the immunoadsorbent with 3.0 M sodium trichloroacetate. The eluate was dialyzed against saline before it was analyzed by SDS-PAGE.

Protein determination

Protein was determined according to the method of Lowry *et al.* [22] with bovine serum albumin as standard.

Results

Irradiation of ecdysteroids

Ecdysteroids in aqueous solution absorb UV-light with a maximum at $\lambda = 250$ nm ($\varepsilon = 11\,500$ M^{-1} · cm^{-1}). In addition they reveal a minor absorption peak with a maximum at $\lambda = 315$ nm ($\varepsilon = 200$ M^{-1} for ecdysone and 20-hydroxyecdysone). In aqueous solution ecdysone was transformed by irradiation with UV-light in the range of 315 nm to a number of unknown compounds which were less polar in TLC. The conversion was a first order reaction as detected by quantitative analysis with TLC. Using this technique the rate of photoinduced conversion of ecdysteroids was measured under various experimental conditions and expressed as $t_{50\%}$, the time in which 50 percent of the ecdysteroid were destroyed photochemically (Table I). The half-life depended on several variables including the ecdysteroid irradiated and the optical system used (*i.e.* the intensity and the wavelength distribution of light and spectral characteristics of the filter).

Photoinduced reaction of ecdysteroids with proteins

When ecdysone was irradiated in the presence of protein, a proportion of ecdysone crosslinked with the protein. The extent of crossreaction of steroid could be determined quantitatively by the use of radiolabelled ecdysone and separation of linked from free steroid after the irradiation. Several separation methods were tested based on differences between free ecdysone and the ecdysone-protein complex: differences in molecular size (gel filtration),
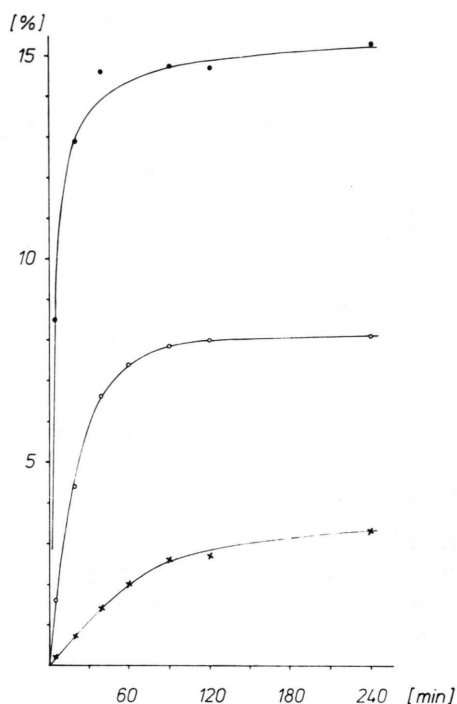

Fig. 1. Photoaffinity labelling of albumin with ecdysone. The progress curves of the photoinduced reaction of radiolabelled ecdysone (1 μM; 4.5 μCi/ml) with albumin (100 μM) were obtained with a constant light source under three different optical conditions: ● in a quartz cuvette without any filter (cut-off 200 nm); ○ in a glass cuvette with an additional special glass filter (cut-off 316 nm); and × in a quartz cuvette with an additional interference filter (cut-off 326 nm). The extent of crosslinking was related to the initial amount of ecdysteroid. Crosslinkage was determined in aliquots from the irradiated solution by liquid scintillation counting of the radioactivity associated to the protein which was separated from the solution by precipitation with methanol.

Table I. Stability of ecdysteroids irradiated by ultraviolet light. Ecdysteroid in aqueous solution (1 μM; 4 μCi/ml) was irradiated under various conditions (type of cuvette, filter). The optical properties of the filter system were characterized by a "cut-off". This term indicates the wavelength at which the sum of absorbancies of filter and front wall of the cuvette were $A = 0.5$. Light of shorter wavelength was absorbed by the filter system. Quantitation of the ecdysteroid was done on aliquots withdrawn at various intervals by TLC and radioscanning. The time in which 50% of the ecdysteroid was destroyed photochemically is indicated as $t_{50\%}$.

Ecdysteroid	Type of cuvette	Additional filter	Cut-off [nm]	$t_{50\%}$ [min]
Ecdysone	quartz	–	200	1.7
Ecdysone	glass	–	304	6.5
Ecdysone	quartz	interference	326	53
Ecdysone	glass	long-pass glass	316	12.9
20-Hydroxyecdysone	glass	long-pass glass	316	15.9
Ponasterone A	glass	long-pass glass	316	28.1

in polarity (charcoal absorption; TLC) and in solubility in certain solvents (methanol; TCA). With two methods (TLC and TCA precipitation) essentially identical results were obtained. While the reproducibility of the results in the charcoal method depended largely on a sufficient protein concentration, gel filtration often led to an overestimation of the crosslinking due to non-covalent binding of steroid to protein. Therefore precipitation of the ecdysteroid-protein complex with TCA was used mostly to quantify the extent of crosslinkage.

The rate of photoinduced crosslinking of ecdysone with albumin as well as the final yield of the ecdysone-albumin complex were dependent on the quality (wavelength and intensity distribution) of the light (Fig. 1). Both, the highest rate and yield were observed with unfiltered UV light. However under these conditions the proteins were not stable due to their light absorption around 280 nm. Alkaline phosphatase as a control (Fig. 2) lost 97% of its enzyme activity within 10 min of irradiation with unfiltered light. On the other hand, no decrease in enzyme activity was detectable during irradiation with filtered light (cut-off 316 nm). When this filter system was used the yield of crosslinking was normally between 0.5 and 5% (related to the steroid applied).

Rate and yield of crosslinkage depended furthermore on the concentration of steroid, the type and concentration of protein, and the light absorbance of the irradiated solution. Only the influence of the
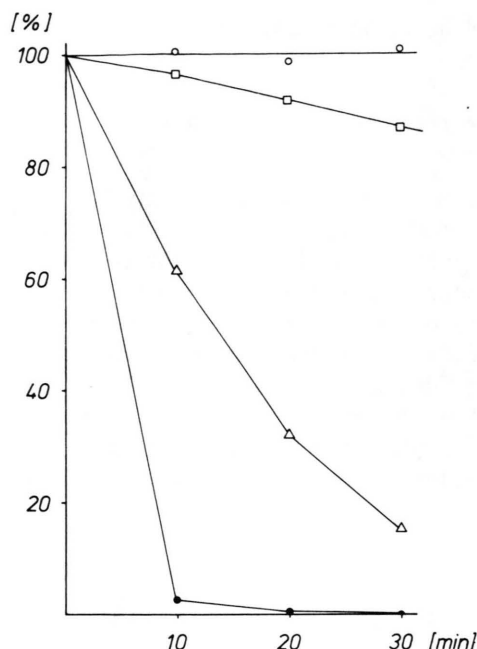

Fig. 2. Enzyme activity of alkaline phosphatase irradiated by ultraviolet light. A solution of alkaline phosphatase (1 mg/ml; 1.1 U/ml in phosphate-buffered saline) was irradiated in the presence of ecdysone (1 μM) under four different conditions: ● in a quartz cuvette without any filter (cut-off 200 nm) and without temperature control; △ in a glass cuvette without filter (cut-off 290 nm) and without temperature control; □ in a glass cuvette with an additional long-pass glass filter (cut-off 316 nm) and without temperature control; ○ in a glass cuvette with an additional long-pass glass filter (cut-off 316 nm) at constant temperature (20 °C). The enzyme activity was determined in aliquots from the irradiated solutions by an optical assay (see Methods).

Table II. Photoaffinity labelling of proteins with ecdysone. Protein (1 mg/ml) was irradiated in the presence of radiolabelled ecdysone (1 μM; 1.4 μCi/ml) for 30 min in a cuvette with cut-off at 290 nm. The crosslinking was determined by TCA precipitation of the protein and liquid scintillation counting. The results are given as steroid covalently bound to protein as percentage of the amount bound to albumin. The concentration of ecdysone crosslinked with albumin by irradiation was 9.5 nM.

Protein	Purity	Crosslinkage (% as compared to albumin)
Larval haemolymph	unpurified	219
Calliphorin	pure	250
Albumin	pure	100
3-Hydroxysteroid dehydrogenase	crude extract	91
Ovalbumin	pure	65
Phosphatase, alkaline	pure	58
Isocitrate dehydrogenase	pure	51
Aldolase	pure	50
Alcohol dehydrogenase	pure	44
Cytochrome c	pure	25
Catalase	pure	11

type of protein among these variables was studied in detail.

Specificity of photoinduced protein-ecdysteroid crosslinkage

The protein specificity of photoaffinity labelling was tested with several proteins following the hypothesis that the yield of crosslinkage should reflect the affinity for ecdysteroids. The crosslinking of albumin was taken as standard reaction and set to 100% since it is known that albumin binds various steroids [1]. Obviously all proteins tested could be crosslinked to ecdysone by photoreaction (Table II). However, the extent of crosslinking depended considerably on the type of protein. Albumin and 3-hy-
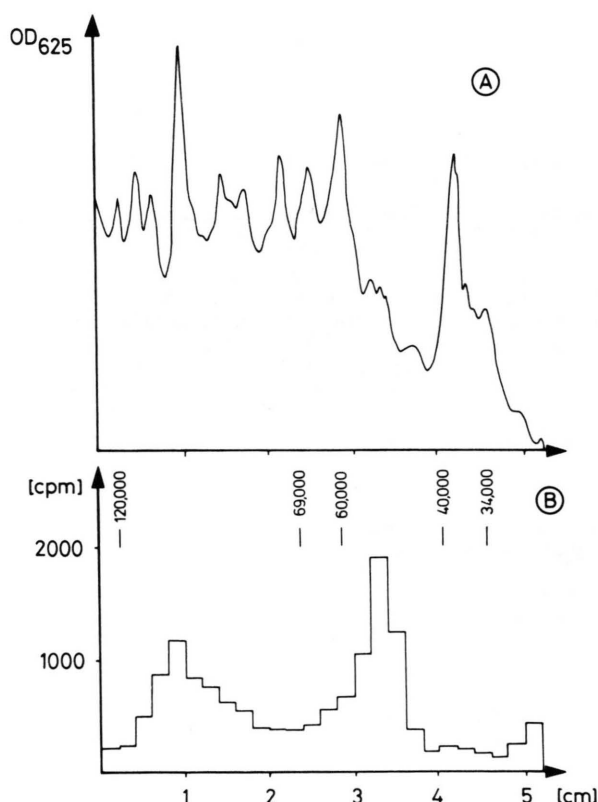

Fig. 3. SDS-PAGE of a cell extract containing 3-hydroxysteroid dehydrogenase after photoaffinity labelling with radioactive ecdysone. The conditions of photoaffinity labelling are mentioned in the legend to Table II. The gel was 10% in polyacrylamide and 0.1% in SDS. Molecular weight standards were run in a separate gel. The proteins were stained with Coomassie blue and scanned in the gel at 625 nm wavelength (A). The radioactivity subsequently was analyzed in gel slices by liquid scintillation counting (B).

droxysteroid dehydrogenase revealed a high percentage of crosslinkage while cytochrome c and catalase gave a low yield. The observation that the crosslinking of calliphorin, as well as of larval haemolymph was much higher with ecdysone suggested that these proteins have a high affinity for ecdysteroids compared to albumin.

The assumption that the photoinduced reaction of ecdysone with proteins reflects an affinity between steroid and protein was further tested by SDS-PAGE analysis of an irradiated sample of 3-hydroxysteroid dehydrogenase. This enzyme is known to react with vertebrate steroids [23] and to a much lesser extent, with ecdysteroids [18]. The enzyme preparation used was a crude extract from *Pseudomonas testosteroni*. In SDS-gel electrophoresis it showed numerous bands following protein staining (Fig. 3A). The radioactivity of crosslinked ecdysone on the other hand exhibited two maxima at molecular weights of approximately 50 000 and 100 000 (Fig. 3B). The sizes of these maxima coincide with the molecular weights of the 3α- and the 3β-specific hydroxysteroid dehydrogenases, respectively (47 000 and 100 000; (*cf.* [24])). From these experiments it was concluded that the photoinduced reaction of ecdysone with various proteins is not absolute specific.

Interaction of ecdysone antibodies with the ecdysone-protein complex

The following analyses were undertaken to see if ecdysone-specific antibodies were able to interact with protein bound ecdysone to form a complex.

Calliphorin when crosslinked to radioactive ecdysone by photoaffinity labelling behaved in gel chromatography as a protein with a molecular weight of 505 000 (Fig. 4). When the ecdysone-calliphorin complex was incubated with anti-ecdysone antiserum DUL-I [20] for 12 h the molecular weight increased to 675 000 as revealed by gel chromatography (Fig. 4). A preincubation of the antiserum with a thousand-fold excess of unlabelled ecdysone followed by incubation with the ecdysone-calliphorin complex resulted in a molecular weight of the radiolabelled protein of 505 000 (data not shown). It is concluded that the addition of ecdysone antibodies led to the formation of a stable ternary complex consisting of the three reactants calliphorin, ecdysone and ecdysone antibodies. The formation of
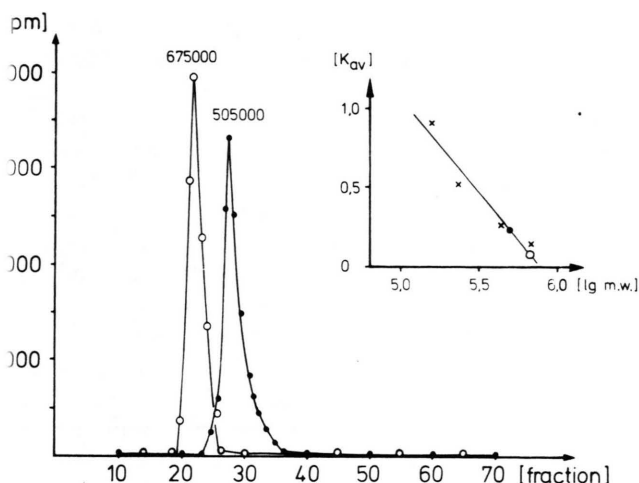
Fig. 4. Gel chromatography of the calliphorin ecdysone complex. The complex was analyzed before (●——●) and after (○——○) mixing with the exdysone-specific antiserum DUL-I. In a separate chromatographic run the column was calibrated with protein standards (inset; the line drawn was calculated by the method of least squares; $r = 0.965$).

the complex appears to be due to a specific interaction of the antibodies with ecdysone since preabsorption of the binding sites of the antibodies with hapten abolished the effect.

Immunoadsorption of the EBP from crude haemolymph

Haemolymph from larvae was irradiated for 30 min with UV-light using the filter system with a cut-off at 316 nm. Free endogenous ecdysteroids which were not crosslinked photochemically to protein were removed from the haemolymph by exhaustive dialysis. The subsequent immunoadsorption of those proteins which were crosslinked to ecdysteroids was achieved by running the haemolymph slowly through a column filled with the ecdysteroid-specific immunoadsorbent DUL-1. Following a washing procedure to remove unbound and unspecifically bound material the specifically bound ecdysteroid-protein complexes were eluted from the column with a solution of trichloracetate. The eluate contained a protein which appeared as a single band in SDS-PAGE (Fig. 5). It is presumed to be the EBP which in SDS-PAGE could not be separated from the subunits of calliphorin.

In a control experiment preabsorption of the binding sites of the immunoadsorbent with a thousand-fold excess of ecdysone prevented immunoadsorption of the EBP from haemolymph.
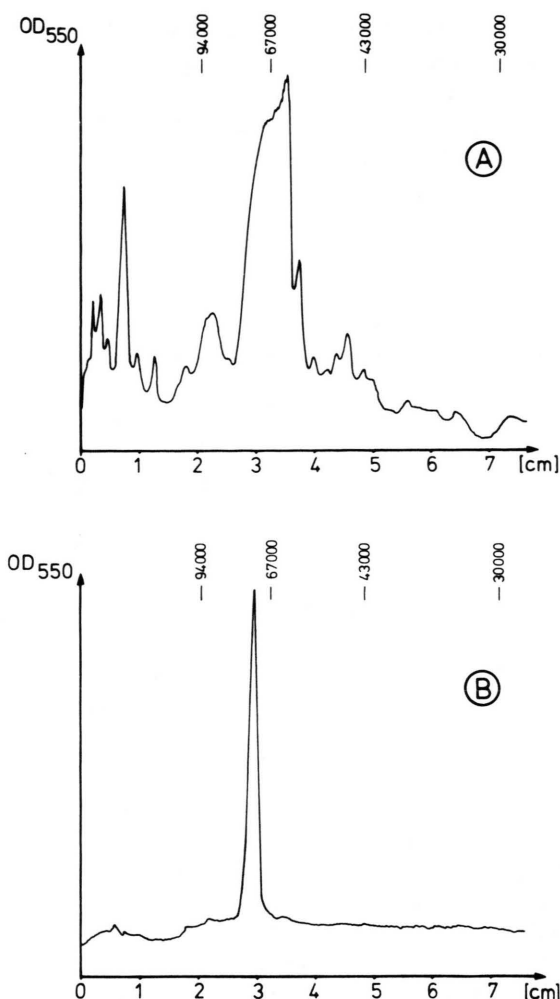

Fig. 5. Immunoadsorption of photoaffinity labelled haemolymph. Untreated haemolymph from blowfly larvae (A) and the ecdysteroid-binding protein isolated from the haemolymph by irradiation and immunoadsorption (B) was analyzed by SDS-PAGE in 10% gels under denaturing conditions. The figure shows densitometric scans of the gels stained with Coomassie blue.

Furthermore no component of the haemolymph was retained by the immunoadsorbent when irradiation of the haemolymph was omitted. No haemolymph component was retained from irradiated haemolymph when an unspecific immunoadsorbent was used prepared accordingly with the γ-globulin fraction of non-immunized rabbits. Thus it is concluded that immunoadsorption is a specific method to isolate ecdysteroid-linked proteins.

The immunoadsorbent was prepared from the γ-globulin fraction obtained from rabbit serum

by ammonium sulphate precipitation. While the resulting immunoadsorbent had a capacity of 1.1 nmol ecdysone per ml of packed absorbent [20], the capacity possibly could be increased by purification of the ecdysteroid-specific antibodies.

Discussion

By photoaffinity labelling of larval haemolymph a protein was covalently and specifically linked with endogenous ecdysteroids. The ecdysteroid-protein complex could be isolated from the irradiated haemolymph using an affinity column with matrix-bound anti-ecdysone antibodies. Three aspects of this work will be discussed here in more detail: photoaffinity labelling of ecdysteroid-binding proteins, the possibility of isolating such proteins by immunoadsorption and the detection of putative ecdysteroid carriers in the blood of arthropods.

Photoaffinity labelling

Photoaffinity labelling has become a major technique for studying molecular interactions in biological systems (review: [25]). In the steroid hormone field it has been used to covalently label proteins which bind steroids, such as enzymes, serum proteins and intracellular steroid receptors (review: [26]). While initial studies were done with steroids to which photoreactive functions were attached synthetically, more recently unmodified natural steroids were used which contain an α,β-unsaturated ketone group [27−31]. Due to a 6-oxo-7-en structure in ring B of the steroid nucleus ecdysteroids belong to this group of photolabile steroids.

Ecdysteroids are photoexcited by absorption of light around 315 nm where they have a minor absorption maximum. A non-bonding electron of the oxygen at C-6 is excited to an antibonding π^* orbital ($n-\pi^*$ transition). According to Benisek [27], the initially produced singlet state is very short-lived and is efficiently transformed to a more stable triplet state (lifetime in the range of msec at 77 K) exhibiting a diradical-like character. Thus the first step in the photochemical reaction possibly involves hydrogen abstraction from suitable donors by the oxygen atom generating a pair of free radicals [27, 30].

Ecdysone when irradiated with UV-light is converted to one (or several similar) compound(s) of lower polarity. While the structure of the product(s) is as yet unknown the change in polarity can be used to quantify the unreacted ecdysone (*e.g.* by TLC). Thereby the effect of irradiation can be measured quantitatively. As expected the process is a first-order reaction and can be used to calculate a $t_{50\%}$ value which indicates the photochemical half-life. Under identical conditions of irradiation the $t_{50\%}$ values of ecdysone, 20-hydroxyecdysone and ponasterone A are different (Table I). Thus to attain the same amount of ecdysteroid crosslinked to a protein different irradiation times have to be used depending on the type of steroid. The $t_{50\%}$ value of ecdysone is strongly affected by the quality of the light, particularly by its wavelength distribution and intensity. It appears that the half-life of the steroid is a suitable measure for the efficiency of the irradiation.

Photoaffinity labelling has been used to label the ecdysteroid receptor(s) in salivary glands and Kc-cells of the fruitfly, *Drosophila melanogaster* [30, 33]. Compared to intra-cellular steroid hormone receptors the serum carrier proteins generally reveal a much lower affinity for steroids. Thus a higher steroid concentration (1000 *versus* 100 nM) had to be used in order to ensure the formation of ecdysteroid-carrier complexes when presumed carrier proteins of ecdysteroids were to be analyzed by photoaffinity labelling. The crucial question which had to be answered experimentally was: is photoaffinity labelling specific at higher steroid concentrations?

Under different conditions of irradiation (Fig. 1) the rate of crosslinkage corresponded to the $t_{50\%}$ value of ecdysone (Table I), supporting the assumption that the photochemical half-life of ecdysone is a useful measure to predict the rate and optimal reaction time of crosslinkage. The final yield of reaction differed with the irradiation conditions. We assume that unfiltered light caused activation of the protein also and thereby could increase the yield and lower the specificity of crosslinking. Irradiation can damage the structure and resultant function of steroid-binding protein [31]. We tested a possible damage of protein structure with alkaline phosphatase as a model protein (Fig. 2). While unfiltered light destroyed enzyme activity, the use of a glass cuvette and an additional glass filter during irradiation at constant temperature prevented any decrease of enzyme activity. For this reason the latter conditions of irradiation appeared to be the most

suitable for photoaffinity labelling of ecdysteroid-binding proteins.

Rather rigorous conditions of irradiation were chosen (cut-off at 290 nm) in which the light might also activate some amino acid residues to test the photoinduced crossreaction of ecdysone with a number of reference proteins (Table II). Ecdysone crosslinked to all proteins. However, proteins which are known to interact with steroids were labelled more efficiently than other proteins (Table II and Fig. 3). The specificity of photoaffinity labelling with irradiation cut-off at 316 nm should be equal or even higher. This would mean that reaction of ecdysteroids with proteins at a steroid concentration of 1 μM reflected sufficiently the affinity between these classes of molecules.

Immunoadsorption of proteins crosslinked to ecdysteroids

Affinity chromatography has been shown to be a powerful tool in the purification of steroid-binding proteins, especially receptors. It has been used to purify the receptors of many vertebrate steroid hormones to homogeneity (review: [34]). An affinity reagent with ecdysteroid chemically coupled to a matrix has been synthesized previously [35] but was as yet not applied successfully to the purification of ecdysteroid-binding proteins from insects.

The approach used in this study was different from the conventional affinity chromatography experiments. Here the steroid-binding protein, which was already linked to the steroid by photoinduced reaction, was applied to an affinity column with matrix-bound anti-ecdysone antibodies. A prerequisite of the experiment was that ecdysone-specific antibodies are able to interact with ecdysone, which is linked to a protein, without the use of a spacer molecule which provides some distance between steroid and protein. Such an interaction had been indicated indirectly by immunohistochemical studies [30]. The experiment shown in Fig. 4 directly demonstrates that upon addition of ecdysone-specific antiserum a ternary complex is formed consisting of a steroid-binding protein, ecdysone and antibodies. The antibodies interacted in a specific manner with protein-bound ecdysone since the increase in molecular weight of the complex (ΔM_r 170 000) coincided with the molecular weight of the antibodies (IgG M_r 150 000) and since preabsorp-

tion of the binding sites of the antibodies with hapten abolished the effect. In a previous study [20] we have already described the preparation of the immunoadsorbent from an antiserum raised against ecdysone-albumin conjugate in the rabbit. The affinity medium was ecdysteroid specific and had sufficient affinity to retain ecdysteroids from crude extracts of insects. With high concentrations of sodium trichloroacetate the ecdysteroids could be eluted. An identical protocol was used in this study to absorb photoaffinity labelled EBP from crude haemolymph. The protein eluted from the affinity column under similar conditions as free ecdysteroids. Appropriate control experiments (preadsorption of immunoadsorbent, use of an unspecific immunoadsorbent, omission of photoaffinity labelling) revealed that immunoadsorption was specific due to an interaction of ecdysone-specific binding sites of the antibodies with ecdysteroids bound to protein.

While we have applied the combination of photoaffinity labelling and immunoadsorption only to the purification of an ecdysteroid-binding protein from the larval haemolymph of blowflies it is our belief that the method can be adapted to other steroid systems.

Ecdysteroid carrier in the haemolymph

Analyses of haemolymph samples from *Calliphora* larvae (by gel filtration and RIA) had shown that a significant amount of ecdysteroids circulated not free but associated to macromolecules (unpublished) corroborating earlier findings by Thamer and Karlson [10]. Thus is was no surprise that by photoaffinity labelling a certain haemolymph protein could be crosslinked to ecdysteroids. Endogenous ecdysteroid (Fig. 5) as well as radioactive exogenous ecdysone (Table I) was crosslinked to a haemolymph protein. In electrophoresis this protein was indistinguishable from calliphorin, which is the major blood protein of blowfly larvae [36] and similar to homologous proteins in the blood of other dipteran species, such as lucilin and drosophilin (LSP 1) [44]. In fact pure calliphorin could also be crosslinked with ecdysone efficiently by photoaffinity labelling. This is an indication that calliphorin can act as a carrier protein for ecdysteroids in the haemolymph of blowfly larvae. However, further experiments which are underway in this laboratory have to prove independently the affinity of calliphorin for ecdysteroids.

As a working hypothesis we assume that calliphorin is a weak binder of ecdysteroids. With regard to these properties calliphorin compares to albumin in the blood of vertebrates.

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