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Xenobiotic Absorption and Binding by Proteins in Hemolymph of the Weevil *Diaprepes abbreviatus*

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A synthetic coumarin, 7-amino-3-phenyl coumarin (coumarin-10), was used to study the uptake of ingested xenobiotics into hemolymph. Larvae were force-fed coumarin-10 in peanut oil, and hemolymph was extracted and analyzed by fluorescence spectroscopy. Coumarin-10 entered hemolymph within 5 min, reaching a steady state of concentration within 1 h. Assayed 2 h after feeding, hemolymph titers of 1–5 ng/μl were proportional to log dose between 10 and 100 ng/mg body weight; hemolymph did not reach saturation. Fluorescence spectra of hemolymph in saline revealed that energy was readily transferred from hydrophobic residues of hemolymph proteins to coumarin-10. Ultracentrifugal density gradients revealed that 94% of absorbed coumarin-10 was bound to sedimenting proteins while 6% bound to lipophorin. Native polyacrylamide gel electrophoresis (N-PAGE) on minigels identified two major proteins responsible for binding. Though readily separated by native electrophoresis, these proteins were not fully separable by HPLC using a wide variety of columns. Gel permeation-HPLC of the sedimenting proteins from hemolymph revealed a single major peak of 480,000 M_r. When upper and lower electrophoretic bands were isolated by preparative N-PAGE, the upper band (band I) yielded subunits of 75,000 and 71,000 M_r, while the lower band (band II) yielded only one size subunit of 75,000 M_r on denaturing (SDS) PAGE. The fluorescent products bound by sedimenting proteins were identified by thin-layer chromatography and scanning fluorescence densitometry as coumarin-10 (80% of total) and a polar metabolite (20%). In addition, lipophorin-containing fractions contained an apolar metabolite (3% of total fluorescence). In vitro binding studies utilizing fluorescent energy transfer demonstrated saturation binding with a K_D of 1.5 μM.

Key words: insect hemolymph proteins, fluorescence spectroscopy, native electrophoresis, root weevils, Coleoptera, Curculionidae, citrus

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INTRODUCTION

Hemolymph proteins may be as crucial in absorption and disposition of xenobiotics in insects as blood proteins such as albumen are in vertebrates, but little has been done to explore this possibility. Although studies a decade ago indicated binding of insecticides to hemolymph proteins or lipoproteins (see [1]), a specific lipoprotein, lipophorin, was not implicated until 1985 [2]. Soon thereafter, a second protein, identified as an arylphorin, was found to bind a number of insecticides in vitro [3].

Genetic transformation of plants, tissue regeneration, and expression of natural products may afford protection for plants against pests [4], but the lack of useful genes and gene products has inhibited progress [5]. Discovery and use of natural products may be hastened by understanding the mechanisms of absorption and distribution in insects, a subject that has received scant attention. Working on a citrus pest, the root weevil *Diaprepes abbreviatus*, we have begun to study hemolymph lipoproteins and proteins that may play a role in uptake and delivery of toxic compounds to sites of intoxication or detoxification.

In initial studies on uptake and binding of xenobiotics in hemolymph, a fluorescent natural product analog, 7-amino-3-phenyl coumarin (coumarin-10)* was employed [6]. Those studies demonstrated in vivo uptake and binding of coumarin-10 in *D. abbreviatus* larvae that were assayed after 1–2 months of dietary exposure. Although most of the coumarin-10 recovered in hemolymph sedimented in a density gradient with proteins other than lipophorin, binding proteins other than lipophorin were not identified.

In the present report, observations are expanded to short-term pharmacodynamic studies, using a newly developed forced-feeding technique. The short-term kinetics of absorption are described, and binding to hemolymph proteins, originally discovered through use of fluorescent energy transfer methods [6], has been confirmed by electrophoresis. The primary proteins responsible for binding coumarin-10 have been identified through native electrophoresis, their molecular weights determined, and in vitro binding to a semipurified preparation of the proteins has been described.

MATERIALS AND METHODS

Insects

Larvae of *Diaprepes abbreviatus* were reared from eggs obtained from field-collected adult weevils [7]. Last instar larvae of 300–800 mg weight (3–5 months old) were used.

Forced-Feeding

Coumarin-10 (7-amino-3-phenyl coumarin; Kodak, Rochester, NY; 95% purity by TLC) was dissolved in acetone at 1.0 mg/50 μl, then diluted to 1.0

*Abbreviations used: Coumarin-10 = 7-amino-3-phenyl coumarin; DFP = diisopropyl fluorophosphate; F = fluorescence; GP = gel permeation; N-PAGE = native polyacrylamide gel electrophoresis; PBS = phosphate buffered saline (0.10 M sodium phosphate/0.15 M NaCl/0.05% EDTA/pH 7.0); SDS = sodium dodecyl sulfate.

ml with peanut oil. This stock solution was then serially diluted with pure peanut oil.

Larvae that had been starved overnight were briefly anaesthetized in ice-water and force-fed under a dissecting microscope by inserting the point of a drawn, fire-polished 20- or 50-µl capillary tube into the mouth between the mandibles and gently injecting coumarin-10 in peanut oil (20 µl/500 mg of body weight) at room temperature. Approximately 2 µl of concentrated blue food coloring were injected immediately after the peanut oil/coumarin-10. Any breach in the digestive system during feeding was immediately indicated by the blue color appearing between gut and cuticle, and any larva so affected was discarded. With practice, the gut was rarely breached. Larvae regained movement during or shortly after feeding.

Hemolymph Collection and Extraction

For quantitative studies, 20 µl of hemolymph were collected from each briefly rechilled larva and immediately diluted in 1.0 ml of PBS. One milliliter of ethyl acetate (ACS grade, Fisher, Orlando, FL) was added, and samples were mixed thoroughly in 100 × 13 mm screw-capped tubes. Tubes were centrifuged at 1,800*g* in a Sorvall (DuPont, Wilmington, DE) SA600 angle rotor for 3 min at 10°C, and the top layer of ethyl acetate was removed with a drawn Pasteur pipet. Extraction of the aqueous phase was repeated twice, and pooled ethyl acetate phases were brought to 3.0 ml in graduated conical tubes prior to determination of fluorescence. To convert fluorescence to coumarin-10 concentration, a standard curve was developed using a dilution series of coumarin-10 in ethyl acetate. From the standard curve, fluorescence was converted to ng/µl of hemolymph.

Electrophoresis

N-PAGE for fluorescence studies was on 4–10% gradient minigels (100 × 70 × 1.5 mm) using the Laemmli system [8], excluding SDS and β-mercaptoethanol. Gels were run for 45 min at 200 V constant voltage and cooled by circulating water bath with a handmade cooling coil. Buffer in the center well reached a maximum of 10°C. Gels were immediately photographed with a Polaroid (Cambridge, MA) MP-3 camera and type 55 negative/positive film with UV filter under a longwave UV lamp directed at the gel from the top at an oblique angle. Gels were stained with Coomassie brilliant blue G-250 (BioRad, Richmond, CA) immediately after photographing [9].

Preparative N-PAGE was performed on a Bio-Rad model 422 electroeluter. Neutral gels (MZE 3328.IV system as per Moos et al. [10], except without SDS or β-mercaptoethanol) of 0.8 cm diameter × 4.5 cm were poured into tubes, and frits, cups, and membranes were added after polymerization. Samples of fractions 7–10 from KBr density gradient ultracentrifugation (see Fig. 4B) were dialyzed against 25% PBS (final absorbance at 280 nm was 12.8; determination made on a Gilford Response spectrophotometer, Ciba Corning Diagnostics [Oberlin, OH] following dilution). Each gel was layered with 50 µl of those samples, electrophoresis was run for 1.5 h at 200 V constant potential, gels were removed, and one gel was stained for 30 min and destained. Cylindrical slices containing each protein were cut from the remaining gels, re-

placed in tubes with cups and membranes, and run at 3 mA constant current for 2 h to elute proteins. Proteins were rerun in neutral [10] or high pH (Laemmli minus SDS, β-mercaptoethanol) N-PAGE minigels, and in the Laemmli SDS-Page system [8] on minigels. SDS-PAGE molecular weight determination of subunits utilized the Laemmli buffer system on large gels (15 × 20 × 0.15 cm) run at 200 V constant potential. Molecular weights were determined by linear regression analysis of log molecular weight vs. distance migrated, relative to measurements taken from Bio-Rad low molecular weight standards run on the same gel.

Chromatography

TLC of coumarin-10 was on silica plates (20 × 20 cm × 250 μm; Fisher) developed in chloroform:ethyl acetate (4:1). Spots were quantified by fluorescence densitometry on a Shimadzu (Columbia, MD) CS-9000 scanner equipped with a xenon lamp and 1 × 10 mm slit width, using excitation of 370 nm and cutoff filter number 2 (100% transmittance above 440 nm).

HPLC equipment included a Spectra Physics (San Jose, CA) SP8800 gradient pump with variable wavelength DuPont (Wilmington, DE) UV detector. For simultaneous detection of protein and coumarin-10, a SpectroVision (Chelmsford, MA) FD-300 fluorescence detector (excitation 390 nm, emission 460 nm) was placed in series with the UV detector. A TSK G3000SW-XL column (0.78 × 30 cm; TosoHaas Co., Philadelphia, PA) was used for GP-HPLC. GP-HPLC standards included bovine serum albumin ($M_r = 66{,}500$) and transferrin (76,000) (Sigma Co., St. Louis, MO); lactate dehydrogenase (140,000), phosphorylase a (370,000), phosphorylase b (185,000), and pyruvate kinase (237,000) (Boehringer, Indianapolis, IN); aldolase (158,000) and ferritin (440,000) (Pharmacia, Piscataway, NJ); and urease (483,000) and β-galactosidase (540,000) (Calbiochem, San Diego, CA). Hydrophobic interaction HPLC was run on a Brownlee labs (Applied Biosystems, Santa Clara, CA) Aquapore HIC-300 (10 × 0.46 cm, 7 μm) column coupled to an Aquapore HIC-GU (3 × 0.46 cm, 7 μm) column. A Rainin (Woburn, MA) Hydrophore SCX column (10 × 1 cm) was used for strong cation-exchange HPLC, and a Bio-Rad HRLC MA7Q column (5 × 0.78 cm) was used for anion-exchange HPLC.

Fluorescence Spectroscopy and In Vitro Binding Studies

Fluorescence spectroscopy and measurements of fluorescence intensity were done on an SLM-Aminco (Urbana, IL) SPF-500C spectrofluorimeter. Samples extracted in ethyl acetate were analyzed for fluorescence intensity at 450 nm with excitation at 370 nm [6].

For binding studies, lipophorin was first removed by density gradient ultracentrifugation and band I/II proteins were further copurified by GP-HPLC (see Results). A saturated solution of coumarin-10 was prepared by thoroughly mixing coumarin-10 in PBS at 0.2 mg/ml at room temperature. Insoluble coumarin-10 was removed by pelleting in a microcentrifuge (Beckman model 11, Fullerton, CA) at full speed for 2 min, and the supernatant was removed and used for binding studies. The concentration of courmarin-10 in 1-ml aliquots of the supernatant was determined by partitioning against ethyl acetate (see Hemolymph Collection and Extraction, above).

After diluting saturated coumarin-10 with PBS to given concentrations, protein was added to give a final absorbance of 0.10 at 280 nm, mixed thoroughly, and an emission spectrum recorded (constant excitation at 290 nm) within 1 min in a water-jacketed cuvette maintained at 25°C. From difference spectra (see Results, Fig. 9), the fluorescence at 340 nm and change in fluorescence at 455 nm with addition of protein to coumarin-10 was recorded, and binding saturation curves estimated (see [11] for a full discussion of the method).

Curve-Fitting

All curves were fitted by computer by finding optimal goodness-of-fit using iteration, with algorithms for linear or nonlinear regression analysis [12] (GraphPad, ISI Software, Philadelphia, PA).

RESULTS

Time-Course and Dose-Response of Coumarin-10 Absorption

Larvae were force-fed coumarin-10 in oil and hemolymph was sampled and coumarin-10 content quantitated 5 min or longer after feeding. Coumarin-10 rapidly appeared in hemolymph: Accumulation was detectible within 5 min, continued to increase throughout the first 50 min, and reached a steady state within 1 h (Fig. 1).

Oral administration of varying doses of coumarin-10 resulted in increasing accumulation in hemolymph with increased dose (Fig. 2), and the relationship was readily described by a semilogarithmic sigmoidal curve fitted by computer by goodness-of-fit. Accumulation rapidly increased with increasing doses between 10 and 100 ng coumarin-10 mg body weight. Within the limits of coumarin-10 solubility in the injected oil mixture (maximum of 2 mg/ml), saturation of the hemolymph by coumarin-10 was not achieved during the 2 h of larval exposure. Accumulations of up to 5 ng/μl in hemolymph compare to 1–3 ng/μl solubility in PBS.

Demonstration of Binding by Fluorescence Spectroscopy

Short-term studies confirmed the initial conclusion from long-term dietary studies [6] that coumarin-10 binds to proteins once it is absorbed into hemolymph. In hemolymph collected from larvae 1 h after feeding, excitation of Trp residues of proteins at 290 nm resulted in a peak of emission from those residues at 340 nm and an emission peak from coumarin-10 at 455 nm (Fig. 3). Direct excitation of coumarin-10 at 380 nm (the excitation maximum of coumarin-10 in aqueous solution [6]) in the same hemolymph indicates that the 455-nm emission was from coumarin-10. Hemolymph from unfed controls showed no significant emission at 455 nm when excited at 290 nm.

Identification of Binding Proteins

With evidence of coumarin binding, identification of the protein or proteins responsible for binding was attempted. Since lipophorin binds apolar insecticides such as DDT [2,3], lipophorin was separated from other hemolymph proteins using a density gradient ultracentrifugation technique [9] adapted from Shapiro et al. [13]. When density gradients were run for 2 h and 16 h (the

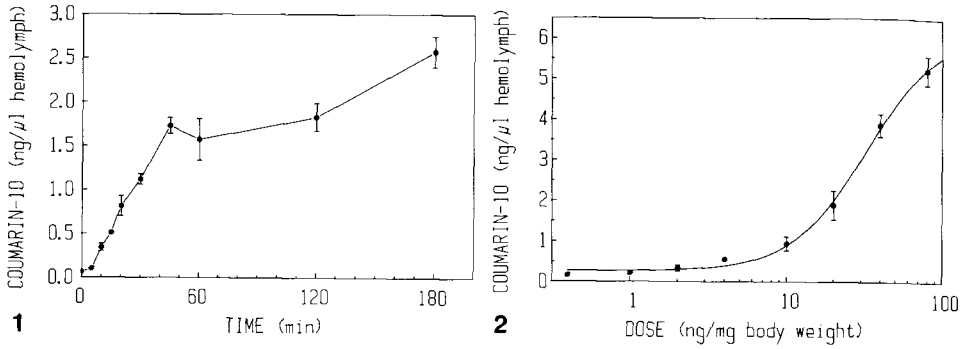

Fig. 1. Time-course of coumarin-10 absorption from gut into hemolymph. Larvae, averaging approximately 500 mg in weight, were force-fed 20 ng/mg body weight of coumarin-10 in peanut oil (20 μl/500 mg of weight). Hemolymph samples (20 μl/larva) were drawn at various times after feeding, extracted with ethyl acetate, and fluorescence in the extracts was converted to coumarin-10 equivalent concentrations (ng/μl hemolymph) as described in Materials and Methods. Mean ± SEM of triplicates.

Fig. 2. Dose-response of absorption from gut. Larvae were fed increasing weight-specific doses of coumarin-10 in proportionate volumes of peanut oil, and hemolymph was collected and analyzed as above, 2 h after feeding. Mean ± SEM of triplicates. The fitted sigmoidal curve was derived from the following equation:

$$Y = 0.268 + \frac{6.257 - 0.268}{1 + \frac{(10^{x})^{1.809}}{(10^{1.517})^{1.809}}}$$

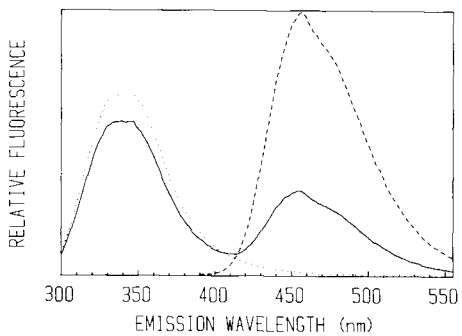

Fig. 3. Emission spectra of whole hemolymph from larvae fed 20 ng/mg body weight of coumarin-10 1 h prior to bleeding, with excitation at 290 nm (———) or 380 nm (------), and of whole hemolymph from unfed larvae, excited at 290 nm (·····).

gradient reaches equilibrium within 16 h [9]), progressive sedimentation of most coumarin-10 fluorescence with nonlipoproteins was evident (Fig. 4). Only about 6% of the total fluorescence extracted by ethyl acetate appeared at the same position as lipophorin (arrows, Fig. 4) in fractions from both 2-h and 16-h centrifugations.

Hemolymph from coumarin-10-fed animals yielded fluorescent bands cor-

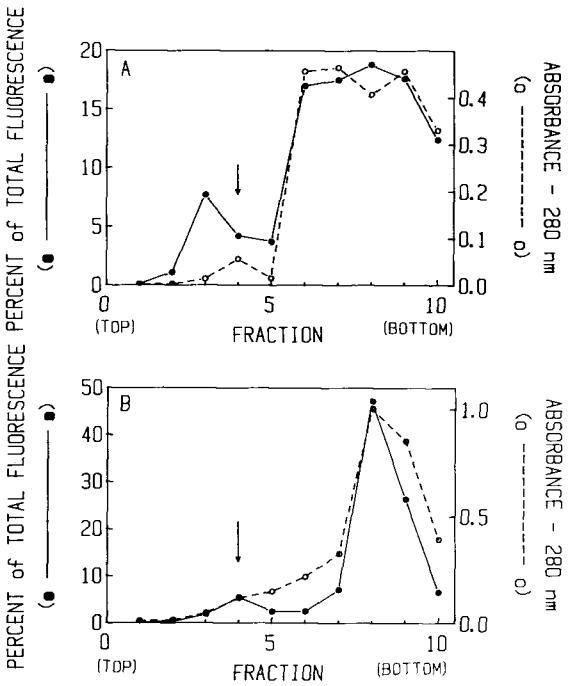

Fig. 4. Density gradient ultracentrifugation of hemolymph from coumarin-10-fed larvae. Fluorescence (●——●), as percent of total in the gradient, was measured after extraction in ethyl acetate; absorbance of fractions at 280 nm (o------o) was measured prior to extraction. Larvae were fed 40 ng/mg body weight of coumarin-10, and approximately 100 µl of hemolymph were removed 1 h later. Hemolymph was then centrifuged in a KBr density gradient for **A)** 2 h or **B)** 16 h [9]. Lipophorin is found in fractions 3 and 4 after a 16- or 18-h centrifugation. Fractions were collected, extracted, relative fluorescence was determined, and background fluorescence was subtracted prior to conversion to percentages. Mean of duplicate gradients. Arrows: Position of lipophorin.

responding to the major hemolymph proteins when subjected to native minigel PAGE (Fig. 5B). Some fluorescence also ran at the front, and some smeared in the stacking and upper separating gels, where lipophorin tends to smear. If gels were run without cooling, Coomassie-stained protein bands remained compact, although very little fluorescence was bound to proteins (results not shown).

The extent of coumarin-10 binding to each of the major proteins in gels was variable. Although the two major proteins usually bound coumarin-10 equally, coumarin-10 sometimes bound predominantly to the upper protein band. However, Coomassie staining of the fluorescent gels indicated that both proteins were present in similar quantities in all cases (Fig. 5A).

When sedimenting proteins from a density gradient (equivalent to fraction 8, Fig. 4B) were chromatographed on a high-resolution GP-HPLC column, only a single major sharp peak appeared (Fig. 6A). All eluting coumarin-10 fluorescence in hemolymph from coumarin-10-fed larvae coeluted with this peak (results not shown, but are superimposable on the absorbance peak), although variable proportions of the total fluorescence also bound reversibly to the column packing. Using a logarithmic curve fitted by least-squares goodness-of-

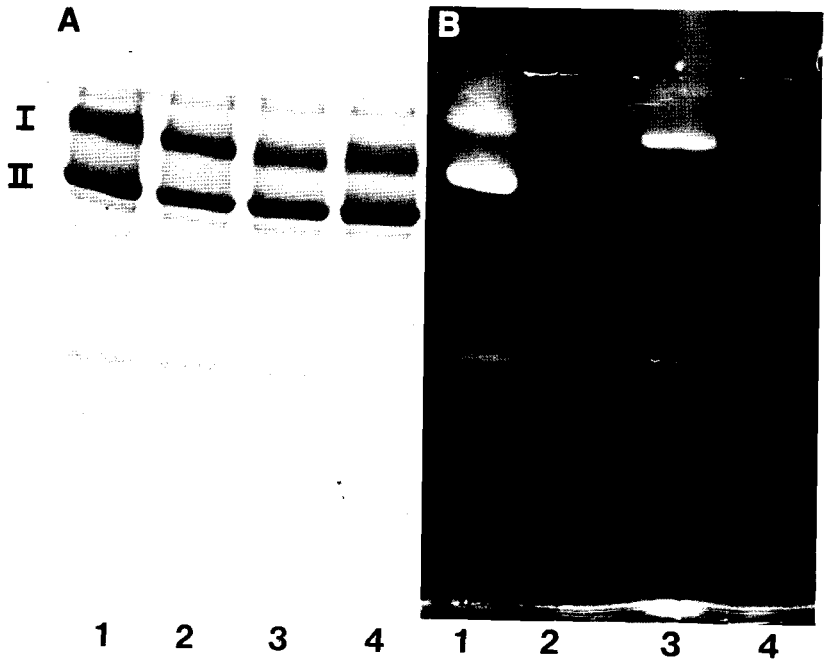

Fig. 5. Native PAGE of hemolymph proteins, showing **A)** coomassie-stained protein bands, or **B)** fluorescence under longwave UV. Hemolymph (0.5 µl/lane) from unfed larvae (**lanes 2,4**) or from larvae fed coumarin-10 (**lanes 1,3**) 1 h prior to collection were run on a 4–10% gradient minigel. Larval weights: lane 1, 680 mg; lane 2, 630 mg; lane 3, 315 mg; lane 4, 290 mg.

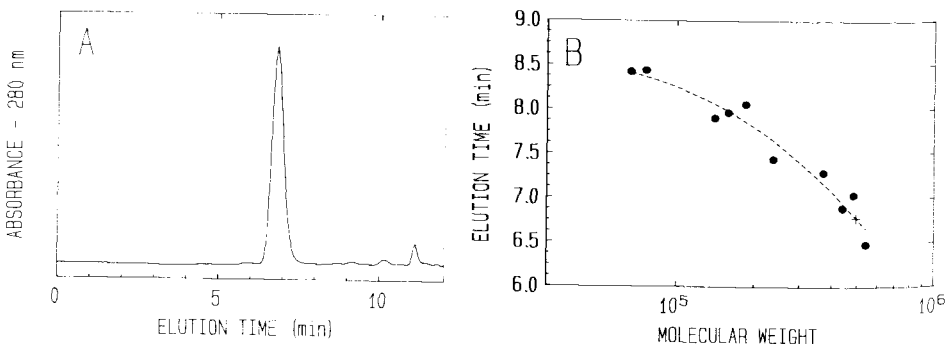

Fig. 6. GP-HPLC determination of molecular weight of the major proteins sedimented in a KBr density gradient. **A:** Elution profile. **B:** Plot of standard-protein elution time vs. molecular weight, with fitted exponential decay curve [12] and major peak of sedimenting hemolymph proteins (+). Sedimenting proteins (fractions 7–10, Fig. 4B) collected from a KBr density gradient were dialyzed extensively vs. PBS prior to chromatography. Flow rate: 1.0 ml/min. Buffer: 0.1 M $NaHPO_4$/0.2 M NaCl/pH 7.0, with 0.004% NaN_3. Standards: bovine serum albumin, transferrin, lactate dehydrogenase, aldolase, phosphorylase b, pyruvate kinase, phosphorylase a, ferritin, urease, and β-galactosidase.

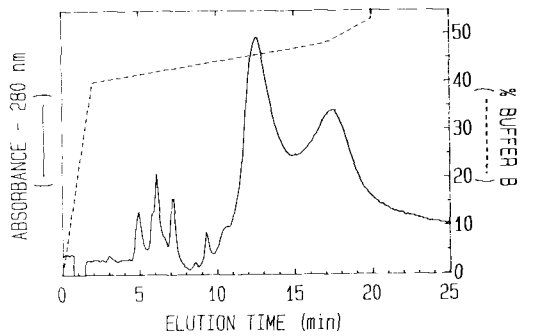

Fig. 7. Cation-exchange HPLC of whole hemolymph. Hemolymph (total of 300 µl) from 6 larvae was pooled into 700 µl of 20 mM sodium phosphate/pH 5.5 containing 9 µl of DFP. Sample was dialyzed against the same buffer for 1 h at 4°C, filtered through a 0.45-µm nylon filter, and 30 µl were injected onto the column. Starting buffer (buffer A): 20 mM sodium phosphate/pH 5.0; buffer B: 20 mM sodium phosphate/1.0 M NaCl/pH 5.0. Flow rate: 1.0 ml/min.

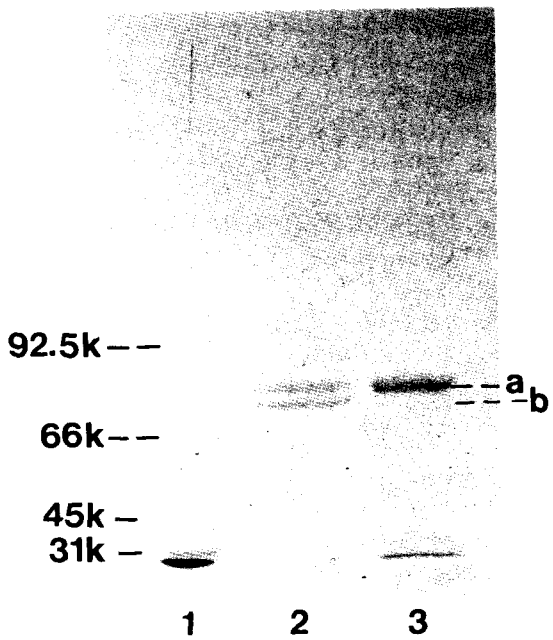

Fig. 8. Coomassie-stained SDS-PAGE on a 7% minigel of subunits eluted from neutral pH N-PAGE gels as bands I and II. Molecular weight markers: phosphorylase B (M_r 92,500), bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), carbonic anhydrase (M_r 31,000).

fit, relative molecular weight was determined to be 480,000 by interpolation (Fig. 6B). That result was almost exactly duplicated on the same column run at 0.3 ml/min instead of 1.0 ml/min.

Though the proteins that bound coumarin-10 appeared as two bands on N-PAGE, they could not be fully separated chromatographically. Despite optimization of shallow gradients in cation- and anion-exchange and hydrophobic interaction HPLC columns, only a broad shoulder could be separated from

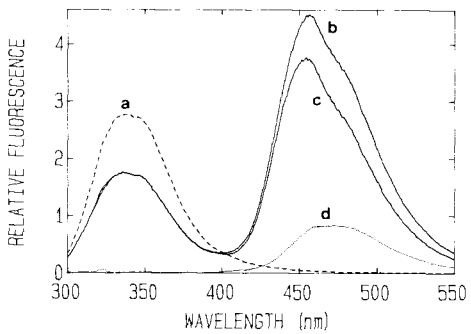

Fig. 9. Emission spectra (290 nm constant excitation) demonstrating energy transfer upon binding of coumarin-10 to GP-HPLC-isolated proteins in vitro. **a:** Protein (final A_{280} = 0.1) without coumarin-10 (------). **b,c:** Protein with coumarin-10 (final concentration: 1.3 μM) in PBS (———), mixed approximately 1 min prior to beginning the wavelength scan. d: Coumarin-10 without protein (·····). c represents the difference spectrum of **b–d**, yielding ΔF.

the main peak. The best separation was achieved using cation exchange HPLC (Fig. 7). The peak at 13 min contained both upper (band I) and lower (band II) N-PAGE bands, while band II predominated in the 17.4-min peak, with only slight band I contamination.

Hemolymph was electrophoresed using preparative N-PAGE on neutral pH tubular gels, and bands I and II were separately eluted from gel sections. Electroeluted band II was nearly homogeneous when rerun on analytical N-PAGE at high pH, while band I subdivided into both bands I and II upon rerunning (results not shown). When eluted band I and II proteins were rerun on SDS-PAGE, band I showed two subunits (a and b), M_r of 75,000 and 71,000, respectively, while band II showed only subunit a (Fig. 8; molecular weights were determined on a standard size SDS gel as described in Materials and Methods).

Binding of Coumarin-10 to Partially Isolated Proteins In Vitro

As in Figure 6A, band I/II proteins were partially copurified by removing lipophorin on a density gradient and collecting the major protein peak eluting from the GP-HPLC column. Resulting proteins I/II were 94% pure by GP-HPLC; 4% of impurities were in the form of aggregates. When these proteins were mixed with coumarin-10 in PBS, Trp fluorescence (340 nm emission) decreased and coumarin-10 fluorescence (455 nm emission) increased (Fig. 9). In addition to the increase in fluorescence intensity, the peak emission wavelength of coumarin-10 in PBS shifted from 470 nm to 455 nm. A difference spectrum (curve c) was calculated by subtracting curve d from b. The change in fluorescence at 455 nm (ΔF_{455}) with addition of protein was then read directly from curve c.

Saturation binding analysis showed decreasing Trp emission (F_{340}) with increasing coumarin-10, while coumarin-10 emission (ΔF_{455}) increased (Fig. 10A). Both curves plateaued at approximately the same concentration of coumarin-10. A binding curve fitted by algorithm for a rectangular hyperbola (Fig. 10A) yielded the following equation:

$$Y = 6.28X/(1.08 + X)$$

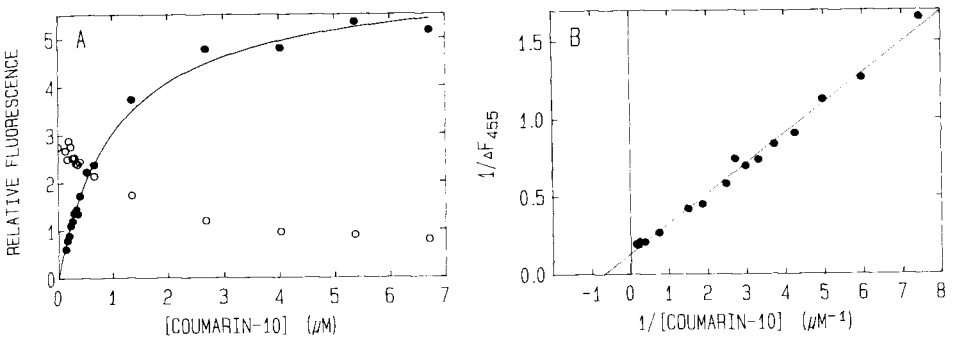

Fig. 10. **A:** Fluorescence intensity (F) of emission at 340 nm (○ ○ ○) or change in fluorescence at 455 nm (ΔF_{455}) (····) with addition of increasing concentrations of coumarin-10 to GP-HPLC-isolated protein. Protein (final A_{280} = 0.1) and coumarin-10 in PBS were mixed as in Figure 9 and emission spectra were recorded at 290 nm constant excitation. **B:** Double reciprocal plot of ΔF_{455} vs. coumarin-10 concentration.

Fluorescence saturation (ΔF_{455}) was thus estimated to be 6.3, with a K_D of 1.1 μM (K_D = coumarin-10 concentration at half-maximal fluorescence).

A double-reciprocal plot of the same data (Fig. 10B) yields similar information: linear regression analysis gave the equation:

$$Y = 0.195X + 0.133$$

The X-intercept ($-1/K_D$) is thus estimated to be -0.68, and K_D = 1.5 μM.

Identification of Metabolites in Hemolymph and Density Gradient Fractions

When whole hemolymph was extracted with ethyl acetate and analyzed by TLC, the position of major spots coincided with coumarin-10 (R_f = 0.46) and a major polar metabolite at the origin (Table 1). A small amount of a contaminant that comprised 3.4% of the original coumarin-10 preparation was present between these two spots (R_f = 0.22). Fraction 9 from an 18-h density gradient

TABLE 1. TLC of Coumarin-10 and Metabolites From Whole Hemolymph and From Density Gradient Fractions*

	Percent of total fluorescence		
R_f	Whole hemolymph	Fraction 4 (lipophorin)	Fraction 9 (subnatant)
0.00	30.0	25.6	18.5
0.22	1.0	0.4	1.5
0.46	65.5	69.0	80.0
0.75	3.5	5.0	N.D.

*Larvae were fed 40 ng/mg body weight of coumarin-10; hemolymph was collected 1 h later and was either extracted with ethyl acetate as above or centrifuged in a density gradient and fractions extracted with ethyl acetate. Ninety-six percent of fluorescence from chromatographed coumarin-10 ran at an R_f of 0.46. Samples in ethyl acetate were run on thin-layer plates as described and scanned (N.D. = not detectable).

centrifugation of hemolymph (see Fig. 4B; larvae bled 1 h after feeding) and fractionated by density gradient showed 80% coumarin-10 and 20% polar metabolite at the origin, compared to 96% and 4%, respectively, in the stock coumarin-10 preparation. Lipophorin (fraction 4, Fig. 4B) also showed an apolar metabolite constituting 5% of total fluorescence ($R_f = 0.75$).

DISCUSSION

These results clearly demonstrate in vivo uptake and binding and in vitro binding of a xenobiotic to proteins in hemolymph of an insect. Three distinctly different physical methods were used to demonstrate binding: fluorescent energy transfer, native PAGE, and density gradient ultracentrifugation. With the present results, the isolation of lipophorin from *Diaprepes abbreviatus* [9], and further isolation of the binding proteins identified in the present study, we are establishing tools to examine a broad range of xenobiotics, the characteristics of their interactions with hemolymph proteins, and pharmacodynamics following digestive uptake.

Earlier fluorescence spectroscopy studies [6] demonstrated coumarin-10 uptake and binding to hemolymph proteins, but those studies involved larvae fed dietary coumarin-10 over a period of 1–2 months. Forced-feeding, combined with analysis by minigel electrophoresis and fluorescence spectroscopy, allows rapid tracing of xenobiotic uptake and binding over broad periods of time. Detectable uptake under test conditions occurs rapidly (within 5 min) and at low doses of coumarin-10.

Although many aspects of this analysis can make use of radiotracers, fluorescent compounds are found in abundance in many plants, including citrus, and do not require derivatization. Some fluorescent light-activated compounds such as the coumarins play significant roles in plant-insect interactions [14–17]. Furthermore, fluorescence has provided a tool useful not only for quantitation, but also for qualitative discoveries. Binding to proteins was first identified by fluorescent energy transfer during long term studies [6]. We have now demonstrated uptake and binding of 94% of absorbed coumarin-10 to nonlipoproteins in vivo. In vitro, binding to these proteins in a hydrophobic environment proximate to aromatic amino acid residues is indicated by 1) the increased intensity of coumarin-10 fluorescence (455/470 nm peak) when excited at 290 nm upon addition of protein to coumarin-10, indicating increased indirect excitation of coumarin-10; 2) decreased protein fluorescence at 340 nm upon addition of coumarin-10, demonstrating reduced emission due to nonradiative energy transfer from Trp residues; and 3) a shift of the emission peak of coumarin-10 in hemolymph from 470 to 455 nm, indicating a decrease in polarity of the coumarin-10 microenvironment with addition of protein (see [18] for a full discussion). The shift is comparable to that observed when coumarin-10 is dissolved in ethyl acetate [6], a solvent of intermediate dielectric constant (6.02 at 20°C) and dipole moment (1.88 at 25°C).

These binding studies fully confirmed the in vivo results. Spectra from coumarin-10 bound in vivo (Fig. 3) match those from coumarin-10 bound in vitro (Fig. 9). With an estimated K_D of 1.5 μM, coumarin-10 shows in vitro binding of moderate affinity at 25°C. The demonstration of energy transfer

from protein residues to coumarin-10 in vitro was conclusive: all small molecules present in vivo had been removed by GP-HPLC, leaving only aromatic amino acid residues in protein to overlap in emission spectrum with the excitation spectrum of coumarin-10, a prerequisite for energy transfer [18]. Emissions from those aromatic residues decreased with increasing coumarin-10 concentration, with clear correlation to increasing coumarin-10 emissions (Fig. 10A).

Migration of fluorescence in high pH native gels was coincident with the migration of two specific proteins, the most abundant proteins in hemolymph of *D. abbreviatus*. Both high and neutral pH N-PAGE systems showed similar protein banding patterns. When used preparatively, both systems yielded a single relatively pure band II protein and a heterogeneous band I protein which contained both band I and II upon reelectrophoresis. On SDS-PAGE, protein eluted from band I contained two sizes of subunit (a and b, Fig. 8), while that from band II contained only subunit a. This indicates that differential mobility of bands I and II is due primarily to their charge and not size, since the band containing only the larger subunit ran faster in both high and neutral pH gels. The native molecular weight of 480,000 indicates probable hexameric structures, consistent with structures of known insect storage proteins [19]. However, positive or negative identification as storage proteins will await further developmental and structural studies.

There is no known functional significance for binding of xenobiotics in hemolymph. Xenobiotic binding may either protect an insect against intoxication or expose the insect to increased toxicity, and has now been observed in three insects: *Diaprepes abbreviatus, Manduca sexta,* and *Heliothis zea.* In *Manduca,* binding was observed following cuticular absorption in vivo [2]. In *Heliothis,* binding was observed through in vitro studies with isolated hemolymph [3]. Some earlier studies, e.g., with *Periplaneta americana* [20,21], also indicated possible binding, and referred to hemolymph lipoproteins as the binding moieties. Those studies varied in their use of in vivo vs. in vitro methods; in vivo studies have varied in introduction of xenobiotics by cuticular vs. oral routes. These various means of exposure of the animal and its hemolymph or isolated proteins to xenobiotics must be directly compared in a single species to permit firm conclusions about function.

Our studies do not delineate the relative importance of binding by lipophorins vs. other hemolymph proteins. We find that 6% of coumarin-10 in the hemolymph binds to lipophorin, with the remainder binding to other proteins. However, only studies using compounds of differing polarities and hydrophobicities will allow us to test the hypothesis of Haunerland and Bowers [3], that partitioning between arylphorin and lipophorin determines the mode of binding of various compounds. We also have yet to characterize fully the binding protein(s) other than lipophorin. The significance of variability in binding by the major proteins during electrophoresis (Fig. 5) is presently unknown. The appearance of two major proteins in native PAGE and the difficulty of separating them with any of several chromatographic techniques are also unexplained.

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