Identification of juvenile-hormone-binding proteins on blotted electropherograms using tritiated juvenile hormones

J. R. Wiśniewski

III. Zoologisches Institut-Entwicklungsbiologie, Universität Göttingen, Berlinerstr. 28, D-3400 Göttingen (Federal Republic of Germany)
Received 30 March 1989; accepted 25 May 1989

Summary. An electroblotting method has been developed to identify juvenile-hormone-binding proteins (JHBPs) of insects. JHBPs were separated by means of one- or two-dimensional native PAGE and then transferred onto ion-exchange membranes. Following incubation in buffer containing ^{3}H-JH, the hormone-binding proteins could be detected by autoradiography or ^{3}H-scanning of the blots. In *G. mellonella* and *L. migratoria* hemolymph, respectively, two and five electrophoretically different JHBP species were detected.
Key words. Juvenile hormone binding protein; juvenile hormone; electroblotting; *Galleria mellonella*; *Locusta migratoria*.

Juvenile-hormone-binding proteins (JHBPs) occur in the hemolymph and in various tissues[1]. The JHBPs in the hemolymph function as JH carriers for the transport of JHs from the sites of synthesis to the target tissues[2]. While the role of intracellular JHBPs is still unclear, it has been presumed that certain JHBPs may act as receptors of JHs[3–7]. For an elucidation of the molecular mechanisms of JH action, the isolation and characterization of JHBPs is an important prerequisite. The identification on blots of hormone-binding proteins and receptors by iodinated human growth hormone, epidermal and insulin-like growth factors, prolactin, and calmodulin has been reported[8–13]. However, no procedure has been available to detect on blots proteins which bind isoprenoid or steroid hormones. I have therefore developed a method in which JHBPs from complex protein samples, after electrophoretic separation, are transferred onto ion-exchange membranes by electroblotting. The selected membranes exhibit, in contrast to nitrocellulose and other blotting materials, very low nonspecific adsorption of JH. Thus following an incubation of the blots in buffer containing ^{3}H-JHs it was possible to detect JHBPs by means of autoradiography or ^{3}H-scanning.

Materials and methods

Racemic [10-^{3}H]-JH III (10.9 Ci/mmol) was purchased from Dupont-NEN (Dreieich, Germany). (10*R*,11*S*)-[12,13-^{3}H]-JH II (49 Ci/mmol) and (10*R*,11*S*)-JH II were a gift of Prof. G. D. Prestwich (Stony Brook, USA). Unlabelled and racemic JH III was obtained from Sigma. Nitrocellulose (0.45 µm) was obtained from Sartorius (Göttingen, Germany). The nylon membrane (Pherobind, 0.45 µm) was from Biotech-Fischer (Reiskirchen, Germany), whereas the DE-81 paper was from Whatman (Maidstone, England). The ion-exchange membranes NA-45 (DEAE) and NA-49 (carboxy-methyl, CM), the aminobenzyloxymethyl-paper (ABM) and aminophenylthioether-paper (APT) were purchased from Schleicher & Schuell (Dassel, Germany).

Hemolymph samples of last instar larvae of *Galleria mellonella* (Lepidoptera) and *Locusta migratoria* (Orthoptera) were collected and stored as described previously[14,15]. Crude preparations of the hemolymph JHBPs were obtained by means of gel filtration on a Sepharose CL-6B column as described earlier[14].

Pieces (10 × 5 mm) of different blotting filters were tested for unspecific adsorption of JHs. Prior to incubation with radiolabelled JHs the blotting filters were preincubated in selected quenching agents at 20 °C for 1 h. Nitrocellulose and nylon membranes were incubated in TK buffer (10 mM tris/HCl, 50 mM KCl, pH 7.4), containing one of the following substances: 5% (w/v) bovine serum albumin, 2% (w/v) gelatin, 2% (w/v) polyvinylpyrrolidone, or 0.05% (v/v) Tween 20, respectively. The ABM and APT papers were diazotized and then inactivated according to Alwine et al.[16] and Symington et al.[17], respectively. The NA-45 and DE-81 filters were incubated in 10 mM tris/H_3BO_3, pH 9.0 or in 1% (w/v) gelatin titrated with tris to pH 9.0. The membrane NA-49 was incubated in 10 mM sodium phosphate, pH 5.9 or in this buffer containing 1% (w/v) gelatin. Following the preincubation the filters were rinsed in the same buffers without quenching agents, and then were incubated separately for 10 min at 20 °C in 0.4 ml of the appropriate buffer containing 10 nM (100,000 dpm) of [^{3}H]-JH III. After incubation with hormone the filters were rinsed four times for 1 min in the appropriate buffer and the wet filters were transferred into scintillation vials for the measurement of adsorbed radioactivity.

Samples containing JHBPs were electrophoresed according to Davis[18] in 5% (*L. migratoria* proteins) or 10% (*G. mellonella* proteins) polyacrylamide (PA) slab gels (140 × 140 × 1.5 mm). The separations of protein samples were always done in duplicate; one of the gels was stained with Coomassie R-250, whereas the other one was blotted. The hemolymph proteins of *L. migratoria* were also analyzed by means of two-dimensional electrophoresis. In the first dimension the hemolymph proteins were

isoelectrofocused in 1% (w/v) Agarose IEF (Pharmacia) gel containing 7.8% (v/v) Servalyt 4–7 (Serva). In the second dimension the proteins were separated in a PA linear gradient gel according to de Bruijn et al.[19]. Immediately after electrophoresis the gels were soaked in three changes (7 min per change) of transfer buffer. 10 mM tris/H_3BO_3, pH 9.0, was used for the transfer to DEAE-membranes (NA-45) and 10 mM sodium phosphate, pH 5.9 for the transfer to CM-membranes (NA-49). The electrotransfer of proteins was carried out in a Transfor 2005 Unit (LKB) at 4 °C for 1 h at 100 V (DEAE-membrane) and 70 V (CM-membrane), respectively. Following the electrotransfer of proteins the DEAE- and CM-membranes were incubated for 15 min at 20 °C in 1% (w/v) gelatin/tris, pH 9.0, and in 1% (w/v) gelatin in 10 mM sodium phosphate, pH 5.9, respectively. Then they were rinsed briefly in the appropriate transfer buffer and overlayered with a 10 nM (100,000 dpm/ml) solution of [^{3}H]-JH II or [^{3}H]-JH III prepared in the corresponding buffer for 30 min at 20 °C. Then the unbound hormone was removed by rinsing the filters in four changes of buffer for 1 min each. The dried blots were analyzed for radioactivity using a TLC scanner (LB 2722, Berthold, Germany) or were autoradiographed using ^{3}H-Hyperfilm (Amersham).

Dot blots were prepared by pipeting 3-µl aliquots of crude JHBPs from *L. migratoria* hemolymph onto 10 × 10 mm squares of the NA-45 membrane. After 5-min incubation at room temperature the membranes were washed twice in 10 mM tris/H_3BO_3, pH 9.0, and then treated like the electroblots as described above. Finally the wet filters were transferred to scintillation vials for measurement of the bound radioactivity.

The JH binding activity of the samples of eluted PA gel slices was determined using the dextran-coated charcoal method described previously[14].

Results and discussion

The detection of JHBPs on protein blots by their binding of ^{3}H-JHs requires the use of blotting matrices which themselves exhibit no or very low adsorption of JHs. Juvenile hormones are known to adsorb readily to glass, plastics and filters[20], and were found in the present study to adsorb heavily to nitrocellulose and nylon membranes (table). Several agents, including bovine serum albumin, gelatin, polyvinyl-pyrrolidone and Tween 20 have been proposed for quenching of unspecific adsorption of antibodies and proteins to blots [for ref. see Beisiegel[21]], but these substances only slightly reduced the unspecific interaction of JH III with nitrocellulose or nylon membranes (table). ABM and APT papers were found to adsorb 1.2% and 5.7% JH III, respectively, when coated with gelatin (table). The lowest adsorption of JH III was exhibited by ion-exchange membranes of types NA-45 and NA-49 preincubated in 1% gelatin (table). The percentage of JH III adsorbed to the NA-45 and NA-49 matrices appeared to be constant within a range of 1 nM – 10 µM JH III (not shown).

As model JHBPs, the hemolymph proteins of the moth, *G. mellonella*, and the locust, *L. migratoria*, which have been characterized previously[19, 22–24], were used in blotting experiments. In the present investigation, gel electrophoresis and elution of sliced gel fractions of *G. mellonella* hemolymph proteins led to the identification of a second JHBP form with lower electrophoretic mobility in addition to the main JHBP band (fig. 1 A). In larval hemolymph of *L. migratoria* three JHBP fractions were detected (fig. 1 B). The labelled hormone could be displaced by a 1000-fold excess of cold hormone (fig. 1) and the hormone binding in all JHBP peaks was therefore specific.

The basic JHBPs from *G. mellonella*[22] were transferred after electrophoresis onto CM-membranes (NA-49), whereas the JHBPs from *L. migratoria* hemolymph, which are retained on DEAE cellulose columns[24], were blotted onto DEAE-membranes (NA-45). A quantitative transfer of protein bands from the gels to the filters was achieved (figs 2 and 3, B). *G. mellonella* JHBP exhibits the highest affinity to JH II[22] and therefore the electroblots of *G. mellonella* proteins were incubated with [^{3}H]-

Adsorption of [^{3}H]-JH III on different blotting materials

Blotting material	Blocking agent(s)	Percent of adsorbed JH III
Nitrocellulose	no[a]	28.5 ± 5.4[b]
	5% bovine serum albumin	19.8 ± 3.3
	2% gelatin	28.5 ± 1.5
	2% polyvinylpyrrolidone	23.3 ± 3.1
	0.05% Tween 20	21.8 ± 3.4
Nylon (Phero bind)	no	14.6 ± 3.0
	5% bovine serum albumin	10.3 ± 2.0
	2% gelatin	12.5 ± 1.5
DBM-paper	10% ethanolamine/0.25% gelatin	5.7 ± 0.6
DPT-paper	10% ethanolamine/0.25% gelatin	1.2 ± 0.1
DE-81 (DEAE-paper)	no	3.0 ± 0.2
	1% gelatin	0.48 ± 0.37
NA-45 (DEAE-membrane)	no	3.2 ± 0.34
	1% gelatin	0.18 ± 0.16
NA-49 (CM-membrane)	no	3.1 ± 0.67
	1% gelatin	0.17 ± 0.09

[a] Buffer only; [b] mean ± SE, N = 5.

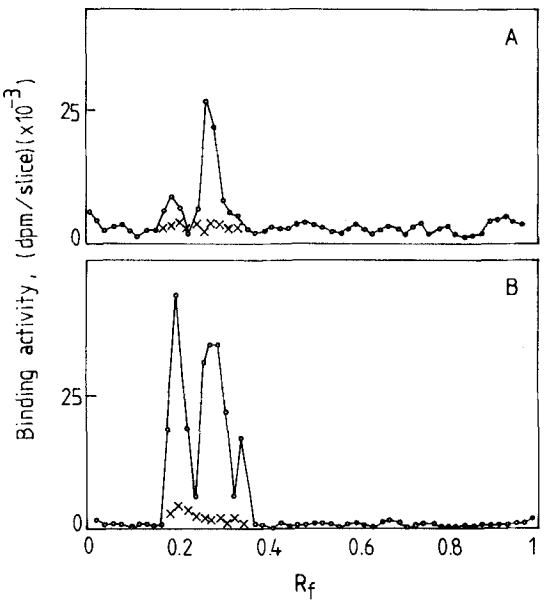

Figure 1. Distribution of JH binding activity after electrophoretic separation of samples of crude JHBPs from the hemolymph of *G. mellonella* (A), or *L. migratoria* (B). The sample of *G. mellonella* JHBPs contained 300 µg protein and was separated in a 10% gel (A), whereas the sample of *L. migratoria* contained 2 µl hemolymph and was separated in a 5% gel (B). The total JH-binding of eluted samples from 2.5-mm slices of the gels (circles) was determined by the charcoal adsorption assay using 10 nM [^{3}H]-JH II (A) or 10 nM [^{3}H]-JH III (B). The unspecific binding was measured in the presence of an excess of 10 µM unlabelled JH III (crosses).

JH II. For the hemolymph of *L. migratoria* the lowest K_D has been found for JH III [19] and the *L. migratoria* proteins on the blots were therefore incubated with [^{3}H]-JH III.

Following incubation with tritiated hormones and subsequent rinsing, the electroblots were first analyzed by a radioactivity scanner and then autoradiographed. Scanning allowed a fast localization of the radioactive spots within a few hours. Autoradiography on ^{3}H-hyperfilm resulted in high resolution but required an exposition time of 0.5–2 weeks. The positions of the ^{3}H-labelled areas on the blots detected by the scanner fully corresponded to the binding activity in the gels which was detected after elution (compare fig. 1 with panels C in figs 2, 3).

The hemolymph of *G. mellonella* has been reported to contain a single JHBP subunit of 32 kDa [22] which during some purification procedures is readily metabolized by hemolymph proteases [23]. In the applied blotting procedure the native form of JHBP as well as its degraded form was detected (fig. 2).

The hemolymph of *L. migratoria* larvae separated in 5% gels revealed five JHBP species on autoradiographs (fig. 3D) some of which were not resolved by scanning (fig. 3C) or by the gel slicing procedure (fig. 1B). The labelled bands represent proteins which specifically bind JH III since no radioactivity was detectable after incubation with radioactive hormone in the presence of a 1000-fold excess of unlabelled hormone (fig. 3, panels C and D, lanes 1). Two-dimensional separation by IEF followed by gradient PAGE allowed the determination of relative molecular masses and isoelectric points of *L. migratoria* JHBPs: A, Mr 660,000, pI 5.7–6.2; B, Mr 500,000, pI 4.6; C, Mr 450,000, pI 5.2; D, Mr 390,000, pI 5.1; E, Mr 390,000, pI 4.6 (fig. 4).

The present method is also suitable for the determination of binding constants. The binding affinity of JH III to the

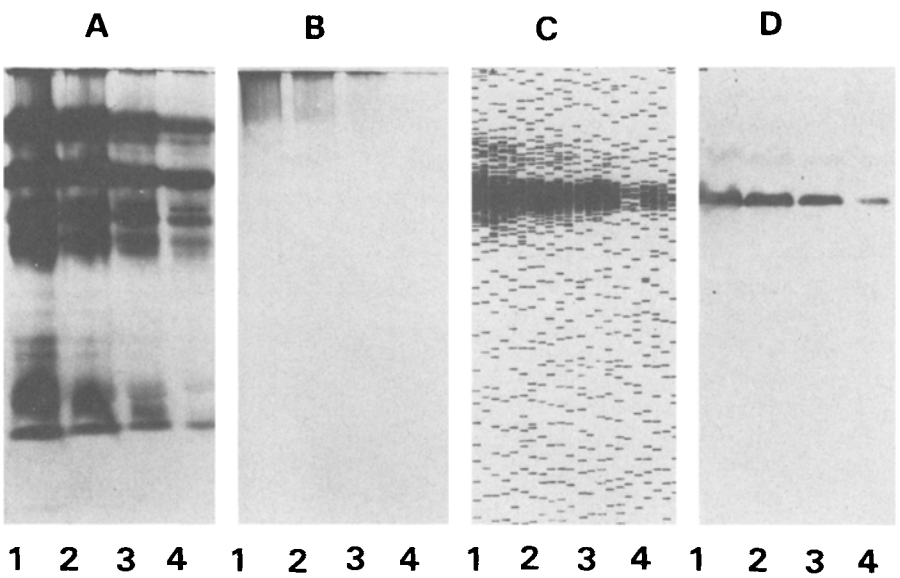

Figure 2. Identification of hemolymph JHBPs of *G. mellonella* larvae on NA-49 membrane. Four dilutions of crude JHBP were electrophoresed in 10% gel. The proteins were then electroblotted onto NA-49 membrane. The protein blots were quenched in 1% gelatin and then incubated in 10 mM sodium phosphate buffer, pH 5.9, containing 10 nM [^{3}H]-JH II. Subsequently the membrane was washed in the buffer and, after drying, radioscanned and autoradiographed. *A* Gel stained with Coomassie R-250; *B* parallel gel stained after electrotransfer ('empty gel'); *C* radioscan of the labelled blot; *D* autoradiogram of the same blot as in C. Lanes 1: 300 µg; lanes 2: 120 µg; lanes 3: 40 µg; lanes 4: 18 µg of protein.

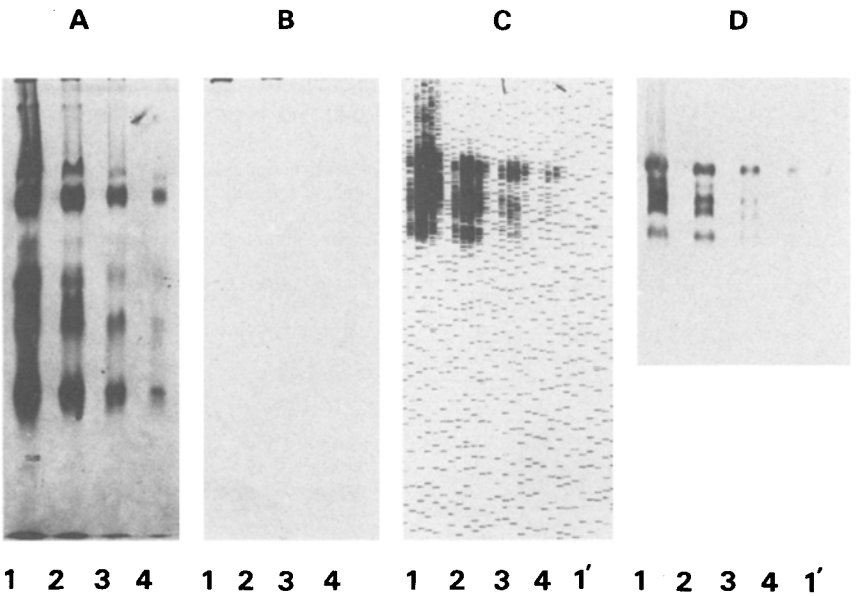

Figure 3. Identification of hemolymph JHBPs of *L. migratoria* larvae on NA-45 membrane. Four dilutions of hemolymph were electrophoresed in 5% PA gel. The proteins were then electroblotted onto NA-45 membrane. The electroblots were quenched in 1% gelatin/tris, pH 9.0, and then incubated in 10 mM tris/H_3BO_3 buffer, pH 9.0 containing 10 nM [^{3}H]-JH III. Subsequently the blots were washed in the buffer and after drying radioscanned and autoradiographed. *A* Gel stained with Coomassie R-250; *B* parallel gel stained after electrotransfer – 'empty gel'; *C* the radioscan of the protein blot on NA-45 membrane labelled with [^{3}H]-JH III (lanes 1–4). Lane 1' corresponds to lane 1, but the electroblot was incubated with tritiated hormone in the presence of a 1000-fold excess of unlabelled JH III (control for unspecific binding); *D* autoradiogram of the same blot as in C (only the upper part of the blot, which contained the radioactive spots was autoradiographed). Lanes 1 and 1': 4 µl; lanes 2: 1.4 µl; lanes 3: 0.4 µl; lanes 4: 0.12 µl of hemolymph.

immobilized JHBPs was determined by competitive displacement of bound [^{3}H]- JH III with increasing concentrations of unlabelled JH III (fig. 5). The data obtained by measuring the radioactivity bound to dot blots (fig. 5 B) were used to determine the K_D value by relationship $K_D = EC_{50}/(1 + L/K_{D1})$, where L is the concentration of [^{3}H]-JH III and K_{D1} = 8.8 nM, the equilibrium dissociation constant obtained for JH III binding by the dextran-coated charcoal assay[14] (fig. 5 B). For the JHBPs from *L. migratoria* a K_D-value of 4.8 nM was obtained. In this experimental approach racemic JH III was used. Thus, if for binding studies the natural (10*R*)-JH III had been used, a K_D-value about two times lower would have been expected[25].

Despite the heterogeneity of JHBPs, the analysis of JH III binding exhibited a single class of binding sites [K_D = 8.8 nM][11]. This indicates that the detected electromorphs have similar or identical binding affinities to JH III. The multiple JHBPs may represent a) products of initial steps of JHBP degradation in the hemolymph, b) isoforms of JHBP(s), or c) different aggregates of JHBP(s). It can also be speculated that these forms may reflect d) initial steps of JHBP(s) processing, as has been recently suggested for *G. mellonella* JHBP[23].

The K_D-value obtained for JH III binding by dot-blotted JHBPs was in the range of the K_D-values found by the conventional charcoal adsorption assay 6.8 nM[26], or by the hydroxylapatite adsorption assay 4.1 nM[24]. This indicates that the blotting procedure described in this paper

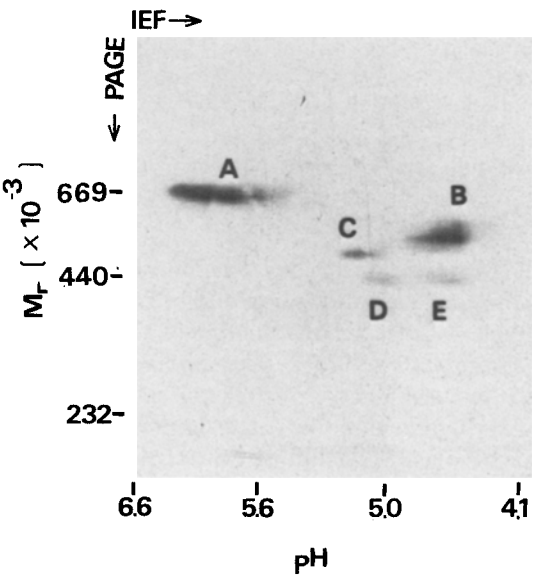

Figure 4. Two-dimensional analysis of the hemolymph JHBPs of *L. migratoria* larvae. 10 µl of larval hemolymph were electrofocused in 1% agarose gel followed by electrophoresis in 5–15% gradient PA gel. After electrophoretic separation the proteins were electroblotted onto NA-45 membrane. The protein blot was quenched in 1% gelatin/tris, pH 9.0, and then incubated in 10 mM tris/H_3BO_3 buffer, pH 9.0 containing 10 nM [^{3}H]-JH III. The dried electroblot was autoradiographed.

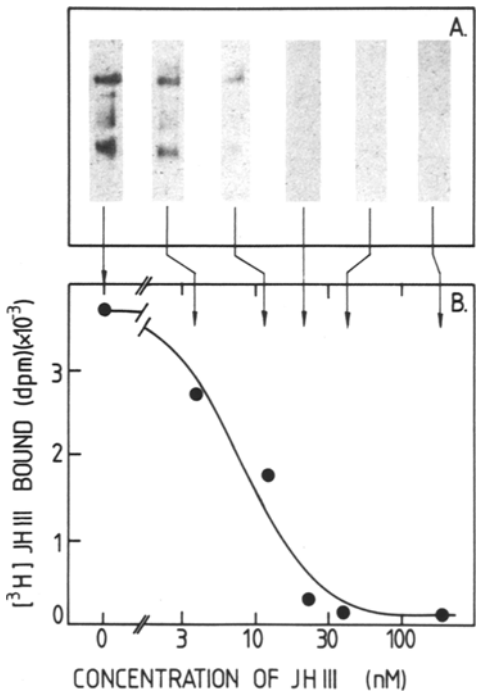

Figure 5. Displacement of bound [^{3}H]-JH III by increasing concentration of unlabelled JH III. *A* Crude JHBPs from *L. migratoria* Hemolymph containing 50 µg protein per slot were electrophoresed in 5% gel. After electrophoretic separation the proteins were electroblotted onto NA-45 membrane. The protein blots were quenched with 1% gelatin/tris, pH 9.0, and then incubated in 10 mM tris/H_3BO_3 buffer, pH 9.0, containing 6.3 nM [^{3}H]-JH III and increasing concentrations of unlabelled JH III. The dried blots were autoradiographed. Only the region of the autoradiogram corresponding to JHBPs in shown; no binding was detected elsewhere. *B* Dot blots (10 × 10 mm squares) of the JHBPs sample used in A (15 µg protein per dot) were incubated together with the electroblots as described in A. The washed dot blots were counted for radioactivity bound. The values are the mean of duplicates and were corrected by subtraction of unspecific binding of JH III to the membrane. The curve is the computed best fit of the data to the Hill equation.

is therefore suitable for the determination of binding constants and a quantification of blotted JHBPs, as has been demonstrated for the binding of ^{125}I-prolactin by prolactin receptors [11], of ^{125}I-insulin-like growth factors I and II by insulin-like growth factor binding proteins [12] and of ^{125}I-calmodulin by calcineurin [13].

In conclusion, electroblotting followed by the localization of JHBPs on the blots will be a useful and convenient method for studying these proteins by means of 1-D and 2-D PAGE. It appears that blotting onto NA-45 and NA-49 membranes may be useful also for the detection of proteins which bind other lipophilic molecules such as steroids, pheromones and retinoids.

Acknowledgments. I thank the Alexander von Humboldt Foundation for a 'Humboldtstipendium' which made this work possible. I gratefully acknowledge the helpful advice of Professor U. Grossbach (Universität Göttingen). I thank Professor G. D. Prestwich (SUNY, Stony Brook) for the gift of JH II and Ms K. Just for the preparation of the photographs. I thank Schleicher & Schuell (Dassel, Germany) for a gift of NA-45, NA-49, ABM and APT filters. Part of the experiments was performed in the Zentrales Isotopenlaboratorium der Universität Göttingen.

1 Koeppe, J. K., and Kovalick, G. E., in: Biochemical Actions of Hormones, vol. 13, p. 265. Ed. G. Litwack. Academic Press, New York 1986.
2 Goodman, W. G., in: Endocrinology of Insects, p. 147. Eds R. G. H. Downer and H. Laufer. Alan R. Liss. Inc., New York 1983.
3 Engelmann, F., Molec. cell. Endocr. *24* (1981) 103.
4 Roberts, P. E., and Wyatt, G. R., Molec. cell. Endocr. *31* (1983) 53.
5 Wiśniewski, J. R., and Kochman, M., FEBS Lett. *171* (1984) 127.
6 Roberts, P. E., and Jefferies, L. S., Archs Insect Biochem. Physiol. *3* (1986) 7.
7 Wiśniewski, J. R., Wawrzeńczyk, C., Prestwich, G. D., and Kochman, M., Insect Biochem. *18* (1988) 29.
8 Haeuptle, M. T., Aubert, M., Dijane, J., and Kraehenbuhl, J. P., J. biol. Chem. *129* (1983) 305.
9 Fernadez-Pol, J. A., FEBS Lett. *143* (1982) 86.
10 Lin, P. H., Selinfreund, R., and Wharton, W., Analyt. Biochem. *167* (1987) 128.
11 Sakai, S., and Murakami, H., Analyt. Biochem. *167* (1987) 406.
12 Hossenlopp, P., Seurin, D., Segovia-Quinson, B., Hardouin, S., and Binoux, M., Analyt. Biochem. *154* (1986) 138.
13 Hubbard, M. J., and Klee, C. B., J. biol. Chem. *262* (1987) 15062.
14 Prestwich, G. D., Eng, W.-S., Robles, S., Vogt, R. G., Wiśniewski, J. R., and Wawrzeńczyk, C., J. biol. Chem. *263* (1988) 1398.
15 Ożyhar, A., Wiśniewski, J. R., Sehnal, F., and Kochman, M., Insect Biochem. *13* (1983) 435.
16 Alwine, J. C., Kemp, D. J., and Stark, G. R., Proc. natl Acad. Sci. USA *74* (1977) 5350.
17 Symington, J., Green, M., and Brachmann, K., Proc. natl Acad. Sci. USA *78* (1981) 177.
18 Davis, B. J., Ann. N. Y. Acad. Sci. *121* (1964) 404.
19 de Bruijn, S. M., Koopmanschap, A. B., and de Kort, C. A. D., Physiol. Ent. *11* (1986) 7.
20 Giese, C., Spindler, K. D., and Emmerich, H., Z. Naturforsch. *32c* (1977) 158.
21 Beisiegel, U., Electrophoresis *7* (1986) 1.
22 Ożyhar, A., and Kochman, M., Eur. J. Biochem. *162* (1987) 675.
23 Ożyhar, A., and Kochman, M., Experientia *42* (1986) 1276.
24 Roberts, P. E., Archs Insect Biochem. Physiol. *2* (1985) 351.
25 de Kort, C. A. D., and Koopmanschap, A. B., Experientia *43* (1987) 904.
26 de Kort, C. A. D., Koopmanschap, A. B., and Ermens, A. A. M., Insect Biochem. *14* (1984) 619.

0014-4754/89/11-12/1124-05$1.50 + 0.20/0