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Ecdysteroid receptors in imaginal discs of *Drosophila melanogaster*

(molting hormone/ponasterone A/20-hydroxyecdysone/nuclear receptors/ecdysone)

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ABSTRACT **[^{3}H]Ponasterone A (PNA) of high specific activity has been used to identify and begin characterization of ecdysteroid (formerly called ecdysone) receptors in cytoplasmic and nuclear fractions of imaginal discs of *Drosophila melanogaster*. The equilibrium K_d of the observed macromolecular binding, 3–4 × 10^{-9} M PNA, is in good agreement with the minimal concentration required for induction of complete morphogenesis *in vitro*, 4.2 × 10^{-9} M PNA. Binding is analog specific and has kinetics consistent with a role in hormone response. On gentle homogenization, <5% of the binding capacity of the cell is released as soluble receptor; the other 95% remains with the nuclear fraction. This nuclear fraction specifically binds [^{3}H]PNA *in vitro*. Greater than 95% of nuclear PNA receptors are released by extraction with 0.3 M KCl. The binding properties of the nuclear receptors are indistinguishable from those of the cytosol fraction or of the whole cell.**

A useful model system for studying development is the hormonally induced morphogenesis in *Drosophila* imaginal discs. Mass-isolated imaginal discs in defined culture medium are induced by physiological concentrations of the natural hormone,‡ 20-hydroxyecdysone, to undergo a complicated morphogenesis, termed "evagination" (2, 3), and to alter the pattern of macromolecular synthesis (4–8) including the synthesis of chitin and specific endocuticle proteins (unpublished observations). Investigation of ecdysteroid action in imaginal discs offers insights into both the mechanism of insect hormone action and the role of gene regulation during morphogenesis and differentiation. By using 20-[^{3}H]hydroxyecdysone, the native hormone, ecdysteroid binding with biochemical characteristics consistent with a role in the biological action of the hormone has been detected in intact imaginal discs (9, 10). Specific binding of ecdysteroids to the nuclear fraction of discs has been demonstrated at the time of the first detected hormonal effect on gene activity, an increase in RNA synthesis (ref. 10; unpublished data). Because steroid hormone action appeared to be generally the same in insects and vertebrates, it seemed likely that in insects, as in vertebrates, the specific binding of hormone in the nucleus would be mediated by a hormone-binding macromolecule or receptor (9, 10).

Although preliminary experiments in our laboratory were both suggestive and tantalizing, we were unable to characterize adequately these putative ecdysteroid receptors. The high level of nonspecific binding at physiological concentrations of 20-hydroxyecdysone (0.1 μM), the high equilibrium K_d, and the low specific activity of available 20-hydroxyecdysone placed significant technical constraints on further work. Ponasterone A (PNA), an ecdysteroid approximately 50 times more active than the native hormone (9), has been shown to be identical to 20-hydroxyecdysone in its action on discs, differing only in the effective concentration (ref. 9; unpublished data). The synthesis of radiolabeled PNA of high specific activity has made it possible for us to demonstrate unequivocally the presence of ecdysteroid receptors in *Drosophila* imaginal discs and for Maroy *et al.* (11) to characterize receptors in *Drosophila* cells in tissue culture.

MATERIALS AND METHODS

Unlabeled Steroids. The unlabeled PNA (2β,3β,-14α,20*R*,22*R*-pentahydroxy-5β-cholest-7-en-6-one) and 20-hydroxyecdysone (2β,3β,14α,20*R*,22*R*-25-hexahydroxy-5β-cholest-7-en-6-one) were generous gifts of D. H. S. Horn, (Commonwealth Scientific and Industrial Research Organization, Australia). The unlabeled ecdysone (2β,3β,14α,22*R*-25-pentahydroxy-5β-cholest-7-en-6-one) was kindly supplied by J. B. Siddall (Zoecon Corporation, Palo Alto, CA).

Preparation of [^{3}H]PNA. Stachysterone C (24-dehydro-25-deoxy-20-hydroxyecdysone) (12) was isolated from a methanol/*n*-butanol extract of the bark of *Stachyurus praecox* kindly supplied by S. Imai. Further fractionation of this initial extract involved (*i*) solvent partitioning with the solvent system cyclohexane/*n*-butanol/water, 6:4:10 (vol/vol) ($K_{org/aq}$, ≈31), (*ii*) partitioning with $CHCl_3$/ethanol/water, 1:1:1 (vol/vol) ($K_{org/aq}$, ≈27), (*iii*) reversed-phase chromatography on Amberlite XAD-2 (elution with 70% methanol in water) (13), (*iv*) silica gel column chromatography (elution with 10% methanol in benzene), (*v*) preparative thin-layer chromatography (20 cm × 1 m silica plates, twice developed with 10% methanol in $CHCl_3$), and (*vi*) high-resolution reversed-phase liquid/solid chromatography (μ-Bondapak C_{18}; elution with 50% methanol in water). Final purification was achieved by crystallization from ethyl acetate/methanol. The identity of the product was verified by chromatography and mass spectrometry.

[24,25-^{3}H]PNA (specific activity, ≈122 Ci/mmol) was synthesized by the Chemical and Radioisotope Division of ICN from stachysterone C by Pt-catalyzed tritiation in dry ethanol using carrier-free tritium gas. [^{3}H]PNA was then purified and characterized by Maroy *et al.* (11). The identity and specific activity of the [^{3}H]PNA were verified by bioassay [disc evagination (8)]. The radiochemical purity of the [^{3}H]PNA was checked regularly by using the procedure of Chang *et al.* (14) for the separation of ecdysteroids. In our hands, PNA has an R_f of 0.41 and 20-hydroxyecdysone has an R_f of 0.23.

[^{3}H]PNA Binding Assays. Imaginal discs were mass-isolated as described (15) from mid to late third-instar larvae of an Oregon R stock of *Drosophila melanogaster*. In some experiments, 10,000–20,000 discs per ml were incubated in Robb's culture medium (16) at 25°C with 0.2 nM [^{3}H]PNA for 90 min. At the end of the incubation, the discs were washed four times in ice-cold Ringer's solution (17) by gentle centrifugation and homogenized to prepare cytoplasmic or nuclear fractions. In other experiments, freshly isolated discs were homogenized, and cellular fractions were exposed to 2–3 nM [^{3}H]PNA. In each

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Abbreviations: PNA, Ponasterone A; TES buffer, 0.01 M Tris/1.5 mM EDTA/7 mM dithiothreitol, pH 7.4.

‡ The nomenclature of this group of compounds has been revised recently (1). The generic term "ecdysone" has been replaced by "ecdysteroid," "α-ecdysone" has been replaced by "ecdysone," and "β-ecdysone" has been replaced by "20-hydroxyecdysone."

case, the discs were rinsed once with 0.01 M Tris/1.5 mM EDTA/7 mM dithiothreitol, pH 7.4 (TES buffer) and homogenized in a Dounce homogenizer at 75,000–100,000 discs per ml of buffer until no unbroken cells could be seen by phase-contrast microscopy. A cytoplasmic fraction (cytosol) was prepared by centrifugation at 100,000 × *g* for 60 min or 17,000 × *g* for 30 min. Both preparations had equivalent PNA binding characteristics. A nuclear fraction was prepared by the method of Williams and Gorski (18) by spinning the homogenate at 1000 × *g* for 10 min, resuspending and rinsing the pellet two to four times in 1–2 ml of TES buffer with gentle centrifugation at 1000 × *g* for 10 min. If discs are prelabeled in culture with ^{3}H-labeled hormone, little or no radioactivity is removed after the second rinse. The final pellet constituted the nuclear fraction.

Aliquots of cytosol (110–200 μl) of isolated nuclei from 50,000–100,000 discs (in 200 μl of TES buffer) or of a 0.3 M KCl/TES extract of the nuclear fraction (110–200 μl) were incubated with 1–3 nM [^{3}H]PNA under various conditions. Bound and free hormone in these samples or in fractions of discs exposed to [^{3}H]PNA in culture were separated on 0.5 × 11 cm Bio-Gel P-10 columns run in TES buffer without dithiothreitol at 0–4°C. Bound hormone was defined as hormone in the excluded volume (macromolecular fraction) the binding of which is competitively inhibited by excess unlabeled PNA or 20-hydroxyecdysone. Free hormone was defined as that eluting in the low molecular weight region coincident with unreacted steroid. Nuclear pellets were extracted with 70% methanol to quantitate any residual radioactivity. The relative size of the nuclear samples was determined from hydrolysis of the RNA in the nuclear pellet (9). Cytosols and 0.3 M KCl nuclear extracts were normalized from Lowry determinations of protein content (19). Radioactivity was measured in a xylene-based scintillation fluid (20) by using an Amersham/Searle Delta 300 counter.

Gel Filtration. Binding components of disc extracts (samples, 0.2–0.3 ml) were examined on a 1.3 × 35 cm Sephacryl S-200 (superfine; Pharmacia) column packed and run at 4–5°C at a flow rate of 50 ml/hr in a running buffer consisting of 0.01 M Tris/1.5 mM EDTA/0.1 M KCl, pH 7.4. The running time was less than 2 hr.

RESULTS

Cytoplasmic binding

Nature of Bound Complex. Specifically bound radioactivity was detected in the macromolecular fraction of imaginal disc cytosol that had been incubated with 3 nM [^{3}H]PNA. The macromolecular [^{3}H]PNA binding was fully inhibited by excess unlabeled PNA (Fig. 1*A*). On a Sephacryl S-200 column, bound [^{3}H]PNA runs as a single peak in the region corresponding to a molecular weight (for a globular protein) between 250,000 and 480,000. The number of binding sites per unit is not known. Fig. 1*B* shows the separation of bound and unbound [^{3}H]PNA on a Bio-Gel P-10 column used for the routine assay of bound radioactivity. The level of binding varied with both the concentration of [^{3}H]PNA and the amount of cytosol. When bound material was rerun on a P-10 column, all of the recovered radioactivity eluted at the void volume (data not shown). There was no detectable radioactivity in the soluble portion of the elution profile. The binding was sensitive to Pronase, heat, and *n*-ethylmaleimide but not to DNase or RNase. Because (*i*) radioactivity in disc extracts is indistinguishable from tritiated and unlabeled PNA samples on thin-layer chromatography (data not shown), (*ii*) it has been shown that imaginal discs in culture do not metabolize 20-hydroxyecdysone (21), and (*iii*) the bound radioactivity has the properties expected of PNA (see below), we conclude that the bound material is PNA.

Analog Specificity of [^{3}H]PNA Binding. Competition experiments demonstrated that binding of [^{3}H]PNA to the macromolecular fraction of disc cytosol is analog specific. When a constant amount of [^{3}H]PNA was supplied together with varying amounts of competitor steroids, effectiveness in preventing the binding of [^{3}H]PNA was PNA > 20-hydroxyecdysone > ecdysone (Fig. 2). The concentration of competing steroid that decreased [^{3}H]PNA binding 50% is in excellent agreement with that required to induce 50% of the morphogenetic response (Table 1).

Kinetics. The kinetics of binding were measured at 25°C, the optimal growth temperature for *Drosophila*, and at 0–4°C. At 25°C, maximal specific binding of [^{3}H]PNA to aliquots of a cytosol preparation was achieved after 25–30 min and was relatively stable for up to 90 min (Fig. 3*A*). The kinetics are second-order (*inset*) with an average association rate constant over three experiments of 1.2×10^7 M^{-1} min^{-1}. The rate of dissociation was measured in experiments in which the reduction in binding over time was determined when 2 μM unlabeled PNA (1 μg/ml) was added to aliquots of cytosol that had been incubated previously with [^{3}H]PNA for at least 30 min at 25°C. Fig. 3*B* shows that the displacement of bound [^{3}H]PNA follows pseudo-first-order kinetics. The dissociation rate constant is 3.6×10^{-2} min^{-1}. K_d estimated from the kinetic rate constants is 3×10^{-9} M. The kinetics of association at 0–4°C are shown in Fig. 3*C*. The same level of binding was achieved after 10–12 hr at 0–4°C as after 30 min at 25°C (159 cpm/mg of protein at 25°C; 161 cpm/mg of protein at 0–4°C; averaged over seven experiments). This observation is consistent with results for whole discs with 20-hydroxyecdysone (9). The equilibrium binding was stable for at least 12 hr at 0–4°C. The binding at 21 hr was fully inhibitible (4445 cpm bound with [^{3}H]PNA alone, 87 cpm bound with [^{3}H]PNA plus 0.2 μM unlabeled PNA). The average association rate constant from two experiments is 1.4×10^6 M^{-1} min^{-1}. In two experiments carried out as in Fig. 3*B* in attempts to determine the dissociation rate constant at 0–4°C, there was no detectable dissociation after 4 hr. Binding was decreased only 16% after 8 hr.

Scatchard Analysis. Fig. 4 *inset* shows a Scatchard analysis of specific binding at 25°C in the presence of increasing concentrations of [^{3}H]PNA. There is apparently one class of binding sites detectable in the concentration range tested. The estimated K_d is 3.3×10^{-9} M, in excellent agreement with the K_d estimated from kinetic experiments. From the *x* intercept, the concentration of ecdysteroid receptors is 0.38 nM, corresponding to only 10–20 molecules per cell. Although this is in good agreement with other estimates of the binding capacity of disc cytosol, it is much lower than the binding capacity of whole discs—500–1000 20-hydroxyecdysone sites per cell (9) and 600–1000 PNA sites per cell (unpublished data)—and represents only 2–4% of the total ecdysteroid-specific binding capacity of the cell.

Of several possible explanations for this discrepancy, two are the degradation of receptors and the presence of endogenous competitor. (*i*) Degradation of receptor: the fact that binding capacity is quite stable in TES buffer (used for homogenization and incubation) for at least 90 min at 25°C (Fig. 3*A*) and for at least 24 hr at 0–4°C (Fig. 3*C*) argues against degradation or inactivation of binding capacity during cell fractionation. (*ii*) Endogenous hormone: Although these discs had been exposed to 20-hydroxyecdysone during larval life, they were isolated from a larval stage at which the *in vivo* titer is low (2). The dissociation rate constant of the 20-hydroxyecdysone receptors detected in whole discs is such that most endogenous hormone would have been removed during the disc isolation procedure. From the level of endogenous hormone in mass-isolated discs (2), the average disc cytosol preparation should contain 6 nM 20-hydroxyecdysone, an amount too small to interfere with [^{3}H]PNA binding (Fig. 2).

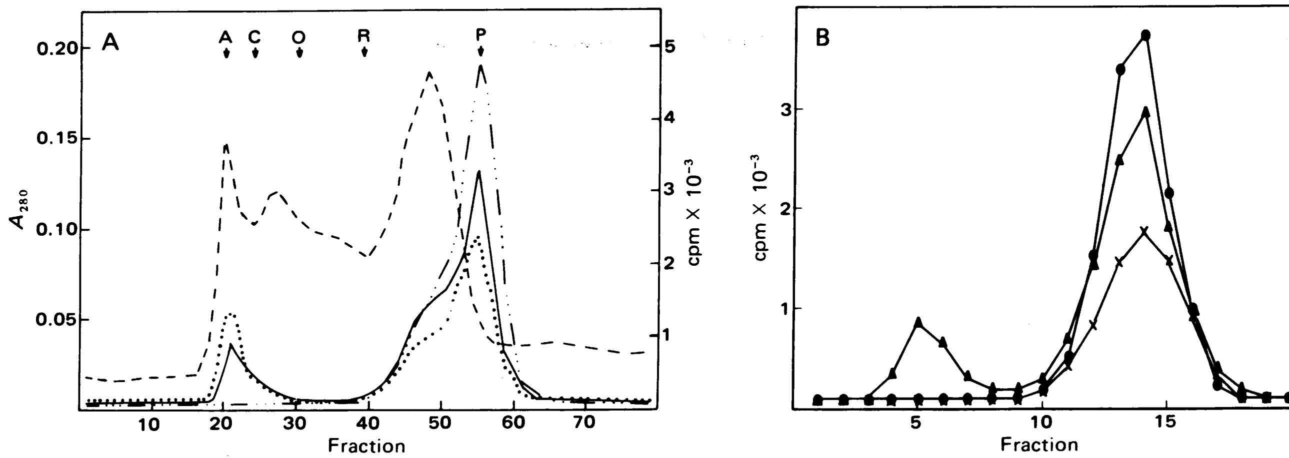

FIG. 1. Macromolecular binding components. (*A*) Elution profile from Sephacryl S-200 gel filtration column. Aliquots (0.25 ml) of a high-speed cytosol preparation were incubated with equal amounts of [^{3}H]PNA for 30 min at 25°C, chilled to 0–4°C in an ice bath and run in the cold on the Sephacryl column. Fraction size was 0.9 ml. One aliquot (—) was incubated with [^{3}H]PNA alone. The other (—···—) was incubated with [^{3}H]PNA plus excess (0.2 μM) unlabeled PNA. - - -, A_{280} profile of the eluate. Arrows, locations of A_{280} peaks, on individual runs and in a mixture, of apoferritin (A; M_r 470,000), catalase (C; M_r 248,000), ovalbumin (O; M_r 45,000), and RNase (R; M_r 12,000) and the cpm peak for free [^{3}H]PNA (P; M_r 460). ·····, Radioactivity elution profile of 0.3 M KCl/TES extract of the nuclear fraction incubated for 30 min at 25°C with [^{3}H]PNA alone. Another aliquot of this nuclear extract incubated with [^{3}H]PNA plus 0.2 μM unlabeled PNA had no detectable radioactivity in the macromolecular region (data not shown). (*B*) Elution profile of Bio-Gel P-10 column used for routine assays. Aliquots (0.14 ml) of a cytosol preparation were incubated with equal amounts of [^{3}H]PNA for 30 min at 25°C, chilled to 0–4°C in an ice bath, and run in the cold on P-10 columns. The fraction size was 0.2 ml. ▲, Incubation with [^{3}H]PNA alone; ●, incubation with [^{3}H]PNA and excess (0.2 μM) unlabeled PNA; ×, free [^{3}H]PNA without disc extract.

As an experimental check for the effects of endogenous hormone, two aliquots of mass-isolated discs were either homogenized at once or incubated for 4–5 hr in Robb's medium in a shaking bath at 25°C before homogenization, a time in excess of that required to remove bound ^{3}H-labeled hormone. Relative to the unincubated discs, binding capacity of the cytosol from preincubated discs was increased 0–20% in three experiments. These results show that, although there may be some low-level endogenous hormone in some disc preparations, this cannot account for the low level of cytosol binding relative to whole cell binding. An alternative explanation is that the unbound receptors are in the nuclear fraction. This has proven to be the case.

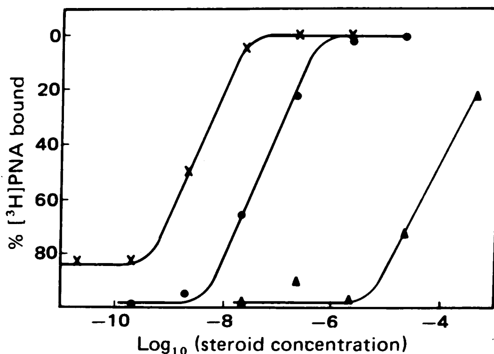

FIG. 2. [^{3}H]PNA binding competition by ecdysone analogs. Aliquots of disc cytosol were incubated with 3 nM [^{3}H]PNA, under equilibrium binding conditions (overnight at 0–4°C), with various concentrations of ecdysone analogs. Binding in the absence of competing steroid was 1000–1600 cpm. To compensate for slight differences in the exact concentration of tritiated hormone from experiment to experiment, results are expressed as percentage of total [^{3}H]PNA bound in the absence of competitor for that experiment. Each point plotted is the average of duplicate determinations in two or three experiments. A limited supply of ecdysone prevented more extensive examination of the effect of higher concentrations. ×, PNA; ●, 20-hydroxyecdysone; ▲, ecdysone.

Nuclear binding

Aliquots of the nuclear fraction of discs were incubated with 1–3 nM [^{3}H]PNA for 30 min at 25°C, washed twice with 1–2 ml of cold TES buffer, and recovered by gentle centrifugation. Greater than 98% of the radioactivity removed was in a low molecular weight form. Additional rinsing in TES buffer removed little or no radioactivity. Under these conditions, approximately 50% of the added radioactivity remained with the nuclei. In competition experiments in which 2 μM unlabeled PNA was present during the incubation, nuclear binding was decreased 90%, indicating competition for a limited number of specific sites. Greater than 95% of the [^{3}H]PNA associated with the nuclear fraction after washing in TES buffer was extractable in 0.3 M KCl/TES. When assayed on columns, virtually all the extractable radioactivity was eluted with the macromolecular fraction—i.e., the radioactivity in the fractions containing low molecular weight material was indistinguishable from background. [^{3}H]PNA bound to the nuclear fraction had similar properties whether the nuclei were exposed to the hormone in whole cells in culture or as isolated nuclei. In both cases, binding was decreased more than 90% when 0.2 μM PNA (0.1 μg/ml) was present during the incubation with [^{3}H]PNA. More than 95% of the nuclear binding was extractable by 0.3 M KCl/TES, and virtually all of the radioactivity in the KCl extract eluted with the macromolecular fraction. For incubations with whole discs, more than 90% of the total specific binding in the cell was located in the nuclear fraction after incubation with [^{3}H]PNA for 60–90 min at 25°C, consistent with the distribution of specific binding of 20-[^{3}H]hydroxyecdysone (unpublished data).

Table 1. Concentrations of ecdysone analogs effective in morphogenesis and in competition for [^{3}H]PNA binding

Steroid	Concentrations, μM: For 50% evagination (9)	Concentrations, μM: For 50% displacement of [^{3}H]PNA (Fig. 1)
PNA	0.001	0.002
20-Hydroxyecdysone	0.042	0.052
Ecdysone	15	72

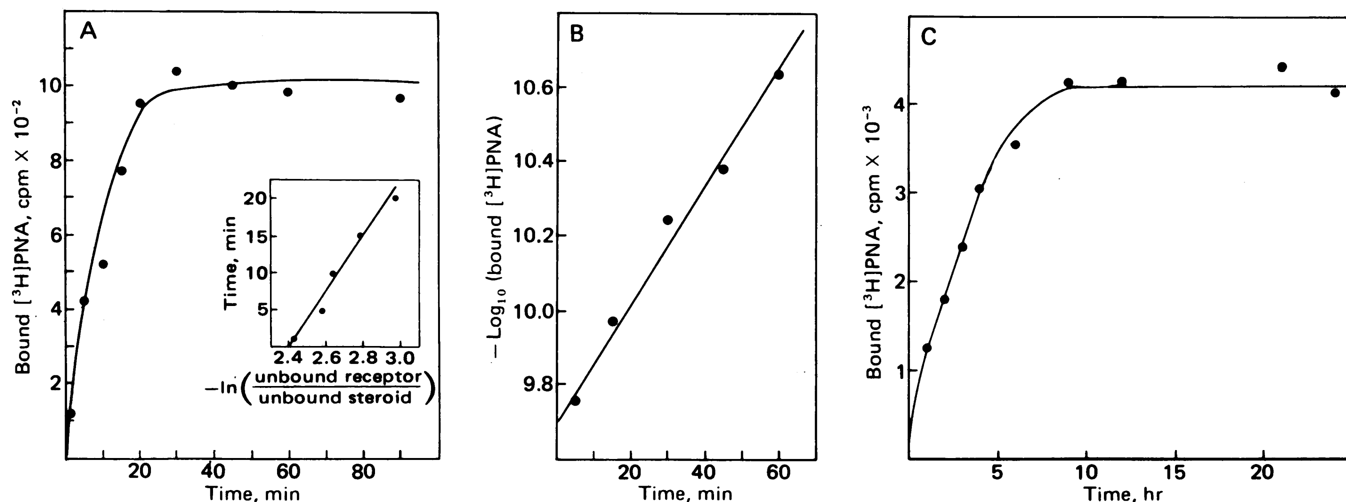

FIG. 3. Kinetics of specific binding of [^{3}H]PNA to disc cytosol. (*A*) Association rate constant at 25°C. Aliquots of disc cytosol were incubated with 3 nM [^{3}H]PNA for the times indicated. Each point is the average of duplicate determinations in one experiment; three experiments gave equivalent results. (*Inset*) Binding data for the first 20 min averaged over three experiments expressed in the form of a second-order reaction. The association rate constant estimated from the slope is 1.2×10^7 M^{-1} min^{-1}. (*B*) Dissociation rate constant at 25°C. Aliquots of disc cytosol were incubated with 3 nM [^{3}H]PNA for 30 min (equilibrium binding conditions); 2 μM unlabeled PNA was added to duplicate samples at 30, 45, 60, 75, and 85 min of incubation. The amount of [^{3}H]PNA bound was assayed after a total incubation period of 90 min. Data are shown in the form of a first-order reaction, −log bound hormone (cpm) vs. time of incubation with excess unlabeled PNA. Each point represents the average of duplicate determinations in two separate experiments. (*C*) Association kinetics at 0–4°C. Aliquots of disc cytosol were incubated at 0–4°C with 2 nM [^{3}H]PNA for the times indicated. Each point is the average of duplicate determinations; two experiments gave equivalent results. The association rate constant, calculated as in 3*A* from the data of the first 6 hr is 1.4×10^6 M^{-1} min^{-1} averaged from the two experiments.

A 0.3 M KCl/TES extract of the nuclear fraction of freshly isolated discs was tested for binding capacity under the same conditions as cytosol. Macromolecular binding was observed that was fully inhibited by 0.2 μM unlabeled PNA. Fig. 5 *inset* shows a Scatchard analysis of an experiment in which aliquots of a 0.3 M KCl/TES extract of nuclei were incubated with increasing concentrations of [^{3}H]PNA. One class of binding sites was apparently present in the concentration range tested. The estimated K_d is 3.8×10^{-9} M. The number of molecules per cell, allowing for uncertainty in exact volumes of fluids left on the pellets, is 1000 ± 300 per cell. The Sephacryl S-200 elution profile of [^{3}H]PNA bound to a 0.3 M KCl/TES nuclear extract was indistinguishable from that of the cytosol (Fig. 1*A*). Clearly, most of the ecdysteroid receptors in imaginal discs mass-isolated from mid to late third-instar larvae are in the nuclear fraction under these conditions.

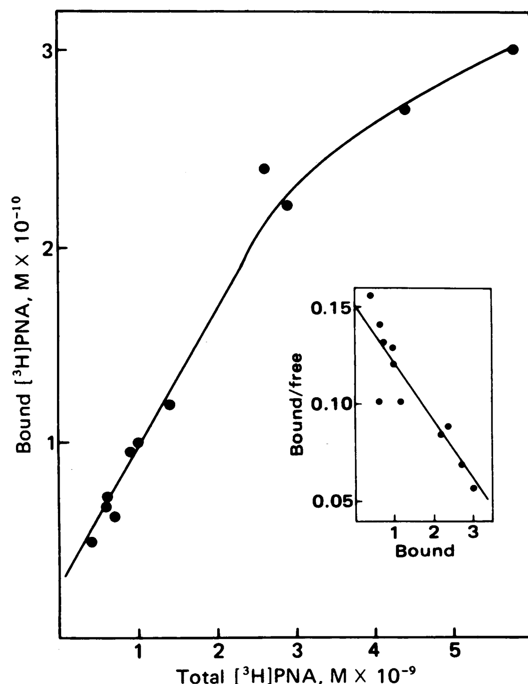

FIG. 4. Specifically bound [^{3}H]PNA was determined for aliquots of disc cytosol in the presence of increasing concentrations of [^{3}H]-PNA. (*Inset*) Scatchard analysis of the binding data.

DISCUSSION

Evidence has been presented for a class of macromolecules in imaginal discs having the characteristics expected of ecdysteroid receptors playing a role in the mediation of hormone action. Preliminary evidence indicates that these receptors are distributed within the cell such that most of the receptors, even those not bound to hormone, are located in or associated with nuclei. At present, no significant differences are found in the binding of hormone to whole discs, to disc cytosol, or to disc

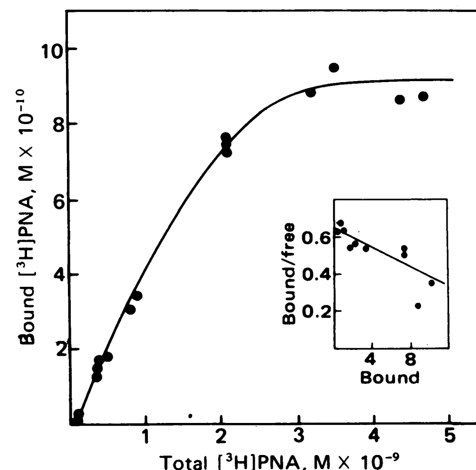

FIG. 5. Specifically bound [^{3}H]PNA was determined for aliquots of a 0.3 M KCl/TES extract of disc nuclei in the presence of increasing concentrations of [^{3}H]PNA. (*Inset*) Scatchard analysis of the binding data.

nuclei, suggesting, but not proving, that nuclear and cytoplasmic receptors may be the same. We conclude that binding of ecdysteroids to these receptors is an integral part of the hormone response mechanism of imaginal discs. The K_d for the nuclear and cytoplasmic binding was $3–4 \times 10^{-9}$ M PNA as compared to the minimal concentration for the induction of complete metamorphosis *in vitro*, 4.2×10^{-9} M PNA. In whole discs the K_d for 20-[^{3}H]hydroxyecdysone binding was 2×10^{-7} M compared to 1.7×10^{-7} M 20-hydroxyecdysone required to induce morphogenesis *in vitro* and 2.5×10^{-7} M determined to be the *in vivo* concentration at the time of morphogenesis (2, 9). Both nuclear and cytoplasmic binding complexes elute at the same molecular weight region on gel filtration columns. Both nuclear and cytoplasmic binding are almost totally competitively inhibited by unlabeled PNA. Furthermore, the relative affinity of ecdysteroids for the cytoplasmic receptors determined in competition experiments with [^{3}H]PNA corresponds to both the morphogenetic activity of these ecdysteroids and their relative effectiveness as competitors for 20-[^{3}H]-hydroxyecdysone binding to whole discs and the nuclear fraction of whole discs (9, 22). Finally, the binding properties of disc ecdysteroid receptors are similar to those found by Maroy *et al.* (11) in the K_c line of *Drosophila* cells. However, there is a significant difference between receptors obtained from discs and from tissue culture cells derived from embryos: receptors in imaginal discs have a nuclear location whereas those from tissue culture cells are found predominantly in the cytosol (11).

Although unbound receptors are clearly located in the "nuclear fraction" of discs, the tentative interpretation that receptors are associated with nuclei rests on a consideration of the exact constitution of this nuclear fraction. The nuclear fraction contains few, if any, unbroken cells. Nuclei are easily prepared from imaginal discs. Each disc is a folded epithelial layer, one cell thick. In hypotonic medium, the shear forces of homogenization tear open virtually every cell, spilling the contents into the medium. Also, these nuclei are quite "clean" relative to an average nuclear preparation. Discs are embryonic tissues with very little rough endoplasmic reticulum associated with the outer nuclear membrane. The isolated nuclei, as seen by phase-contrast microscopy, are essentially free of cytoplasmic blebs. Nevertheless, the nuclear fraction does contain membranes and has an occasional piece of trachea or cuticle contaminant. Redistribution of receptors during the homogenization procedure cannot be ruled out, although such redistribution does not occur in the tissue culture cells. Finally, the nuclear receptors are not soluble in TES buffer but are removed by 0.3 M KCl.

At present it appears that the ecdysteroid receptors in imaginal discs differ from those in *Drosophila* cell lines (11) and from steroid receptors in vertebrates in that soluble receptors are not released when cells are disrupted. There is a significant physiological difference between mass-isolated imaginal discs and tissue culture cells derived from embryos—the imaginal discs have been exposed repeatedly to hormone during the preceding 4 days of embryonic and larval life whereas the cell lines have not been exposed to hormone. These discs may be in a developmental state different from that of cell lines, particularly in regard to their readiness to respond to the next hormonal signal. Such a developmental difference might involve the distribution of the hormone receptors in the cell.

Some differences between mammalian and insect hormone response systems might be related to differences in the physiology of the organisms and the properties of the molecules used as hormones. In contrast to some vertebrate steroid hormones, both PNA and 20-hydroxyecdysone, the natural hormone, are water soluble at physiological concentrations. No hemolymph carriers are needed to ensure solubility or regulate effective concentration, and, presumably, no cytoplasmic carrier would be required to conduct the hormone to nuclear target sites. No temperature-dependent transition of a cytoplasmic binding to a nuclear location analogous to that commonly found in vertebrates is observed for 20-hydroxyecdysone binding in imaginal discs. In intact discs and in cell fractions, levels of binding equivalent to those reached at 25°C are achieved at 0–4°C. Furthermore, the kinetic curves of specific whole-cell binding and of the binding present in the nuclear fraction of the cells at 0–4°C are superimposable (unpublished data). For imaginal discs, there is no detectable lag between binding and the presence of the bound hormone in the nucleus. It is perhaps not surprising that the specifics of steroid hormone action in a poikilothermic organism with a short life cycle might differ from those of mammals and birds in such ways.

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1. Goodwin, T. W., Horn, D. H. S., Karlson, P., Koolman, J., Nakanishi, K., Robbins, W. E., Siddall, J. B. & Takemoto, T. (1978) *Nature (London)* **272**, 122.
2. Borst, D. W., Bollenbacher, W. E., O'Conner, J. D., King, D. S. & Fristrom, J. W. (1974) *Dev. Biol.* **39**, 308–316.
3. Fristrom, J. W., Logan, W. R. & Murphy, C. (1973) *Dev. Biol.* **33**, 441–456.
4. Petri, W. H., Fristrom, J. W., Stewart, D. J. & Hanly, E. W. (1971) *Mol. Gen. Genet.* **110**, 245–262.
5. Raikow, R. & Fristrom, J. W. (1971) *J. Insect Physiol.* **17**, 1599–1614.
6. Fristrom, J. W., Gregg, T. L. & Siegel, J. (1974) *Dev. Biol.* **41**, 301–313.
7. Siegel, J. G. & Fristrom, J. W. (1974) *Dev. Biol.* **41**, 314–330.
8. Nishiura, J. T. & Fristrom, J. W. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2984–2988.
9. Yund, M. A. & Fristrom, J. W. (1975) *Dev. Biol.* **43**, 287–298.
10. Fristrom, J. W. & Yund, M. A. (1976) in *Invertebrate Tissue Culture*, ed. Maramorosch, K. (Academic, New York), pp. 161–178.
11. Maroy, P., Dennis, R., Beckers, C., Sage, B. A. & O'Conner, J. D. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 6035–6038.
12. Imai, S., Murata, E., Fujioka, S., Matsuoka, T., Koreeda, M. & Nakanishi, K. (1970) *Chem. Commun.* **1970**, 352–353.
13. Schooley, D., Weiss, G. & Nakanishi, K, (1972) *Steroids* **19**, 377–383.
14. Chang, E. S., Sage, B. A. & O'Conner, J. D. (1976) *Gen. Comp. Endocrinol.* **30**, 21–33.
15. Fristrom, J. W. (1972) *Results Probl. Cell Differ.* **5**, 109–154.
16. Robb, J. A. (1969) *J. Cell Biol.* **41**, 876–884.
17. Ephrussi, B. & Beadle, G. W. (1936) *Am. Nat.* **70**, 218–225.
18. Williams, D. & Gorski, J. (1971) *Biochem. Biophys. Res. Commun.* **45**, 258–264.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193** 265–275.
20. Anderson, L. E. & McClure, W. O. (1973) *Anal. Biochem.* **51**, 173–179.
21. Chihara, C. J., Petri, W. H., Fristrom, J. W. & King, D. S. (1972) *J. Insect Physiol.* **18**, 1115–1123.
22. Yund, M. A. & Fristrom, J. W. (1975) in *ICN-UCLA Symposia on Molecular and Cellular Biology: Developmental Biology*, eds. McMahon, D. & Fox, F. C. (Benjamin, New York), Vol. 2, pp. 404–408.