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JUVENILE HORMONE TITERS IN THE HEMOLYMPH DURING LATE LARVAL DEVELOPMENT OF THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.)

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The juvenile hormone (JH), as with many other developmental hormones in insects, must reach its target tissues by way of the hemolymph. Thus, the hemolymph concentration bears an important relation to effects of JH (see de Wilde, de Kort and de Loof, 1971), although tissue retention of hormone or of "covert effects" of hormone may be of significance as well (*e.g.*, Ohtaki, Milkman, and Williams, 1968; Nijhout, 1975). It is generally accepted that the JH titer is "high" during most of premetamorphic development, yet the effects of JH are made manifest only at discrete times during this development—when ecdysone induces a molt. Ligation studies of larvae of the tobacco hornworm *Manduca sexta* suggest that in a larval molt, the effects of JH on epidermal commitment occur simultaneously with initiation of the molting process by ecdysone (Truman, 1972; Truman and Riddiford, 1974). Observations in these studies and in others (Wigglesworth, 1934; Fukuda, 1944) suggest indirectly that in premetamorphic molts the JH titer, in fact, may be very low at the time of prothoracicotropic hormone (PTTH) release, just prior to the critical period for ecdysone secretion. Other reports suggest that substantial titers of ecdysone and of JH do not occur simultaneously even in a larval molt (Patel and Madhavan, 1969).

Much has been deduced about JH titers from indirect measurements of cytological, size, or activity changes in the corpora allata (CA) (see Doane, 1973), but there are difficulties with such methods (Williams, 1961; Johnson and Hill, 1973a) and direct determination of hemolymph titers is desirable. The tobacco hornworm is especially suited for such an investigation of hemolymph titers of JH during larval development. The "gating" of PTTH release by photoperiod (Truman, 1972) allows one to select developmentally synchronous groups of animals from which to obtain hemolymph for analysis. Titers observed can be correlated precisely with developmental events relative to the release of PTTH and ecdysone. Finally, the JH content of hemolymph samples can be analyzed in a bioassay system using assay animals of the same species and stage as those which provided the hemolymph.

This report describes the determination of JH titers in hemolymph during the fourth and fifth instars of wild-type *Manduca* larvae, by means of a sensitive and quantitative JH bioassay utilizing larvae of the *black* mutant (Safranek and Riddiford, 1975).

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Materials and Methods

Experimental animals

Both wild-type and *black* mutant larvae of *Manduca sexta* were reared in a short day photoperiod (12L:12D) at 25° C, as described by Truman (1972). Times of day will be referred to arbitrary zeitgeber time (AZT) (Pittendrigh, 1965; Truman, 1972), with lights-off and the beginning of a new day set at 24.00 AZT. Fourth instar larvae were staged as described by Truman (1972).

The black *mutant larval pigmentation assay*

Unligated fourth instar larvae of the *black* mutant (Safranek and Riddiford, 1975) show melanization of the cuticle similar to that in wild-type fourth instar larvae neck-ligatured during a critical period following initiation of the last larval molt (Truman, 1972). Topical application of JH can prevent melanization, and larvae are maximally sensitive to JH when head capsule slippage begins (Truman, Riddiford, and Safranek, 1973; Safranek and Riddiford, 1975). Synchronous fourth instar *black* larvae within a half hour of this slippage were used for the assay; under our conditions this event occurred between four and nine hours after lights-on.

Larvae were anesthetized by submersion in water for 15–20 minutes prior to assay. For application of a test solution, a disposable microliter pipette (Micropet, Clay-Adams) was used. Anesthetized larvae were swabbed dry, and one or two μl of the test solution was applied by spotting on one side between the third and fourth abdominal spiracles. After a few seconds, the solvent had evaporated, and larvae were returned to individual plastic cups without food. Larvae were scored on the third or fourth day after the hormone application, since the full extent of melanization was not apparent until a day or so after ecdysis to the fifth instar.

Selection of larvae for hemolymph samples

Larvae were selected carefully so as to be similar to one another with respect to age, time of day, and developmental stage or weight. Only Gate II larvae (Truman, 1972) were used for all extractions during the fourth instar (except for larvae on day 0, immediately following ecdysis from the third instar). Table I summarizes the selective criteria used for larvae at each time-point during the fourth and fifth larval instars. For extractions from ligated larvae, day 2 fourth instar larvae (Gate II) were neck- or abdomen-ligatured at 23.30 AZT prior to the initiation of molting, and the portion anterior to the ligature cut away. Ligated larvae were kept at 25° C until blood collection.

Collection of hemolymph

Larvae were anesthetized with carbon dioxide for 5–15 minutes. Until the time of head capsule slippage, the tip of the abdominal horn was cut off to bleed larvae, whereas with older larvae the tips of the second or third pair of abdominal

TABLE I

Criteria used to select Manduca sexta *larvae for hemolymph collection.*

Stage	Description of Stage	Time (AZT) of Weighing	Weight Range (gm)	Time (AZT) of Blood Collection
IV, d0	Newly ecdysed (day 0) fourth instar larvae	—	—	17.00–22.00
IV, d1	Day 1 fourth instar larvae, about 27–30 hrs before time of PTTH release	19.30–21.30	0.40–0.60*	19.30–21.30
IV, d2, −10h	Day 2 fourth instar larvae, about 10 hrs before the time of PTTH release	13.30–14.30	0.60–0.85	13.30–14.30
IV, d2, $-\frac{1}{2}$h	Day 2 fourth instar larvae, about 0.5 hr before the time of PTTH release	22.30–23.30	0.70–1.10	23.15–23.45
IV, d3, +3h	Day 3 fourth instar larvae, about 2.5-3 hr after the time of PTTH release	22.00–23.00	0.75–1.10	02.45–03.15
IV, d3, +12h	Day 3 fourth instar larvae, about 12 hrs after initiation of molting; spiracle apolysis just evident	—	—	12.15–13.00
IV, d3, +18h	Day 3 fourth instar larvae; spiracle apolysis advancing; headcap well-tucked or just beginning to slip	—	—	18.00–19.00
IV, d3, +23h	Day 3 fourth instar larvae; headcap slipped at least past the most anterior ocellus	—	—	23.00–23.30
IV, d4	Day 4 fourth instar larvae, new fifth instar mandibles tanned pale orange to dark brown; no air in old headcap	—	—	18.30–19.30
V, d0	Newly ecdysed (day 0) fifth instar larvae	24.00	1.0–1.6	24.00
V, d1	Day 1 fifth instar larvae, about 12–18 hrs after ecdysis	17.00–20.00	1.8–2.4	17.00–20.00
V, d2	Day 2 fifth instar larvae, about 36–48 hrs after ecdysis	19.00–20.00	2.5–3.5	19.00–20.00
V, d5	Mature fifth instar larvae, about day 4 or 5; no evidence of gut purging or exposure of dorsal vessel	15.00	9.0–10.9	15.00

* During day 1, Gate I and Gate II larvae can be distinguished with 90–95% success on the basis of their weights (Dr. H. F. Nijhout, University of Washington, personal communication).

prolegs were severed. By gentle pressure, hemolymph free of gut contents was expressed from the cut opening into an ice-cold calibrated test tube. Blood either was extracted immediately after collection, or was rapidly frozen in dry ice. Frozen blood was stored for up to two weeks at − 15° C and then thawed in ice water immediately before beginning the extraction. No differences between frozen and unfrozen samples were observed.

Glassware and reagents

All reusable glassware used in the extractions as well as Pasteur pipettes used for transferring solutions and for preparing micro-columns were doubly washed, rinsed with distilled water, doubly rinsed with acetone (Nanograde, Mallinckrodt), and oven dried. Precaution was taken to insure that disposable glassware including microliter pipettes (Micropet, Clay Adams) and micro-test tubes (6 × 50 mm culture tubes, Kimble) were kept free from contamination with juvenile hormones.

Methanol (Matheson, Coleman & Bell), anhydrous diethyl ether (MCB or Allied Chemical), and n-hexane (bp 68.7° C, range 4° C, Baker) were of A.C.S. specifications. The cyclohexane used was spectrophotometric grade (Baker). All solvents were tested periodically to insure that they remained free from JH contamination by carrying out the complete extraction procedure, or portions of it, on the appropriate volume of solvent, and assaying the resulting "extract". Activity IV alumina was prepared from neutral aluminum oxide (M. Woelm-Eschwege, distributed by Alupharm Chemicals) as described by the suppliers.

Preparation of micro-columns

Pasteur pipettes (inner diameter = 5 mm) were plugged with small pieces of glass wool (previously doubly soaked in Nanograde acetone and dried). The bores of the pipettes were filled with a 2.5 cm column of activity IV alumina followed by a 2 cm column of anhydrous sodium sulphate (Merck). Micro-columns were prepared in batches of six or seven, washed with 20–30 ml hexane and stored (filled with hexane) until use. The hexane washes were "extracted" and assayed to insure that columns were free from JH activity.

Extraction and assay procedures for hemolymph extracts

Seven ml of hemolymph were shaken with 25 ml of ether : methanol (4 : 1) on a vortex mixer. In a few instances, the volume of hemolymph was less than 7 ml, but never less than 6.0 ml. The mixture was centrifuged at about 150 *g* for 5 minutes (Sorvall GLC–2 centrifuge), and the upper clear phase was removed to a round-bottomed flask. The remaining phase and precipitate were re-extracted twice with ether : methanol (4 : 1) (total volume 50 ml), as before. The combined organic extracts were then concentrated in a rotary evaporator with bath temperature never exceeding 30° C. Eight ml of hexane were added to the residue, and, after vigorous mixing, the hexane phase was removed and passed through a micro-column (prepared as described above) and the eluant collected. The hexane extraction and column "clean-up" were repeated twice (total volume 16 ml) after which a final 5.0 ml of hexane were passed through the column. Eluants were pooled (total volume about 29 ml), concentrated, and the final residue transferred in hexane to a micro-test tube. Solvent was evaporated under a stream of nitrogen gas.

A volume of cyclohexane equal to 1/250 of the initial volume of hemolymph was added to the N_2-dried extract (*i.e.*, 24 to 28 μl). Two μl per assay animal of this solution—the "undiluted extract"—constituted the initial (1×) dilution and provided to each animal an amount of extracted JH activity equivalent to that

TABLE II

Scoring system for the black *mutant larval pigmentation assay for JH.*

Score	Description*
0	Larvae totally black, including head capsule (hc), thorax (thor) and abdomen (abd); no evidence of localized green spot at the site of hormone application.
+1	Larvae black except for a green spot at the site of hormone application; often green banding extends from the spot to the dorsal midline above the spot.
+2	Larvae black except for green spots on both sides of the segment at the site of application; spots often connected by a green band.
+3	Larvae black with green patches on segment of application and at least one adjacent segment, but may include as much as a three or four segment wide green region centering around the site of application; green region may extend dorsally on the affected segments; sometimes thor muddy colored; occasionally hc partly green.
+4	Larvae green; dorsal transverse stripes on abd moderately heavy and extend as far as the spiracles except in a one to three segment wide area centering around site of application; oblique black stripes present.
+5	Larvae nearly completely green; oblique black stripes pale, at most a few black spots; transverse stripes absent or very pale, restricted to the dorsal midline; some pink pigmentation visible in epidermis.

* See Figure 1 for a photograph of larvae representative of each response category.

from 0.5 ml of hemolymph. From the undiluted extracts, dilution series in cyclohexane were prepared. Assays were performed as described above, with applications of 2 μl cyclohexane serving as controls.

Extraction and assay of a known amount of C18JH indicated that the efficiency of the extraction procedure was close to 100% (see Fig. 2).

Juvenile hormones

Synthetic methyl 10,11-epoxy-3,7,11-trimethyl-*trans*-2,*trans*-6-dodecadienoate (C16JH) was generously provided by Dr. A. M. Ajami or purchased from Eco-Control. Synthetic methyl *cis*-10,11-epoxy-7-ethyl-3,11-dimethyl-*trans*-2-*trans*-6-tridecadienoate (C18JH) (*ca.* 90% purity) was provided by Dr. Kondo of Sagami Chemical Research Center. Stocks of hormones were stored at −15° C.

RESULTS

Scoring system for the black *mutant larval pigmentation assay*

From the responses of over 250 *black* larvae to various doses of C16JH, the scoring system in Table II was devised. Examples of the responses described in Table II are shown in Figure 1.

Two sorts of "false positives" occurred with low frequency and were eliminated from all tabulations. The first type comprised larvae with a bluish (rather than

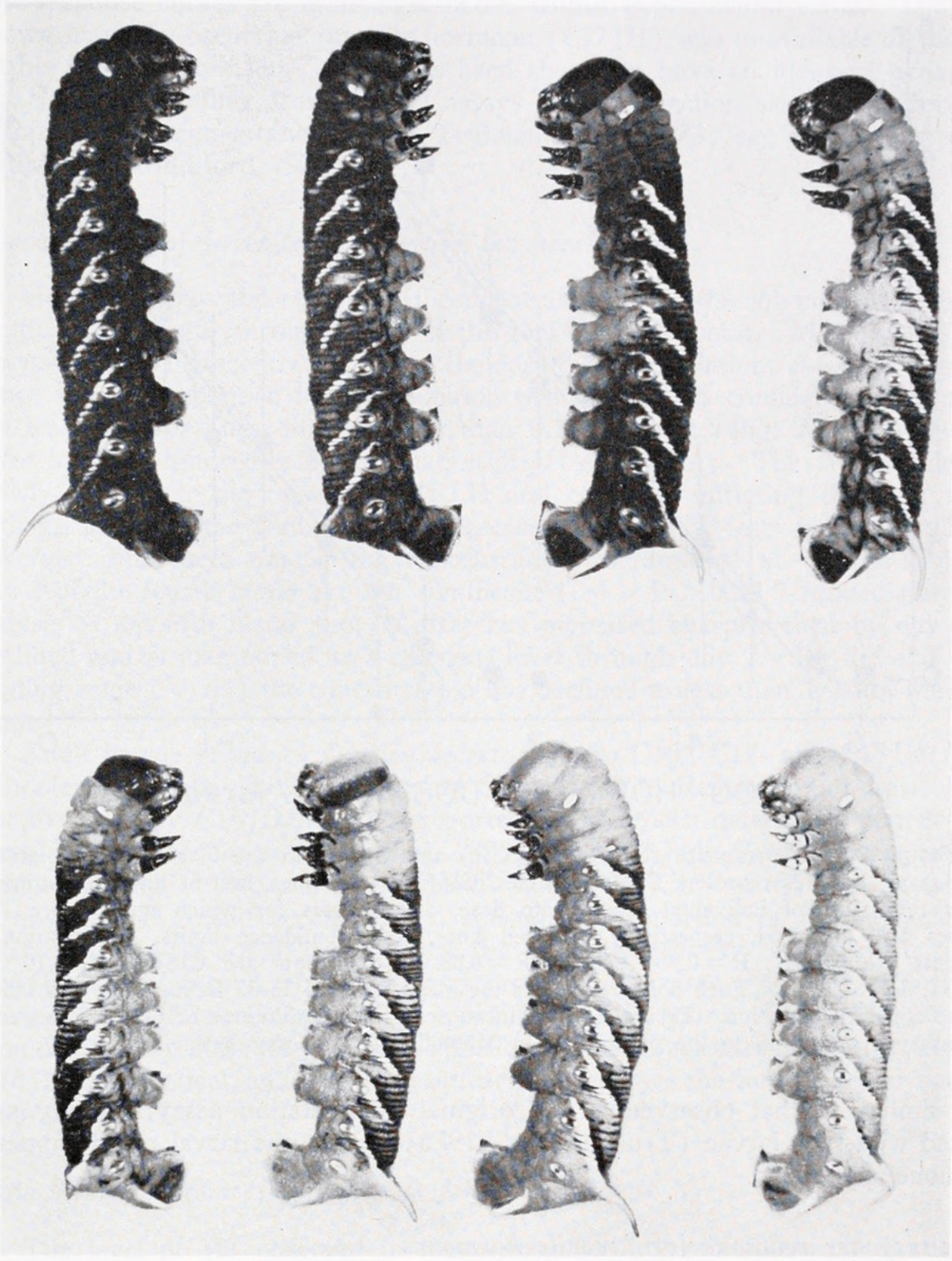

FIGURE 1. Fifth instar *black* larvae following application of various amounts of juvenile hormone at the time of head capsule slippage in the fourth instar. Assay scores are, left to right: upper row, 0, +1, +1 or +2, +3; lower row, +3, +4, +4, +5.

green) "wound" spot at or near the site of hormone application. In response to irritation by cyclohexane, the cuticle had apparently been damaged. If such larvae were gently stretched, the "wound" cuticle would tear. Tearing did not occur with comparable stretching of a "+1" larva. The second type of false-positive

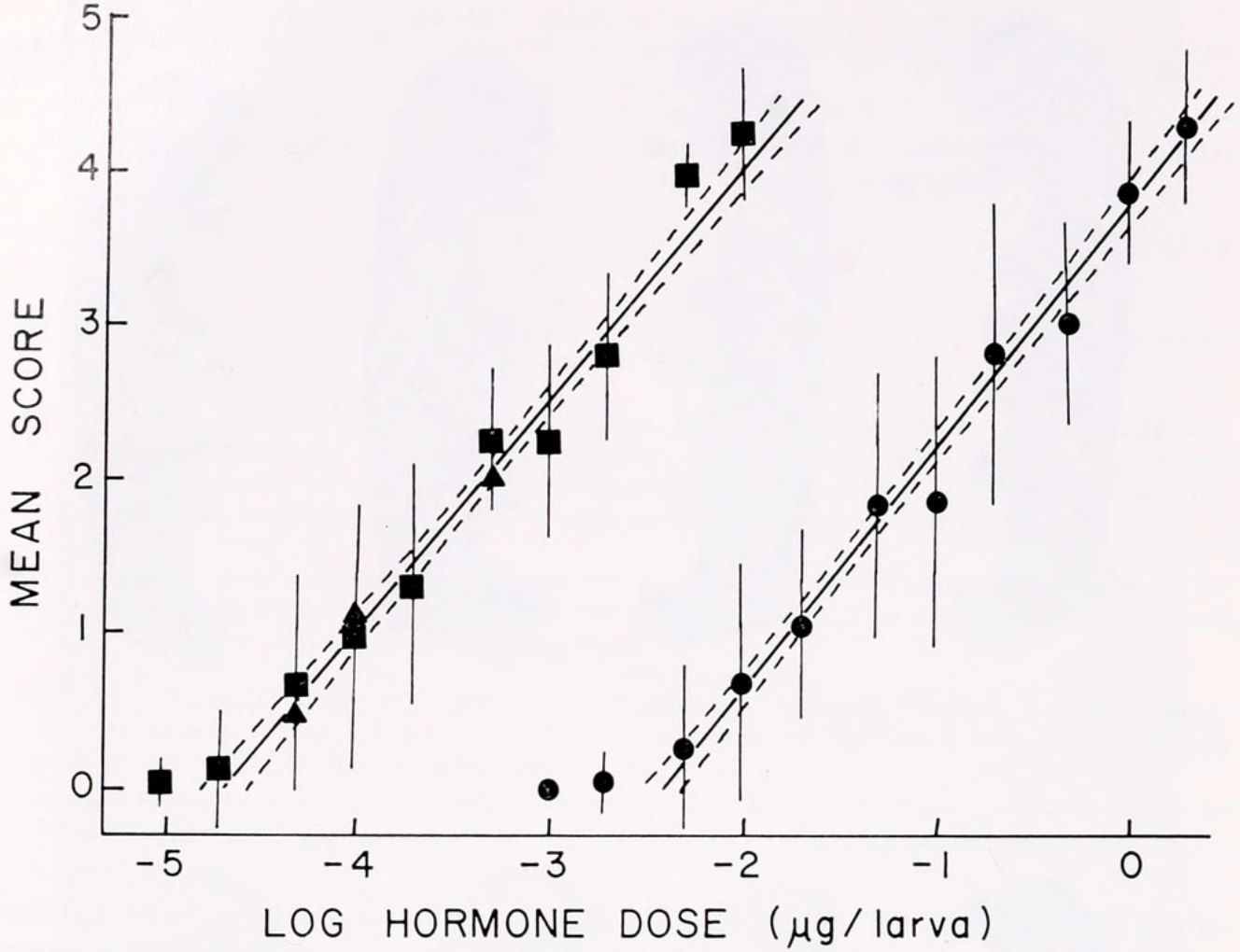

FIGURE 2. Dose-response relations for C16- and C18JH in the *black* mutant pigmentation assay. Squares represent C18JH; circles, C16JH; solid lines, best-fit lines determined by linear regression of individual scores onto dose, at all doses for which mean score > 0.1 (slopes 1.50 and 1.54, respectively); dotted lines, 95% confidence limits. Correlation coefficients are C18JH, $R = 0.91$; C16JH, $R = 0.88$. X-intercepts are C18JH, 2.1×10^{-5} µg; C16JH, 3.8×10^{-3} µg. Each point represents the mean score of 25–37 larvae; the bars indicate ± one standard deviation. Triangles show mean scores for 3 dilutions of C18JH "extracted" and assayed according to the procedures in Methods (9 ± 1 assay larvae per point).

was similar to that observed in the original pigmentation assay utilizing neck-ligated wild-type larvae (Truman *et al.*, 1973) in that the larval cuticle appeared hormone application.

Dose-response relations for juvenile hormones

Figure 2 shows the mean scores obtained for different doses of C16- and C18JH. The lines drawn through each set of points are the least squares lines calculated by regression of individual scores onto the $\log_{10}$ hormone dose. The correlation coefficients ($R = 0.91$ and $R = 0.88$ for C18- and C16JH, respectively) indicate that these lines well represent the experimental data. As expected from results in other *Manduca* JH assays (Nijhout and Riddiford, 1974; Riddiford and Ajami, 1973), C16JH is considerably less active than C18JH. In spite of a nearly 200-fold difference in the activities of these two hormones, the slopes of their respective

dose-response curves are indistinguishable, within experimental error. The third known naturally-occurring juvenile hormone (C17JH) was unavailable at the time of this investigation, but C17JH has been shown to have an identical activity to C18JH in three other *Manduca* JH assays (pupal injection assay, Riddiford and Ajami, 1973; pigmentation assay, Truman *et al.*, 1973; egg maturation assay, Nijhout and Riddiford, 1974).

Concentration of juvenile hormone in the hemolymph

Figure 3 shows the relative JH concentration in the hemolymph from ecdysis to the fourth instar through the first third of the fifth instar. Mean scores were converted to JH concentrations using the dose-response relations shown in Figure 2. Since extremes obtained by extrapolation within the 95% confidence intervals for the least squares lines differ by less than 0.15 log unit (Fig. 2), this aspect of error has been ignored in the calculation of JH equivalents. The JH titer declines slowly until after the release of PTTH and ecdysone initiating the molt to the fifth instar; then the concentration decreases sharply. T-tests indicated that the observed differences among the concentrations determined at various times on day 2 of the fourth instar are not significant ($0.6 > P > 0.3$). Immediately after ecdysis to the fifth instar the JH titer has increased sharply, then by day 1 has declined and is maintained at a constant level through day 2. By the end of the feeding stage (V, d5) the concentration has declined to less than 1/16 of the V, d2 value.

Adult female *Manduca* CA can secrete *in vitro* C16-, C17- and C18JH (Judy, Schooley, Dunham, Hall, Bergot, and Siddall, 1973; Reibstein and Law, 1973), but to date only C16JH has been reported to have been detected in fourth instar larval blood of *Manduca* (Judy *et al.*, 1973; Dr. K. J. Judy, Zoecon Corp., personal communication). Because a very small amount of C17- or C18JH, in addition to any C16JH, could account for a substantial proportion of the extracted JH activity, it is best not to assume that the extracted activity is equivalent only to C16JH. In Figure 3, the right-hand ordinate expresses the JH concentrations in ng/ml C18JH equivalents. JH concentrations in terms of C16JH equivalents would be nearly 200-fold greater. The slopes of the standard curves for C16- and C18JH are identical, and thus the *relative* JH titers are the same whether based on C16- or C18JH.

Rate of degradation of endogenous juvenile hormone

The level of JH observed in hemolymph at intervals following ligation can provide an estimate of the actual rate of "degradation" (including excretion or inactivation) of JH in the hemolymph, since ligation removes the sources of JH (the CA). At varying times after ligation, blood was collected, extracted and assayed just as for intact larvae. The JH titer in intact larvae of the same age (stage IV, d2,-½h in Table I) was considered to be the "0 hour" level of JH. Figure 4 shows the relative concentration of JH in the hemolymph after ligation. The line drawn through the points indicates a half-life ($T_{\frac{1}{2}}$) for endogenous JH in the hemolymph of about 1.5 hours.

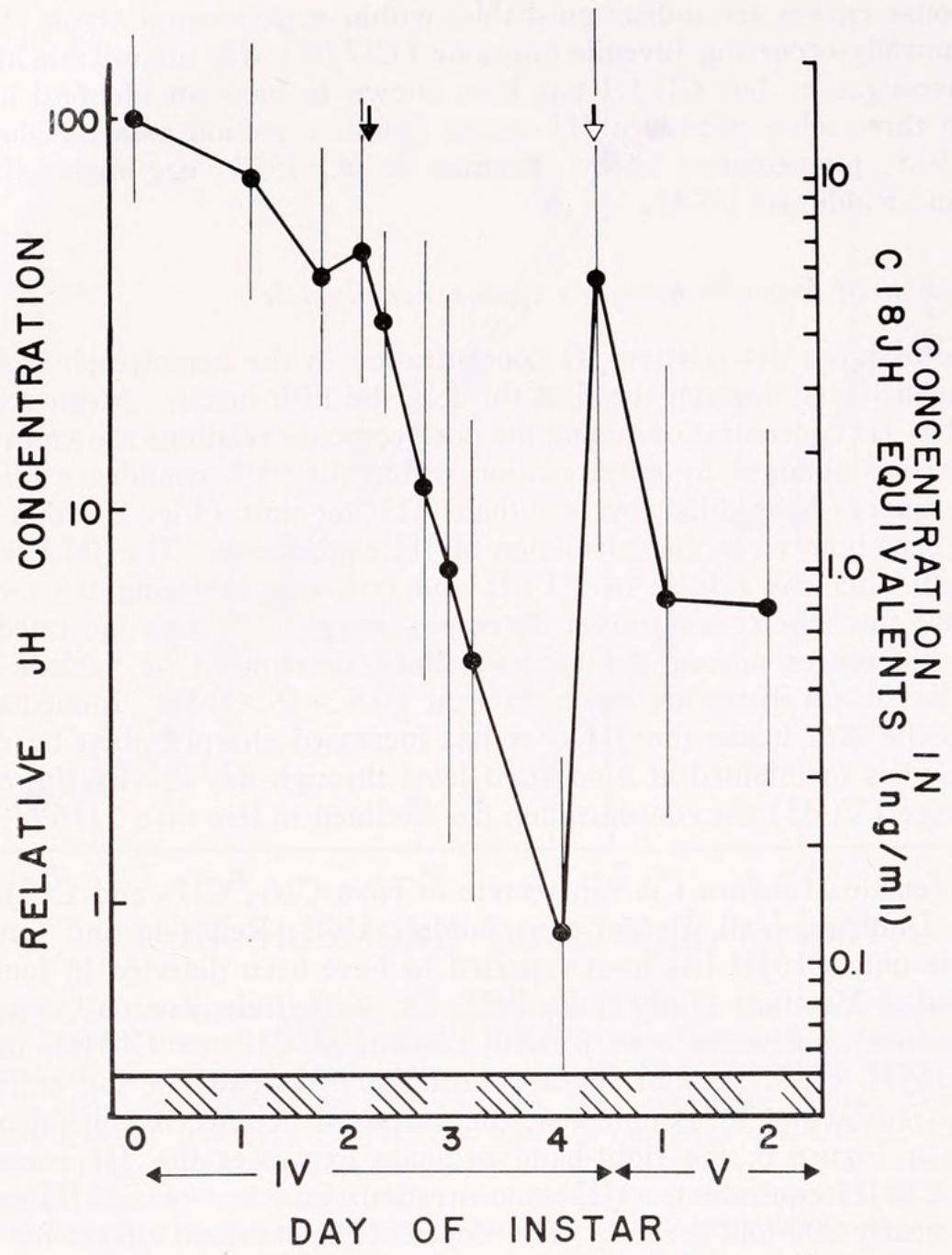

FIGURE 3. Juvenile hormone titer in *Manduca* larvae after ecdysis to the fourth instar through the first third of the fifth instar. The JH concentration at each stage was calculated from the mean scores of initial dilutions of extracts. Each point is based on 3 to 5 extractions and about 25 assay larvae; bars indicate ± one standard deviation; filled arrow, mean time of PTTH release for Gate II larvae as determined by Truman (1972); open arrow, mean time of ecdysis of Gate II larvae to the fifth instar; cross-hatch represents darkness.

Knowing the half-life, one can estimate the rate constant "a" in the expression for the rate of decay, $V_D = dx/dt = -ax$, where "x" is the amount of JH and "t" the time. Integration of the rate expression, from $x = x_0$ to x, and $t = 0$ to t, yields the expression, $x = x_0e^{-at}$, where "x_0" is the initial amount of JH at $t = 0$. Since $x = x_0/2$ at $t = T_{\frac{1}{2}} = 1.5$ hours, then $a = 0.46$ hours^{-1}. Thus the rate expression is $V_D = -x\ (0.46)$ hours^{-1}. Assuming that this rate constant is characteristic of intact fourth instar larvae during the latter part of day 2, one can

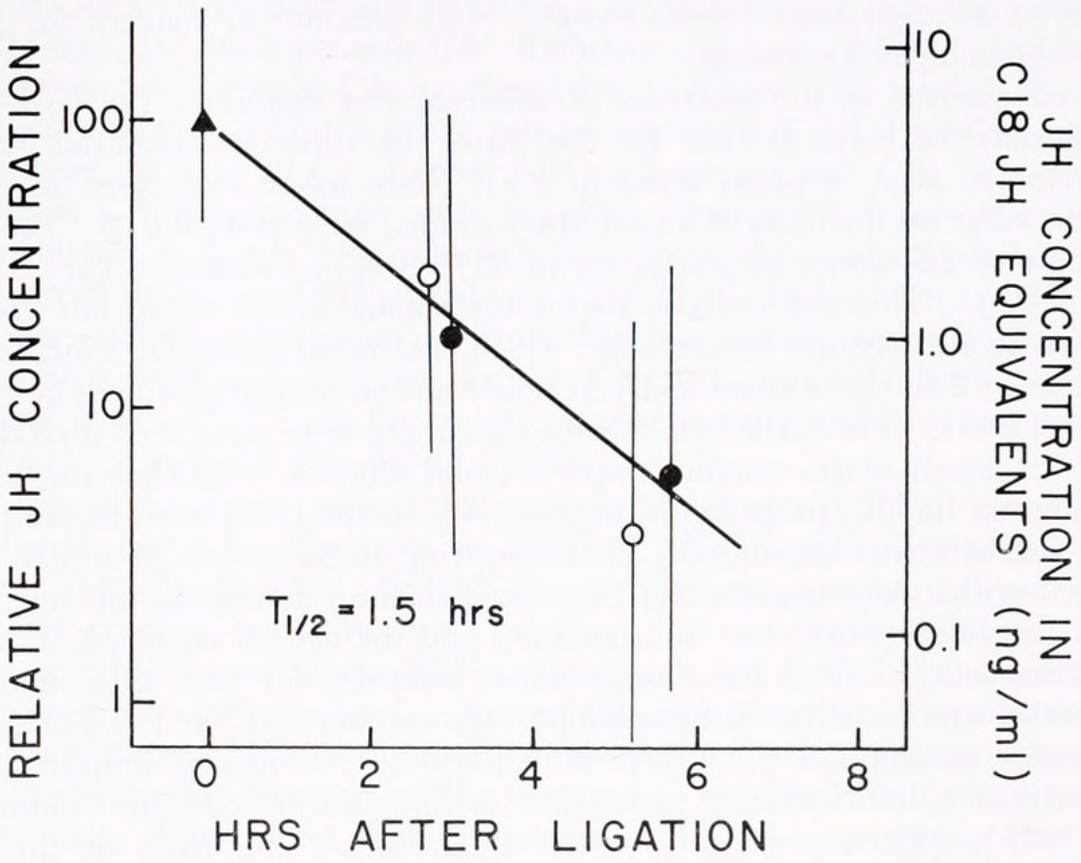

FIGURE 4. Decay of juvenile hormone activity in hemolymph following neck- or abdomen-ligation of fourth instar larvae. JH concentrations were calculated from mean scores of initial dilutions of extracts. Triangles represent unligated day 2 fourth instar larvae (stage IV, d2, -1/2h); filled circles, day 2 fourth instar larvae abdomen-ligated at 23.30 AZT; open circles, day 2 fourth instar larvae neck-ligatured at 23.30 AZT. Points for ligated larvae are based on two extractions and 11–16 assay larvae; bars indicate ± one standard deviation. By 20 hours after ligation, no detectable JH activity remained in either neck- or abdomen-ligated larvae.

estimate the rate of production (synthesis and secretion) of JH which would be required to maintain the titer at the fairly constant level observed during day 2 (Fig. 3). At such a steady state, the rate of production, V_S, must be equal and opposite to the rate of decay, V_D. Thus during this period, $V_S = -V_D = x$ (0.46) hours^{-1}, where "x" is the steady state level of JH. The concentration of JH during this period in intact larvae was approximately 6 ng/ml C18JH equivalents. Total blood volume of larvae which weighed 0.7–1.0 gm was estimated to be roughly 0.4 ml. From these data the steady state level of JH was determined to be 2.4 ng C18JH equivalent per larva, corresponding to $V_S = 1.1$ ng/hour per pair CA (C18JH equivalents). In terms of C16JH equivalents, $V_S = 2 \times 10^2$ ng/hour per pair CA.

DISCUSSION

The use of *black* larvae (instead of neck-ligated wild-type) and the revised scoring system (Table II) facilitated the removal of false positives and resulted in log-linear dose *vs.* response curves over most of the range of the assay, particularly in the lower range, with standard hormones. The departure from

log-linearity at high doses (mean score > 4.5) was not of concern for purposes of assay of hemolymph extracts.

For comparison of the activities of different test materials, the dose-response relations may be characterized by the dose (or dilution) required to give a criterion score (*e.g.,* a mean score of 2.5). Since assay responses are assigned numerical value on the basis of an arbitrary graded scale from 0 to 5 (Table II), a mean score of 2.5 would be analogous to 50% maximal response (or "I.D. 50") (Staal, 1972). This approach is useful for standard hormones, but could only be applied to hemolymph extracts for which the initial assayable dilution gave a mean score ≥ 2.5. Less active extracts could not be compared unless larger initial volumes of blood were extracted. Instead, extracts were compared directly on the basis of the mean scores obtained with a given dilution. By choosing the initial (1×) dilution for this purpose, the applicability of the assay even to the relatively inactive extracts was maximized. In comparing mean scores of initial dilutions of extracts with a dose-response relation established for standards, one must assume that the extracted (unknown) substance(s) and the chosen standard behave similarly in the assay. Since the dose-response relations for both C16- and C18JH were parallel and since larval hemolymph may contain C16- and C17JH, this was a reasonable assumption. As further justification for this assumption, extracts were analyzed by two different approaches making use of assays of dilution series (Fain, 1975). Extract activities were compared on the basis of the dilution required to yield an essentially "0" score (mean score < 0.15). Also, only "+1" responses were considered and extracts were compared on the basis of the dilution corresponding to the 50% point in the distribution of this response. The relative JH titers calculated by these methods were substantially in accord with those calculated directly from mean scores of initial dilutions (Fig. 3). Thus, under the conditions employed, mean scores of initial dilutions of extracts of larval blood provide reasonable estimates of relative JH titers.

In Figure 4, the half-life ($T_{\frac{1}{2}}$) of endogenous JH in the hemolymph after ligation was estimated to be about 1.5 hours. This value agrees with that determined by Johnson and Hill (1973b) for the decay of endogenous JH in the hemolymph following surgical removal of the CA in adult male *Locusta,* and with that of Reddy and Krishnakumaran (1972) for the loss of JH activity in early last instar *Galleria* larvae following injection of C18JH. In *Manduca,* an even shorter half-life (about 0.5 hour) for both endogenous (Nijhout, 1975a) and exogenous (Slade and Zibitt, 1972) JH has been observed in fifth instar larvae, and for exogenous JH in pupae (Ajami and Riddiford, 1973). This rapid decay of endogenous JH from the hemolymph contrasts with the apparent persistence of the effects of JH in isolated abdomens of young fifth instar (Nijhout, 1975a) and of fourth instar *Manduca* larvae and in fragments of fourth instar epidermis cultured *in vitro* (Fain and Riddiford, 1973; Fain, 1975). Fourth instar epidermis of *Manduca* remains capable of a larval response to exogenous β-ecdysone, both *in vivo* and *in vitro,* for many hours after removal of CA.

When CA from adult female *Manduca* were cultured *in vitro* with radio-labelled methionine, both C16- and C17JH—labelled in the ester methyl—were recovered from the medium (Judy *et al.,* 1973). The data from this study provide information on average rates of synthesis and secretion over a 24 hour period. These rates are

about 0.045 ng C16JH/hour and 0.065 ng C17JH/hour per pair CA or a total of about 0.11 ng "JH"/hour per pair CA. Reibstein and Law (1973), using homogenized *Manduca* adult female CA incubated for 12 hours with labelled S-adenosylmethionine, reported at best a linear rate of synthesis of JH (labelled material in the "JH zone" of their TLC) of about 0.04 ng/hour per pair CA. But in cultures of *Manduca* CA supplemented with possible precursors for the sesquiterpenoid skeleton, higher rates of JH production have been observed, as well as changes in the ratio of the various JH's produced (Dr. H. K. Dahm, Texas A & M University, personal communication). Thus, the biosynthetic capacity of CA under some *in vitro* conditions may be restricted by sub-optimal substrate availabliity (Pratt and Tobe, 1974), and the *in vitro* synthetic rates and products may not be indicative of those *in situ* (see Ajami, 1974).

The rate of synthesis required to maintain the observed JH titer during day 2 of the fourth instar in *Manduca,* in terms of C18JH equivalents (1.1 ng/hour per pair CA) is only about 10-fold greater than the average rate of synthesis of total JH reported by Judy *et al.* (1973). But the required rate of synthesis in terms of C16JH equivalents (200 ng/hour per pair CA) is very much greater than the *in vitro* rates observed. Quantitative unambiguous determination of the amounts of different JH's in the hemolymph and of the *in vivo* secretory products of the CA during development will be required to resolve definitively the questions raised by these differences. Yet the results presented here suggest that C17- or C18JH may account for most of the observed JH activity in the hemolymph of fourth instar larvae.

Titers of JH in principle may be regulated in a variety of ways (see de Wilde *et al.,* 1971). Recent findings have stressed the importance of changing rates of degradation (or inactivation) of JH in altering hormone levels in *Manduca* (Weirich, Wren, and Siddall, 1973; Sanburg, Kramer, Kézdy, Law, and Oberlander, 1975; Sanburg, Kramer Kézdy, and Law, 1975), especially since a JH-binding protein in the hemolymph of *Manduca* larvae (Kramer, Sanburg, Kézdy, and Law, 1974) affords bound JH nearly complete protection *in vitro* against hydrolysis by general esterases, but not by JH-specific esterases (Sanburg, Kramer, Kézdy, and Law, 1975; Sanburg, Kramer, Kézdy, Law, and Oberlander, 1975). Although JH-specific esterases may be important in elimination of JH from the hemolymph at the end of the feeding phase of the fifth instar, the relation of such esterases to JH titer changes during the fourth instar is uncertain. If the JH activity in young fourth instar larvae were due primarily to C17- or C18JH, the JH concentration at this time (*e.g.,* day 2) would be about 2×10^{-8} M. Nearly all of this JH should be associated with JH binding protein, if the hemolymph concentration and dissociation constant of binding protein in fourth instar larvae are the same as reported for fifth instar larvae (concentration: 8×10^{-6} M; $K_d = 3 \times 10^{-7}$ M) (Kramer *et al.,* 1974). Almost no detectable JH-specific esterase activity has been observed in fourth instar hemolymph, although general esterase activity is present and appears to increase slightly on day 3 of the instar (Sanburg, Kramer, Kézdy, and Law, 1975). Since the rate of inactivation of JH on day 2 is already quite rapid ($T_{\frac{1}{2}} = 1.5$ hrs), the decline in the JH titer that follows the initiation of the last larval molt may reflect changes in the rate of production of JH by the

CA, rather than alterations of inactivation rates. The observations of Williams (1961) on CA activity of fourth instar *Cecropia* larvae support this suggestion.

In terms of the relative JH concentration during the last two larval instars, one can conclude the following. The JH concentration is highest immediately after ecdysis to the fourth instar, and the level remains high until after the endocrine initiation of the molt to the fifth instar. During this period there may be small (and perhaps physiologically significant) fluctuations in the JH titer. Following the initiation of molting, the JH concentration drops considerably (about 30-fold) over the last two days of the fourth instar. Immediately after ecdysis to the fifth instar, the JH concentration has risen significantly to nearly the level on day 2 of the fourth instar. But by the next day (day 1), it has decreased and remains at a plateau level through the following day (day 2). Nijhout (1975a) has confirmed a comparable low titer on day 2 of the fifth instar. By the end of the feeding phase of the fifth instar, the concentration has dropped to essentially undetectable levels as was observed also by Nijhout and Williams (1974).

Truman (1972) obtained primarily precocious pupations after neck ligature of day 2 fourth instar *Manduca* larvae at the time of PTTH release; but when neck-ligature occurred 1.5 hours later, he observed larval molting. Therefore, he concluded that following PTTH release a surge of JH was necessary for the larval molt. Yet the data presented here indicate that a relatively high and constant titer of JH persists throughout the day preceding PTTH release. Nijhout (1975b) has shown by ecdysone perfusions that the level of JH in young fifth instar *Manduca* larvae is sufficient to allow complete or nearly complete larval molting in response to exogenous ecdysone. In the present study, the JH titer in young fifth instar larvae of comparable age (stage, V, d2) was 6–8-fold lower than that observed late in day 2 of the fourth instar. A transient decline in the JH titer of 8-fold or more at the time of PTTH release in the fourth instar is unlikely to have been masked by averaging of differences among individual larvae. The photoperiod synchronization of initiation of molting (Truman, 1972) and selection of developmentally comparable larvae for extractions argue against such artifacts of averaging. In addition, nearly all of larvae neck-ligatured at 23.30 AZT eventually pupate (Fain, 1975), so extracts were made at a time appropriate to have detected a drop in JH if one occurred. It is evident also that substantial titers of both JH and ecdysone occur together at the time that the last larval molt is initiated. The significance of the formation of precocious pupae after neck ligation at the time of PTTH release will be discussed in another paper (Fain and Riddiford, in preparation).

Williams (1961) showed that during larval development in *Hyalophora cecropia* the CA undergo cyclical changes in activity. Similarly, Krishnakumaran and Schneiderman (unpublished, cited in Patel and Madhavan, 1969) found that the extractable JH activity (in *Galleria* Units) in whole larvae of *Samia cynthia ricini* was high immediately following ecdysis to the fourth instar and decreased during the course of the instar up to the time of ecdysis. As with Cecropia CA activity (Williams, 1961), the JH titer in young fifth instar Cynthia larvae was considerably higher than that at the end of the fourth instar. The present observations on *Manduca* are in accord with these findings.

By contrast, Bartelink (unpublished, cited in de Wilde *et al.*, 1971) found that the JH concentration (in *Galleria* Units) in hemolymph of *Philosamia cynthia* larvae was essentially constant throughout the fourth instar and dropped about 10-fold at the beginning of the fifth instar. This discrepancy between Bartelink's findings and our observations on *Manduca* may reflect differences in experimental animals, in the assays employed (*Galleria* wax wound *vs. Manduca* pigmentation), or in the procedures used to select larvae for blood collection. With *Manduca* it is possible to select developmentally synchronous groups of larvae before, during and after initiation of molting (Truman, 1972). Thus, in the present study, averaging of developmental differences in JH titer was minimized.

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Summary

1. A sensitive and quantitative bioassay for JH is described based on the JH inhibition of cuticle melanization in larvae of the *black* mutant of *Manduca sexta*. The assay is sensitive down to about 2×10^{-5} μg (20 pg) C18JH.

2. Using this assay, the JH titers in hemolymph of larvae of *M. sexta* from ecdysis to the fourth instar through the first third of the fifth instar have been estimated. The JH titer is highest immediately after ecdysis to the fourth instar and remains relatively high until after initiation of the last larval molt. During the time that molting is initiated, the JH concentration is about 2×10^{-8} M in terms of C18JH equivalents. Following initiation of molting the titer drops about 30-fold.

3. Immediately after ecdysis to the fifth instar, the JH titer is again high (nearly the same as seen at the initiation of the molt), then by day 1 has declined sixfold and remains constant through day 2. In mature fifth instar larvae, the titer has dropped again to essentially undetectable levels.

4. The rate of degradation of endogenous JH in the hemolymph was estimated following neck- or abdomen-ligation of fourth instar larvae. After either type of ligation, JH activity was lost from the hemolymph with a half-life of about 1.5 hours.

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