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THE SOURCE AND ACTION OF HEAD FACTORS REGULATING JUVENILE HORMONE ESTERASE IN LARVAE OF THE CABBAGE LOOPER, *TRICHOPLUSIA NI*

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Abstract—Brain (median or lateral regions) or suboesophageal ganglion (SOG) homogenates of Day 1 fifth instar larvae of *Trichoplusia ni* induced the appearance of haemolymph juvenile hormone esterase (JHE) when injected into Day 1, Day 2 or early Day 4 fifth instar ligated hosts. Brain and SOG homogenates of late fourth instars also induced JHE when injected into Day 1 hosts, whole late fifth instar and pupal tissue did not. The pattern of JHE induction by early fourth through Day 3 fifth instar brain and SOG homogenates correlated with natural haemolymph JHE activity occurring at these times. Implantation of late fourth and Day 1 fifth instar brains and/or SOG into similar age hosts similarly induced JHE activity while prothoracic and abdominal ganglia did not. The relative levels of induction following implantation were SOG < brain < brain + SOG. JHE activity which appears in the haemolymph following injection of brain homogenates appears to be largely due to a single enzyme which has an isoelectric point indistinguishable from that of the natural haemolymph enzyme. Evidence is presented which suggests that inhibitory as well as stimulatory brain factors are involved in JHE regulation.

Key Word Index: Noctuidae, *Trichoplusia ni*, juvenile hormone, juvenile hormone esterase, juvenile hormone regulation, neurohormone

INTRODUCTION

IN RECENT years, there has been a great deal of research on routes of juvenile hormone (JH) degradation, and one major route appears to be hydrolysis of JH by JH esterases (JHEs). Little has been published, however, on modes of control of JHE. At some times during development, the appearance of JHE may be due to induction by JH (WHITMORE *et al.*, 1972). SPARKS and HAMMOCK (1979a) obtained JHE induction in both normal and neck or thoracico-abdominal ligated postwandering *Trichoplusia ni* following the application of JH or several JH analogues (JHA or juvenoids). Recently, this phenomenon has been reproduced in *Galleria mellonella* (MCCALEB and KUMARAN, 1980) and *Manduca sexta* (RIDDIFORD and HAMMOCK, unpublished observations). Based on these and other experiments, SPARKS and HAMMOCK (1979a) developed a hypothesis that the second (postwandering) JHE peak may be induced in response to a burst of JH which occurs before pupation.

There is also evidence that a head factor other than JH is involved in some aspects of JHE regulation. KRAMER (1978) found that the head seems to mediate JH or juvenoid of JHE in adult diapausing *Leptinotarsa decemlineata*. RETNAKARAN and JOLY (1976) found in *Locusta migratoria* that the A and B neurosecretory cells might be involved in JHE regulation since cautery of these cells reduced haemolymph JHE activity, but the possible involvement of other tissue was not reported. VINCE and GILBERT (1977) cite unpublished data which indicate JHE may be under neurohumoral control. Neck ligation prior to the prewandering JHE peak caused this peak to be reduced in *G. mellonella* (REDDY *et al.*, 1979) and obliterated in *T. ni* (SPARKS and HAMMOCK, 1979a). Application of JH or juvenoids to unligated larvae at this time suppressed JHE in *G. mellonella* (REDDY *et al.*, 1979). Conversely, it induced a 20% increase in JHE in *T. ni* (SPARKS and HAMMOCK, 1979a). Both teams hypothesized that an unknown "head factor" was responsible for inducing the prewandering peaks. As used by these workers, "induction" refers only to the appearance of haemolymph JHE following experimental treatment.

WING and LEVINSON (unpublished observations) obtained induction of JHE in *T. ni* larvae by injection of homogenates of whole heads from prewandering larvae into similar age ligated abdomens. MCCALEB and KUMARAN (1980) implanted brains and subesophageal ganglia from Day 1 last instar *G. mellonella* into similar age hosts and obtained an increased JHE titre.

Though the above studies suggest head nerve tissue is involved in JHE regulation, no studies have tested other nerve tissue, bioassayed active tissue output or JHE source responsiveness over developmental time, or conducted biochemical studies to determine whether the experimentally induced enzyme is the same as that occurring naturally. The present study was designed to provide such information.

MATERIALS AND METHODS

Experimental animals

T. ni were reared on an artificial diet at 28 ± 2°C with a photoperiod of 14L : 10D (SHOREY and HALE,

1965; SPARKS *et al.*, 1979a). Fourth instar larvae preparing to moult to fifth instars were selected between 6 and 10 hr after lights on (ALO). The selection criteria were a pale green colour and/or signs of head capsule slippage. Such selected larvae usually ecdysed between 1 hr before and 5 hr after lights off (ALOF). L5D1 and L5D2 experimental larvae consisted of both Gate 1 and Gate 2 larvae, since there is little or no difference in haemolymph JHE levels through L5D3 in larvae of either gate (SPARKS *et al.*, 1979a). L5D3 and L5D4 larvae and Day 1 pupae consisted only of Gate 1 animals.

Haemolymph collection and assay

Haemolymph was collected by piercing the integument near the base of the last proleg and bleeding 2–5 larvae. Ten μl of the pooled haemolymph were diluted 100-fold with sodium phosphate buffer (pH 7.4, I = 0.2 M) containing 0.01% phenylthiourea. The JH I or III esterase activity was then monitored at 5×10^{-6}M substrate concentration by either the charcoal precipitation assay (SANBURG *et al.*, 1975) or the partition assay (HAMMOCK and SPARKS, 1977) using C-10 [^{3}H]JH (New England Nuclear). Due to decomposition of the epoxide moiety of the [^{3}H] methoxylabeled substrate for the charcoal assay rates were consistently 10 × lower than with the partition assay. All rates are reported in units of JH III hydrolysis in the partition assay. Enzymatic hydrolysis rates were linearly dependent upon time and protein concentration. Haemolymph and fat body lacetate dehydrogenase (LDH) activity was measured by the method of BERGMEYER and BERNT (1974) as an index of fat body lysis.

Tissue homogenisation and implantation techniques

Brains, suboesophageal ganglia (SOG), prothoracic ganglia or abdominal ganglia of donor larvae were dissected free of adhering tissues, rinsed in double distilled water and stored frozen in 0.5 ml homogenising tubes (Kontes). The following day, they were homogenised in 200–300 μl of double distilled water and centrifuged at 4000 ***g*** at 4°C for 15 min. Preliminary experiments indicated that freezing test tissue overnight did not significantly reduce JHE inducing activity as compared with fresh tissue ($t_{0.05}$). The supernatant was then evaporated to dryness under a gentle stream of nitrogen and stored dry at 0°C until just prior to injection. The dry supernatant was redissolved in double distilled water to give a concentration of approx. 4 brain equivalents/3 μl. Each host larva received 3 μl of brain extract. The injection was made through the dorsum of the last abdominal segment with a 10 μl Hamilton syringe.

In some experiments dissected brains were placed in a depression slide resting on a piece of dry ice. The brain was cut into 1 median and two lateral regions with the median region being approximately equal to the combined size of the two lateral regions. These two groups were separately homogenized as described above.

Alternatively, brains, SOG, or brain–SOG complexes were dissected and rinsed twice with double-distilled water. They were then temporarily placed in a depression slide in double distilled water while a small incision was being cut in the base of a first proleg of an etherised host. A small hook was used to pull the incision apart while the brain was inserted with tweezers. The wound was sealed with Carter's rubber cement.

Evaluation of larval response to homogenates and implants

In injection experiments involving 'standard' recipient larvae, L5D1 larvae were ligated at 12 hr ALO, injected 2 hr later and bled 5 hr thereafter. For larval age dependent studies, the above procedure was identical except that the initial ligation was performed at various times during the fourth or fifth instar.

For dose–response studies two methods were employed. In the first, an extract of 60 brains was serially diluted to yield concentrations of 8, 4, 2, 1, and 0.5 brain equivalents/2 μl double distilled water. An alternative method was an addition of different numbers of brains to a fixed volume (22.5 μl) of solvent to achieve the same brains/2 μl concentrations given above. No significant difference was observed between these methods ($t_{0.05}$). Time dependent effects of brain homogenate treatment were assayed by injecting L5D1 'standard' larvae with 4 brain or SOG equivalents from L5D1 4 hr ALOF donors as described above. The larvae were then bled for assay at various times after injection. A similar time dependent experiment for brain implantations was performed. For inhibition experiments a sample of diluted haemolymph was preincubated with a 1×10^{-3}M solution of *O,O*-diisopropyl phosphorofluoridate (DFP), an aliphatic trifluoromethyl ketone: 1,1,1-trifluoromethyltetradecan-2-one (TFT), or *O*-ethyl-*S*-phenyl phosphoramidothiolate (EPPAT) for 10 min at 30°C before substrate addition.

Isoelectric focusing

The isoelectric points of the JHE in haemolymph samples obtained following injection with 4 brain equivalents were compared on the same gel with JHE from normal L5D2 4 hr ALO larvae. The samples were focused on an LKB Multiphor using either a precast LKB Ampholine® PAG plate (5% polyacrylamide with 2.2% ampholytes, pH 3.5–9.5) or a similar narrow range plate cast in this laboratory (3% polyacrylamide, 5% crosslinking, with 6.3% Pharmacia ampholytes, pH 4.0–6.5).

The gels were run and analysed as described earlier (WINTER *et al.*, 1977; SPARKS and HAMMOCK, 1979b), except that pH paper (pH range 0.0–6.0, Microessential Laboratories) laid in strips across the entire focusing gel was found to give a rapid, reproducible indication of the homogeneity of the linear gradient in different gel tracks. Actual measurement of isoelectric points were made by slicing 0.5 cm sections from empty gel tracks and eluting overnight in 0.4 ml of double distilled water. The ampholine solutions were then measured for pH at 10°C using a Corning model 125 pH meter and a Corning triple purpose AG/AgCl electrode. Esterase activity was determined on 0.25 cm gel sections.

Statistical analysis

The *t*-test was used when comparing means from two treatments. For comparisons which involved more than two treatments the data was first subjected

to an analysis of variance. If the '*F*' value was significant (0.05) the treatment differences were then located by Kramer's modification of Duncan's new multiple range test (KDNMR) (STEELE and TORRE, 1960). Variability around means are reported as standard deviations.

RESULTS

Effect of ligation on the prewandering JHE peak

Ligation at the beginning of the prewandering JHE peak causes a highly significant subsequent decline in haemolymph JHE as compared with the control larvae ($t_{0.01}$) (Table 1) and is consistent with data found by SPARKS and HAMMOCK (1979a). Starvation causes a similar rapid decline in haemolymph JHE ($t_{0.01}$. Also, the JHE titre in L4D2 larvae ligated at the beginning of the intermoult period and assayed 7 hr later dropped by 50% as compared with the unligated control. This effect cannot be an artifact of starvation due to ligation because control larvae do not feed during the intermoult period.

Dose and time dependence

The standard host larvae responded to the L5D1 3 hr ALOF brain homogenate in a dose-dependent fashion (Fig. 1). The dose of 4 brain equivalents was chosen for subsequent experiments because it effected a significantly greater fold increase in JHE than low doses ($KDNMR_{0.05}$) and required less time for dissection than the 8 brain dose. Esterase activity was detected within 3 hr following injection, peaked after 5 hr and then declined.

In subsequent experiments, larvae were routinely bled 5 hr after injection because this time interval resulted in the highest observed induction; moreover, this induction was significantly different from that observed after long periods ($KDNMR_{0.05}$) (Table 2). High levels of haemolymph JHE were also obtained 5 hr after implanting a single L5D1 3 hr ALOF brain into standard recipient larvae (Table 3), and the JHE activity was also significantly greater than that observed after long intervals ($KDNMR_{0.05}$). The higher JHE activity in brain implanted larvae was not an artifact of implantation-induced fat body lysis since haemolymph LDH activity was not significantly different ($t_{0.05}$) in brain implanted and sham operated larvae (Table 3).

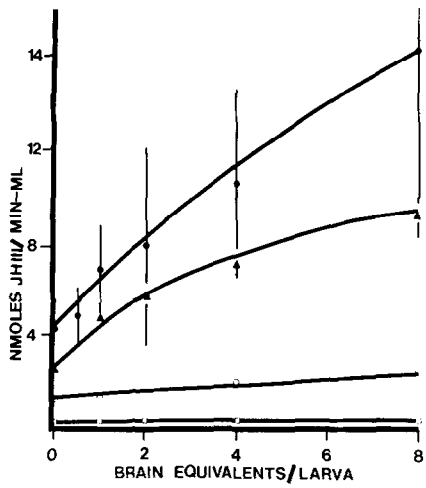

Fig. 1. Induced JHE activity in late L5D1 larvae when injected with various doses of brain extracts from L5D1, 3 hr ALOF donors: uninhibited haemolymph (0); haemolymph incubated prior to assay with 1×10^{-3}m DFP (▲); TFT (0); or EPPAT (□). At least 20 larvae were used for each data point on the dose–response line while 5–11 larvae were used for each point on the inhibitor curves. Regression analysis indicated a highly significant (F = 0.01) increasing trend in response with increases in dose. Also, the standard 4 brain dose caused significantly greater JHE induction than water injected controls ($KDNMR_{0.05}$). The entire experiment was repeated using a different assay procedure with similar results. The percentage inhibition caused by DFP, TFT and EPPAT was 20, 96, and 92%, respectively, for water injected control larvae and 20, 98, and 96% for larvae injected with 4 brain equivalents. These values are similar to the inhibition caused by these three compounds when incubated with normal L5D2 haemolymph (30, 96, and 94%, respectively). The natural L5D2 haemolymph was diluted so that it had an enzymatic activity per unit volume similar to that in the brain induced haemolymph.

Isoelectric focusing and effect of esterase inhibitors

A single, sharp peak of JHE activity was detected on analytical isoelectric focusing of the haemolymph of ligated larvae given L5D1 brain extracts. The isoelectric point of this activity was indistinguishable from that of the natural L5D2 haemolymph JHE when the two samples were run on adjacent tracks of the same gel (pH 4–6). By this method, enzymes with

Table 1. Decrease in haemolymph JHE activity of L5D1 larvae ligated at 12 hr ALO

Time of assay postligation (hr)	% JHE in ligated vs controls*	n†	% JHE in starved vs controls‡	n†
5	45.6 ± 6.8	3	37.6 ± 17.2	3
7	39.9 ± 15.6	3	30.1 ± 1.9	2
10	4.5 ± 2.5	4	—	—
15	2.2 ± 0.8	4	—	—
18	0.4	1	—	—

* For each time of assay, there was significantly less JHE activity in ligated larvae as compared with non-ligated controls ($t_{0.01}$).

† Each replicate consists of a pool of haemolymph from 2 to 5 larvae. The entire experiment was performed four months previously with similar results.

‡ For each time of assay there was no significant difference in the activity in ligated larvae as compared with starved larvae ($t_{0.01}$).

Table 2. Time dependence of JHE induction in L5D1 (12 hr ALO) ligated larvae injected 2 hr postligation with 4 brain equivalents from L5D1 (3 ALOF) larvae

Time of assay postinjection (hr)	Fold increase over ligated water injected larvae*	n†
3	1.8 ± 0.9	6
5	2.6 ± 1.1	6
8	1.8 ± 0.7	4
11	1.6 ± 1.1	5
16	1.1	1
19	1.1	1

*JH Esterase levels at 3–8 hr postinjection are all significantly different than ligated water injected control larvae ($KDNMR_{0.05}$).

†Each replicate consists of a pool of haemolymph from 2 to 5 larvae.

an isoelectric point of ±0.03 pH units can be readily distinguished. The inhibition caused by DFP, TFT and EPPAT was similar for haemolymph from L5D1 recipient larvae whether injected with water or 4 brain equivalents. The inhibition was, in both cases, also similar to that found in diluted normal L5D2 3 hr ALO haemolymph. In each case, DFP was a weak inhibitor while TFT and EPPAT were strong inhibitors (Fig. 1). DFP is a weak inhibitor of *T. ni* JHE, but a strong inhibitor of α-naphthyl acetate esterases (α-NAE). TFT causes minimal inhibition of α-NAE, but it is a potent, reversible inhibitor of JHE, while EPPAT inhibits both enzymes (SPARKS and HAMMOCK, unpublished).

Source of head induction factor

Both lateral and median regions of L5D1 brains and the SOG gave high induction of JHE in standard recipient larvae. The prothoracic ganglion and abdominal ganglia gave no detectable induction. These data indicate that the induction of JHE is not an artifact of nerve tissue, and that the inducing factor is localised in two nervous tissues, the brain and SOG (Table 4).

Brain titre of inducing factor

The apparent titre of the inducing factor in the brain consists of two peaks (Fig. 2). The first peak occurs during L4D2 at about 9 hr ALO. The second peak occurs during L5D2 at about 5 hr ALO. The level of induction at both peaks was significantly greater than that occurring at low points ($KDNMR_{0.05}$). At the time of these two peaks, there are two peaks of haemolymph JHE (SPARKS *et al.*, 1979a), although the L4D2 peak is very small using JH III as a substrate. SPARKS *et al.* (1979a) found another major JHE peak during L5D4 (postwandering), but brains of that age and Day 1 pupae contain no detectable inducing factor. Brain homogenates from L5D3 5 hr ALO larvae caused JHE to be significantly lower than that in water injected controls ($t_{0.05}$).

Table 4. Activity of several tissues when assayed for presence of JHE inducing factor

Tissue*	Fold increase over water injected controls†	n
Whole brain‡	2.3 ± 0.8[a]	16
Median brain region‡	1.5 ± 0.2[b]	5
Lateral brain region‡	1.3 ± 0.1[b]	5
Suboesophageal ganglion‡	2.5 ± 0.8[a]	9
Prothoracic ganglion		
L5D1 3 ALOF‡	1.0 ± 0.16[c]	6
L5D2 6 ALO	1.1 ± 0.03[c]	2
L5D3 6 ALO	0.9 ± 0.2[c]	3
Abdominal ganglia‡	1.2 ± 0.3[b,c]	4

*All brain SOG treatment gave significantly ($t_{0.05}$) greater JHE than water injected larvae, prothoracic and abdominal ganglia treatments did not.

†Means followed by different letters are significantly different ($KDNMR_{0.05}$).

‡Tissue taken from L5D1 (3 hr ALOF) donors, unless otherwise indicated, homogenized and injected into L5D1 14 hr ALO larvae ligated 2 hr previously.

Brain implantation

Because late fourth instar and Day 1 and early Day 4 fifth instar larvae showed either high haemolymph JHE or high brain content of inducing factor, brains from these developmental stages were removed and reimplanted into ligated hosts of the same age. The results in Table 5 show that L4D2 11.5 hr ALO and L5D1 14 hr ALO brains apparently released an inducing factor. The resulting fold increase in JHE was the same as that induced by injection of a homogenate

Table 3. Time dependence of JHE induction and lactate dehydrogenase (LDH) activity in L5D1 (12 hr ALO) ligated larvae receiving implantations of a single L5D1 (14 hr ALO) brain at 2 hr postligation

Time of assay postimplantation (hr)	Fold increase over ligated sham-operated larvae*	n†	LDH activity‡ Sham		Implant	
			Fat body	Haemolymph	Fat body	Haemolymph
3	3.5 ± 0.5	4	21.4 ± 7.8	0.14 ± 0.08	32.4 ± 3.2	0.11 ± 0.03
5	2.9 ± 0.7	3	10.5 ± 5.3	0.11 ± 0.01	10.3 ± 5.6	0.12 ± 0.03
8	2.8 ± 0.8	4	—	—	—	—
11	1.6 ± 0.0	4	—	—	—	—
16	1.9 ± 0.8	4	—	—	—	—

*Esterase levels at 3–16 hr postimplantation are all significantly different from ligated sham-operated control larvae ($KDNMR_{0.05}$).

†Each replicate consists of a pool of haemolymph from 2 to 5 larvae.

‡μ mole NADH oxidized/min/mg haemolymph or fat body protein.

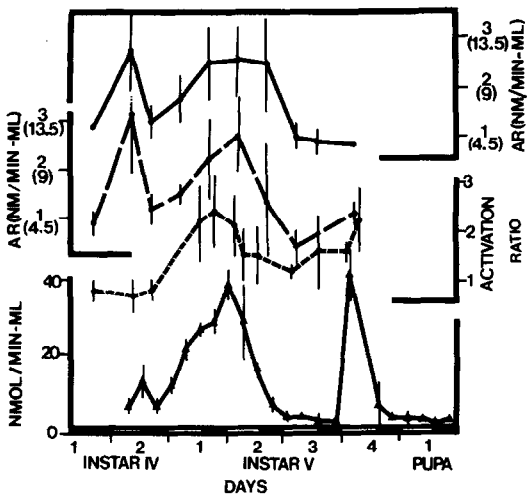

Fig. 2. Titre of brain (upper ●--●) and SOG (●——●) content of inducing factor and responsiveness of different age hosts (●---●) to brain homogenate inducing factor. Natural haemolymph JHE titre (JHE III substrate, lower ▲——▲). AR = activation ratio, the fold increase in haemolymph JHE activity over water-injected controls. JHE activity is expressed as nmol/min. JH III hydrolysed per ml haemolymph.

of 4 brains ($t_{0.05}$). Induction was similar whether based on JH I or III esterase activity. L5D4 larvae receiving L5D4 6 hr ALO brains showed lower JHE activity than sham-operated controls (Table 5).

SOG titre and implantations

The assay of SOG content revealed a pattern similar to that of the brain, i.e. two peaks occurred at the same times as the natural late fourth instar and early fifth instar JHE haemolymph peaks (Fig. 2). The induction occurring at both peaks was significantly greater than that occurring at low points ($KDNMR_{0.05}$). Reimplantations of single intact SOG on L4D2 and L5D1 resulted in a significant increase ($t_{0.05}$) in haemolymph JHE as compared with sham-operated controls (Table 5). The resulting fold increase in JHE was the same as that induced by injection of a homogenate of 4 SOG ($t_{0.05}$).

Brain–SOG complexes

Implantation of a disconnected brain and SOG into the same L5D1 standard larva resulted in significantly higher JHE activity than that caused by implantation of the SOG alone or brain alone ($KDNMR_{0.05}$). Implantation of an intact brain–SOG complex resulted in significantly higher JHE than that in standard larvae receiving a disconnected complex ($KDNMR_{0.05}$) (Table 5).

Host responsiveness to inducing factor

The responsiveness of different age hosts to L5D1 3 hr ALOF brain homogenates is given in Fig. 2. Peak responsiveness occurs during L5D1 about 5 hr ALOF and during L5D4 about 7 hr ALO, and corresponds well with the two fifth instar peaks of haemolymph JHE found by SPARKS *et al.* (1979a). The level of response at these two peaks was significantly greater than that occurring at low points ($KDNMR_{0.05}$). Late fourth instars did not appear to be responsive to a dose of 4 brain equivalents of L5D1 brain homogenates.

DISCUSSION

As reviewed in the Introduction and demonstrated by this study, there is evidence for a JHE inducing factor(s) produced by the head in prewandering 5th instar *T. ni* larvae, and JH seems to induce JHE in postwandering larvae. A previous study provided evidence that in 5th instars the natural pre- and postwandering haemolymph JHE activity, as well as postwandering JHE activity artificially induced by JH or juvenoid application, were all due to a single enzyme (SPARKS and HAMMOCK, 1979b). Based on similar isoelectric focussing data presented here, as

Table 5. Effect of brain or SOG implantation on the activity in neck-ligated larvae

Tissue	Age (day, hr) of donors and recipients*	Fold increase over sham†	nmol/min-ml III cleaved in recipient larvae	n‡
Brain	L4D2 11.5 ALO	2.2 ± 0.9§	7.2 ± 1.5	5
	L5D1 14.0 ALO	2.9 ± 1.3c§	12.9 ± 5.3	7
	L5D4 6.0 ALO	0.7 ± 0.2	6.8 ± 0.8	6
Suboesophageal ganglion	L4D2 11.5 ALO	1.8 ± 0.6§	8.7 ± 1.5	8
	L5D1 14.0 ALO	2.0 ± 0.6c§	10.4 ± 5.3	6
	L5D4 6.0 ALO	0.8 ± 0.2	7.1 ± 0.9	9
Brain, SOG disconnected	L5D1 14.0 ALO	4.6 ± 0.7b§	17.3 ± 2.3	6
Brain + SOG complex	L5D1 14.0 ALO	8.3 ± 2.6a§	30.6 ± 9.2	6

*Tissues were implanted 2 hr after ligation and the haemolymph was assayed 5 hr thereafter.

†Means followed by the same letter are not significantly different ($KDNMR_{0.05}$).

‡Each replication consists of a pool of haemolymph from 2 to 5 larvae.

§Significantly greater JHE activity than sham-operated control ($t_{0.05}$).

well as studies with three esterase inhibitors, this same esterase is apparently induced in isolated abdomens by head homogenates. These results should be directly applied only to *T. ni* at this time because general esterases contribute very little to JH hydrolysis in *T. ni* in contrast to *M. sexta* where they are reported to be moderately active (SANBURG *et al.*, 1975) and *G. mellonella* where they are very active (RUDNICKA *et al.*, 1979).

The inducing factor found in the brain and SOG by this study has not yet been characterized, and several possibilities exist as to its nature. (1) The tissues contain a toxin which causes histolysis of tissues containing JHE, (2) the tissue contains JHE, (3) the tissues contain JH which induce the observed JHE, (4) the tissue homogenates yield nutritional factors which induce JHE by preventing starvation effects, (5) the inducing factor is a neurohormone.

If the inducing factor is an intracellular toxin released by homogenisation, then it is unlikely that the implantation of the intact brain would induce JHE, which it did. The fat body in standard ligated larvae does not contain enough JHE to totally account for the haemolymph JHE induced, even if total cell lysis of this tissue occurred (WING *et al.*, unpublished). Dissection failed to reveal extensive fat body lysis and, should this phenomenon occur, one would expect to see high levels of cytosolic lactate dehydrogenase in the haemolymph which was not observed. Finally, for the brain content of the toxin to rise and fall concomitantly with the L4D2 and early fifth instar haemolymph JHE peaks would seem highly coincidental.

It is unlikely that the injected brain homogenates provided the JHE since the haemolymph JHE levels following injection of brain homogenates were at least 800 times greater than that which could be explained based on the brain or SOG content of JHE activity (unpublished observations). It is also unlikely that the induction was caused by JH in the brain homogenates since DAHM *et al.* (1976) stated that the CA are the only head organs which produce JH, and since JH will not induce JHE in prewandering larvae without another head factor (SPARKS and HAMMOCK, 1979a). It is similarly unlikely that brain homogenates are acting solely by supplying a key nutrient because the biomass injected was very small, homogenates of the prothoracic ganglia and abdominal ganglia were inactive, brain and SOG tissues from some stages were inactive, and implants as well as homogenates from critical stages were active.

Having eliminated the other four possible interpretations of the data, our working hypothesis is that the inducing factor is neurohormonal. This hypothesis is further supported by finding that the inducing factor from both the brain and the SOG, as well as the responsiveness of the fat body to this factor, correspond well with the JHE titre at those times when the head is thought to be involved in JHE regulation. This hypothesis is also based on preliminary evidence demonstrating the inducing factor's instability when exposed to heat or trypsin.

SPARKS *et al.* (1979a) noted that there appeared to be a small haemolymph JHE peak at late L4D2 but were hesitant to draw a firm conclusion. This study shows that the late L4D2 brain both contains (Fig. 2) and secretes (Table 5) an inducing factor. Also, the ligation data on late L4D2 shows that the head is involved in the appearance of JHE at this time. However, when late L5D1 brains were injected into late L4D2 hosts, there was no induction. This result is explained by noting the brain content of the inducing factor of late L5D1 larvae appears less than the smaller-sized L4D2 brains (Fig. 2). Further, the L5D1 implantation and homogenisation both indicate the total output of a secreting L4D2 brain is probably greater than its instantaneous brain content at homogenisation on L5D1. If the large amount of inducing factor secreted in a normal L4D2 can induce only a very small haemolymph JHE peak (SPARKS *et al.*, 1979a), it is not surprising that L5D1 brain homogenates gave no effect. Data of SPARKS and HAMMOCK (1979a) provided evidence that JH was the major head factor involved in inducing the postwandering JHE peak. Consistent with this conclusion is that injection of L5D4 5 hr ALO brain homogenates gave essentially no JHE increase over water-injected controls, though there was high JHE in the fat body at this time (WING, unpublished observations). The absence of the inducing factor along with high JHE in the fat body was also found in Day 1 pupae (Fig. 2; WING, unpublished observations).

There are data from several sources which evoke the hypothesis that a head inhibitory factor is involved in JHE regulation. In the present study, injection of L5D3 5 hr ALO (when natural JHE has fallen very low) brain homogenate gave a significant ($t_{0.05}$) reduction of JHE as compared with the water injected control (Fig. 2). Also, the implantation of L5D4 5 hr ALO brains or SOG (when postwandering JHE peak is declining) gave a significant ($t_{0.05}$) reduction in JHE 5 hr later as compared with the sham-operated control (Table 5). Thus, brains at some times may contain and release a factor which inhibits the appearance of haemolymph JHE. If an inhibitor is acting upon the JHE source during late L5D2 and early L5D3, its effect cannot remain for the postwandering JHE peak to occur on early L5D4 and, indeed, no such factor was detected in brain homogenates during late L5D3 to early L5D4 (Fig. 2). These data provide an explanation for the observation of SPARKS and HAMMOCK (1979a) where larvae neck-ligated on L5D2 (during the haemolymph JHE decline) had more JHE 12 hr later than unligated larvae. Also, REDDY *et al.* (1979) found that ligated Day 0 last instar *G. mellonella* showed a rise in haemolymph JHE on Day 4 (as in normal larvae) whereas starved, unligated control larvae did not. In these ligation studies, the higher JHE in ligated larvae cannot be due to ligation-induced fat body autolysis since the data in Table 1 do not indicate such a ligation-induced artifact.

One unexpected result is that the factor was detected in both lateral and medial regions of the brain as well as the SOG (Table 4). The induction is clearly not an artifact of nervous tissue, and involvement of more than one neural region is not unheard of in insect endocrinology. The roles of these various neural regions in production, transport storage and release should be elucidated. The very high level of induction following implantation of the brain–SOG complexes (Table 5) supports a hypothesis of production,

transport, and release involving both nervous centres, though other hypotheses are still plausible.

A summary of our current hypothesis for JHE regulation in *T. ni* is that in the late fourth instar the head produces a factor which causes the relatively insensitive fat body to release only a small amount of JHE. Possibly this inducing factor primes the fat body to subsequently respond to inducing factor released during the fifth instar by producing large amounts of JHE. During late L5D1, increasing amounts of inducing factor from the head stimulate an increasingly sensitive fat body to produce and release JHE culminating in the prewandering haemolymph JHE peak on early L5D2. During the early wandering stage, the brain produces decreasing amounts of inducing factor while production of an inhibin decreases the sensitivity of the fat body to the residual inducing factor. The fat body becomes increasingly sensitive to induction by JH during the postecdysone release period. The early prepupal burst of JH leads to the production and release into the haemolymph of the postwandering peak of JHE. A late prepupal inhibitory factor secreted by the brain and SOG may then prevent further release of JHE.

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