CHOLINESTERASE IN THE BRAIN OF THE CECROPIA SILKMOTH DURING METAMORPHOSIS AND PUPAL DIAPAUSE[1]

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In giant silkmoths, pupal diapause results from the failure of neurosecretory elements in the brain and corpora cardiaca to provide the hormonal stimulus required for initiation of further development; the latter resumes, months later, when the neurosecretory system recovers its function after exposure of the pupa to appropriate temperatures and photoperiod (Williams, 1946, 1952, 1956; Williams and Adkisson, 1964). For further resolution of the control of diapause and development, a key problem is to define the physiological processes, within the brain, that establish and later efface its endocrine impotence.

As one aspect of this problem, we have examined the behavior of cholinesterase (ChE) in the brain of the Cecropia silkmoth. Our inquiry is based on the findings of Van der Kloot (1955), who reported a disappearance of ChE and spontaneous electrical activity in the brain at the time of pupation, and their subsequent reappearance together with the brain's recovery of endocrine activity. The changes were found to be limited to the brain, since the thoracic and abdominal ganglia retained normal levels of enzymatic and electrical activity throughout diapause. On the basis of the close temporal correlations observed, and the possible functional roles of ChE, Van der Kloot recognized that its behavior could account for the neuroendocrine inactivation and reactivation of the brain. As is apparent from current reviews on insect endocrinology and development (*e.g.,* Wigglesworth, 1964; Gilbert, 1964), these observations have remained the most promising leads to date on the control of neurosecretion and diapause in lepidopterous insects.

However, the aforementioned conclusions have recently been questioned as a result of two brief reports (Schoonhoven, 1963; Tyshtchenko and Mandelstam, 1965) containing electrophysiological observations supplemented by certain limited enzymatic data. The observations were made on diapausing pupae belonging to several families of Lepidoptera, and including a number of Cecropia. Since the electrophysiological findings described in these two papers conflict with one another, as well as with the observations of Van der Kloot, the net result has been to create a rather uncertain picture of the extent of neural activity during diapause. From the biochemical standpoint the picture is somewhat clearer but still equivocal. Through the use of a manometric technique, Schoonhoven detected hydrolytic activity in brains of diapausing pupae of a geometrid moth, *Bupalus piniarius* L.; Tyshtchenko and Mandelstam, by histochemical methods, detected hydrolytic activity in brains of diapausing pupae of the silkmoth *Antheraea pernyi* Guer. These

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activities were ascribed to ChE. Though rather suggestive of ChE, both reports omit mention of the biochemical details and controls required to confirm that the activity was in fact enzymatic, and that it was due to the action of ChE rather than that of other esterases known to occur in insect brain.

In the present study, we demonstrate by histochemical and quantitative methods that substantial ChE activity persists throughout diapause in brains of Cecropia and other silkmoths. Our findings provide comparative biochemical information on the properties and specificity of ChE in silkmoth brain, and show that diapause cannot be attributed to generalized absence of ChE, as seemed possible heretofore. But, for reasons to be discussed subsequently, the present findings do not preclude a regulatory role for one or more forms of esterase in the control of neurosecretion and diapause. These findings have been announced previously in abstract (Shappirio, Eichenbaum and Locke, 1965).

Materials and Methods

1. *Experimental animals*

Brains from the following species of silkmoths were used: *Hyalophora cecropia* (L.), *Samia cynthia* (Dru.), and *Antheraea polyphemus* (Cram.). For convenience, these will be identified henceforth as Cecropia, Cynthia, and Polyphemus, respectively. Most of the Cecropia were reared by us under nylon nets on wild cherry trees. Other Cecropia, as well as all the Cynthia and Polyphemus, were purchased from dealers in the northeastern and midwestern United States. The insects were managed as described previously (Williams, 1946; Shappirio and Williams, 1957). In addition to larvae and prepupae, we utilized: (a) "unchilled pupae" kept at 25° C., in which diapause was found to persist for more than five months after pupation; (b) "chilled pupae" stored at 6° C. to favor the prompt return of endocrine activity, followed by the resumption of development, upon their return to 25° C.; and (c) "developing adults" derived from previously chilled pupae allowed to terminate diapause at 25° C. Developing adults were staged by use of the morphological and physiological criteria summarized by Schneiderman and Williams (1954). In additional experiments, adult moths were used four days after their emergence at 25° C.

2. *Initial preparation of tissue*

For histochemical and quantitative studies, brains were excised from insects anesthetized in carbon dioxide (Williams, 1946) and then briefly rinsed in Ringer (Ephrussi and Beadle, 1936). In the case of larvae and prepupae, as well as diapausing pupae, the optic nerves were severed just distal to the melanin granules lying at their base; other nerves were severed as close as possible to the surface of the brain. In experiments on developing adults and adults, the optic lobes and tracts were included with the brain itself, but antennal and other nerves were transected at the surface of the brain and thus excluded from analysis. Also excluded was the subesophageal ganglion, by means of transverse cuts midway across both roots connecting this ganglion to the brain. Further details on tissue preparation are provided below.

3. *Histochemistry*

The size and fragile texture of silkmoth brains posed problems in tissue preparation which were sufficiently overcome only after extensive trials with various techniques. The methods giving most reliable results are described here. Brains were in certain cases prefixed in 10% formalin (pH 7.0) for 1–2 hours at 2° C., and then rinsed in Ringer for an equal time. Alternatively, postfixation was employed as a variant in technique and to control for possible formalin-induced fixation artifact; postfixation was accomplished by exposure of cryostat sections, mounted and sectioned as described below, to acetone at 2° C. for one hour before staining.

To provide a suitable matrix for sectioning, unfixed or fixed brains were embedded in small pieces of fresh mouse liver (approximately 5-mm. cubes) and immediately frozen in isopentane cooled to a viscous state by liquid nitrogen. The resulting frozen block was then sectioned in a Universal cryostat (−15° C.) at thicknesses of 8–10 μ. When possible, serial sections were placed in sequence on several microscope slides.

For detection of ChE activity in cryostat sections, we employed Gomori's (1952) modification of the Koelle (1951) method. In this procedure, acetylthiocholine (AThCh), a thioester analogous to acetylcholine (ACh), serves as substrate. Hydrolysis of the thioester yields thiocholine, which is precipitated at sites of reaction in the form of copper thiocholine sulfate; the latter is then converted to copper sulfide for easier visualization of reaction sites within the section. In our studies, an incubation time of 55–75 minutes at 20° C. proved optimal. After staining and conversion to copper sulfide, sections were lightly counterstained with aqueous Ehrlich hematoxylin, and mounted in glycerin jelly.

In histochemical studies involving inhibitors, cryostat sections were incubated for 30 minutes in Ringer containing the desired inhibitor concentration, before exposure to reaction medium which also contained inhibitor at this concentration. Control sections, serial when possible, were incubated and stained in parallel but without inhibitor.

4. *Quantitative enzymatic methods*

To obtain more detailed information on the properties of ChE in Cecropia brain, and to survey its behavior during the life history, we exploited the sensitive spectrophotometric method introduced by Ellman (Ellman *et al.*, 1961). This procedure also uses AThCh as substrate. Thiocholine generated by hydrolysis reacts with 5,5′-dithio-*bis*-2-nitrobenzoate (DTNB), incorporated in the reaction medium, to yield a bright yellow color attributable to the thionitrobenzoate anion. The reaction was followed at 412 mμ by means of a Beckman Model DU spectrophotometer. Rapid assay of ChE activity in individual brains was possible with this method.

In most experiments, each freshly excised brain was homogenized in a microsize tissue grinder kept at 0° C., to yield 0.5 ml. of homogenate in 100 m*M* potassium phosphate, pH 8.0. This volume was adequate for duplicate or triplicate assays. In routine measurements of ChE activity in brains from animals at successive stages in the life history, we used a reaction volume of 1.02 ml. at 25° C., containing the following reagents at the final concentrations shown: 0.75 m*M* AThCh; 1.0 m*M* DTNB; 100 m*M* potassium phosphate, pH 8.0; and homogenate.

For other types of experiments, designed to examine the effects of pH, substrate concentration, and other factors, this protocol was modified as appropriate for the individual experiments noted under Results.

In experiments using inhibitors, the reaction was initially followed for 5 minutes in the absence of inhibitors, after which inhibitor was added to yield the desired final concentration. When the reaction had stabilized, which occurred within 5 minutes, the rate was recorded for a further 5 minutes. In all experiments, correction was made for changes in absorption due to endogenous thiols, which were very slight. Correction was also routinely made for changes in absorption due to non-enzymatic hydrolysis of substrate, which was 10% or less of the total reaction rate under routine conditions of assay described in the preceding paragraph. Suitable experiments established that the reaction rate, thus corrected, was proportional to the enzyme concentrations used, was linear during the period of assay, and was limited by the hydrolytic step of the reaction rather than by the steps involved in color development. Since pH 8.0 lies close to the limit of buffering action of the $HPO_4^{=}/H_2PO_4^{-}$ system, we also verified that this pH was maintained within 0.05 unit in the course of reactions at the most rapid rates encountered in this study.

5. *Chemicals*

AThCh and its homologues, propionylthiocholine and butyrylthiocholine (PrThCh and BuThCh, respectively), were purchased as the iodides from Sigma Chemical Co., St. Louis, Missouri. DTNB, eserine sulfate, and *tris*-(hydroxymethyl)-aminomethane (Tris buffer) were also Sigma products. The two Burroughs Wellcome anticholinesterase compounds, 62C47 and 284C51j, were generously furnished by Burroughs Wellcome, Inc., Tuckahoe, N. Y. These code names denote, respectively, 1.5-*bis*-(4-trimethylammoniumphenyl)-pentane-3-one diiodide, and 1,5-*bis*-(4-allyldimethylammoniumphenyl)-pentane-3-one dibromide. *Iso*-OMPA (tetraisopropyl pyrophosphortetramide) was obtained from Koch-Light Labs., Colnbrook, Bucks., England. Other chemicals were of analytical reagent grade.

Results

1. *Histochemical observations*

Many general microanatomical features of the pupal brain in Cecropia are typical of the arthropod brains and central ganglia described in the treatise of Bullock and Horridge (1965). The pupal brain is bilobate in structure, with most cell bodies being located peripheral to large regions of neuropile. The latter consists of fine nerve fibers and cytoplasmic processes of glial cells. It constitutes the principal region of synaptic contact, and occupies substantial regions of the central, lateral, and ventral portions of each lobe, as well as the interior part of the tissue connecting the lobes.

When formalin-prefixed or acetone-postfixed cryostat sections were treated as described under Methods, an intense deposit of histochemical reaction product was observed in neuropile, as illustrated in Figure 1. Except for differences in fragmentation of tissue, which was greater after formalin treatment, prefixation and

postfixation yielded an identical histochemical pattern. No reaction product was observed when AThCh was omitted from the reaction medium. Similar findings were made in each of 15 unchilled and chilled Cecropia pupae which were judged to be diapausing in terms of physiological and morphological criteria (Schneiderman and Williams, 1954).

With our material, the histochemical method did not afford sufficient resolution to permit us to ascertain the localization of ChE more intimately in the neuronal or glial elements of neuropile. However, in favorable preparations, reaction product was visualized in bands which apparently correspond to major prevailing directions of fiber tracts.

Initial insight into the specificity of the histochemical reaction in the neuropile was gained with two agents, eserine sulfate and *iso*-OMPA; the former is a general

FIGURE 1. Section of brain from diapausing pupa, stained for ChE. The photograph depicts an 8 μ cryostat section, postfixed in acetone, which was stained histochemically using AThCh as substrate and counterstained with Ehrlich hematoxylin. The darkly stained region contains histochemical reaction product (CuS) and shows the localization of ChE activity ($\times$ 72).

inhibitor of ChE's and the latter is relatively selective for mammalian butyrylcholinesterase (BuChE) when applied at appropriate concentrations. In brains of unchilled and chilled Cecropia pupae, the neuropile-associated esterase was found to be abolished by eserine sulfate at 10^{-5} *M* and to be unaffected by *iso*-OMPA at 10^{-4} *M*.

Our observations on brains of post-diapausing Cecropia are limited in number, but permit several conclusions. When pupae chilled for 16 weeks were placed at 25° C., a period of 7–10 days elapsed before the first externally visible sign of adult development, namely, retraction of the leg epithelium from its overlying cuticle. This signals the second day of adult development (Schneiderman and Williams, 1954). In the present study, the histochemical pattern was found to remain identical with that encountered in brains of diapausing pupae, when obser-

vations were made during the first four or five days after transfer of the chilled pupae to 25° C. Subsequently, an increased area of deposition of reaction product was observed, accompanied by a slight though seemingly significant increased intensity of staining in neuropile. At the outset of adult development, esterase activity was no longer confined to neuropile, but had spread laterally and ventrally to encompass regions of neuronal or glial cell bodies. The esterase reaction in post-diapausing insects was inhibited fully by eserine sulfate at 10^{-5} *M*.

2. *Properties of ChE in pupal brain as revealed by quantitative methods*

In order to provide a meaningful basis for evaluating the behavior of ChE in relation to the onset and termination of pupal diapause, and to afford comparative biochemical insight, experiments were carried out to define optimal conditions of assay for ChE and to determine the specificity of the enzyme or enzymes detected.

a. *Effect of pH on ChE activity*

A series of measurements was performed on the same homogenate of brains pooled from unchilled diapausing pupae of Cecropia, using 100 m*M* phosphate or Tris–HCl buffers in the range pH 6.5 to 9.0. Although the activity with Tris was invariably lower than with phosphate (see below), in both cases enzyme activity increased from pH 6.5 to 8.0 but showed little increase in the range 8.0 to 9.0. For spectrophotometric assays, pH 8.0 was selected since this afforded a maximal rate with relatively low non-enzymatic hydrolysis of AThCh. The rate of the latter reaction increases markedly with further increase in pH, and at pH 9.0 exceeds the enzyme-catalyzed rate when the latter is kept within a range appropriate for meaningful assay. In experiments involving different pH's, suitable controls confirmed that the absorptivity of the thionitrobenzoate anion was essentially constant between pH 6.5 and 9.0.

b. *Effect of composition and concentration of certain buffers*

At pH 8.0, the reaction rate was found to be essentially similar when assays on the same homogenates were carried out in the presence of 100 m*M* phosphate or 70 m*M* bicarbonate (the latter charged at 25° C. with 5% carbon dioxide in oxygen). Only one-quarter of this rate was observed with 100 m*M* Tris at the same pH. The lower rate with Tris appears to be an inhibition rather than a failure of activation, since combination of phosphate and Tris did not elevate the rate above that observed with Tris alone. At 10 m*M*, the rate with phosphate was nearly 50% lower than at 100 m*M*. In view of these findings, a concentration of 100 m*M* was used in routine assays.

c. *Relationship of ChE activity to substrate concentration*

To the best of our knowledge, this important relationship has not been considered in previous studies on ChE in silkmoth brains. Figure 2 illustrates the striking dependence of ChE activity upon the concentration of the substrate, AThCh. A final substrate concentration of 0.75 m*M* was found to yield the most rapid reaction rate (Fig. 2). Above this concentration, one can readily observe that

activity decreases progressively, yielding a graph of a shape typical for acetylcholinesterases (AChE's) in vertebrate and invertebrate preparations (Augustinsson, 1949, 1963).

In order to permit comparison between data obtained under conditions found optimal for assay of ChE in the present study, and the results of Van der Kloot's (1955) investigation, the substrate-activity curve for pupal brain ChE was also

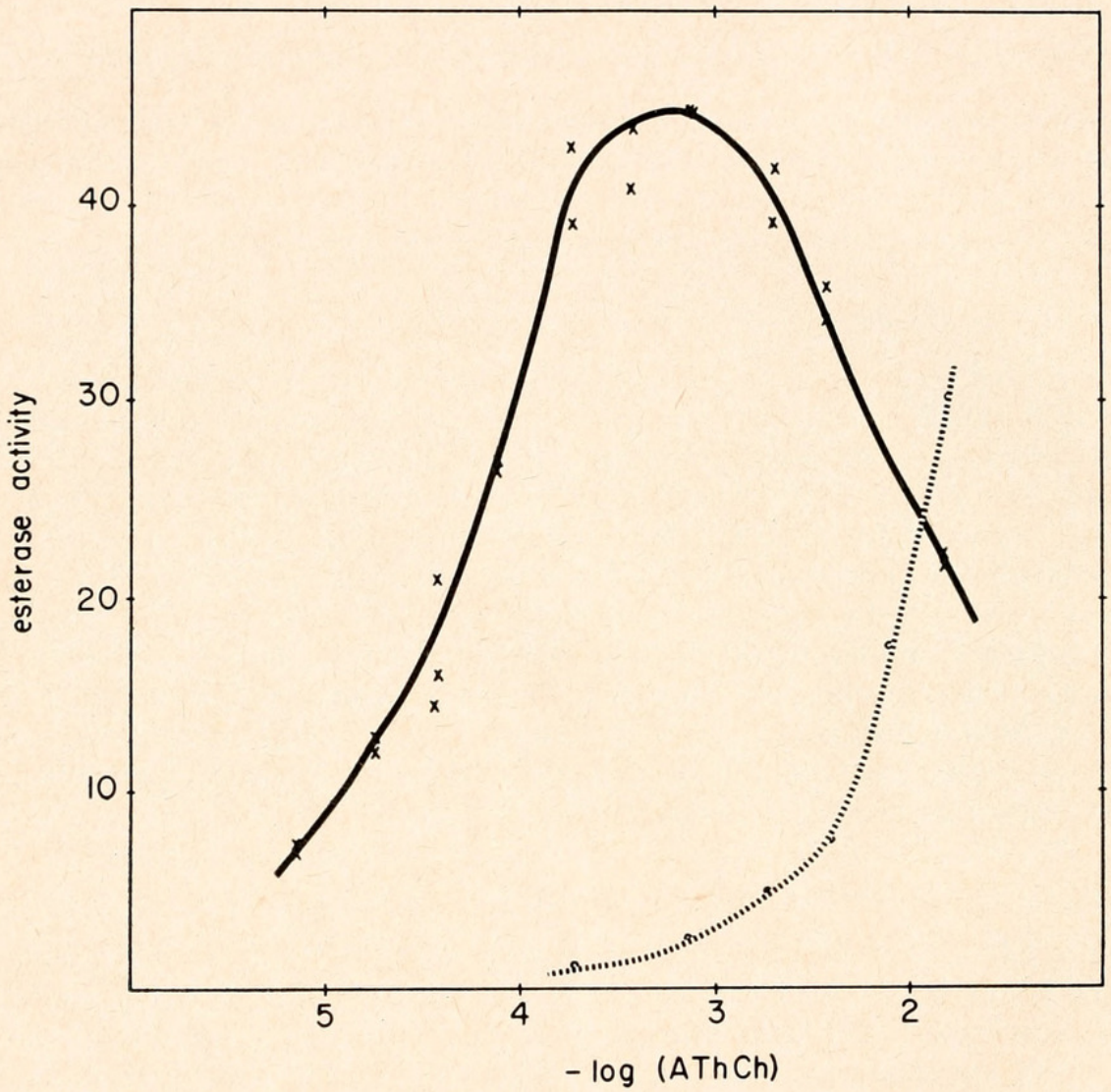

FIGURE 2. ChE activity as a function of AThCh concentration. The solid line describes enzymatic activity, which was corrected for spontaneous hydrolysis of AThCh at substrate concentrations above 10^{-4} *M*. The broken line describes the rate of spontaneous hydrolysis of AThCh. To provide sufficient material for the experiment illustrated, brains of six unchilled Cecropia pupae were pooled before homogenization. Enzyme activity is shown in relative units: one unit corresponds to a change in absorbance of 0.001 per minute.

determined under our "routine" assay conditions, but supplemented by sodium chloride to yield a final concentration of 0.5 *M*. The latter had been employed in Van der Kloot's experiments owing to the findings of Chadwick *et al.* (1953) showing that it yielded optimal ChE activity in homogenates of brains from flies. Our experiments with Cecropia showed that the substrate-activity curves with and without extra salt possess similar shapes. However, the addition of salt alters

both the optimal substrate concentration and the reaction rate at any given substrate concentration. In the presence of added salt, the substrate optimum was slightly higher, and when comparisons were made at the substrate optima with and without salt, that in salt was 22% lower.

d. *Effects of selective esterase inhibitors*

Information on the specificity of the ChE reaction in pupal brains was gained with a variety of agents known to be selective on the basis of studies with vertebrate and invertebrate preparations. The histochemical observations, described earlier, were confirmed by the finding that eserine sulfate at 10^{-5} *M* completely abolished enzymatic activity. The Burroughs Wellcome compounds, 62C47 and 284C51j, also inhibited the reaction fully when tested at final concentrations of 10^{-6} *M*. In contrast, *iso*-OMPA at 10^{-4} *M* failed to inhibit ChE in homogenates of brain from unchilled and chilled pupae.

Additional studies with inhibitors were carried out on brains from larvae, prepupae, developing adults, and adult moths. In all cases, eserine at 10^{-5} *M* was fully inhibitory. The agents 62C47 and 284C51j also fully inhibited ChE in larval brains and in brains from animals at the outset of adult development, when tested at 10^{-6} *M*. *Iso*-OMPA at 10^{-4} *M* was non-inhibitory in larvae, prepupae, and developing adults at early stages; but the brains of adult moths were slightly affected, a concentration of 10^{-4} *M* inhibiting their ChE by 8–10%.

e. *Reactivity of brain ChE with homologues of AThCh*

Figure 3 illustrates the relative reaction rates obtained at a graded series of concentrations of PrThCh and BuThCh. The homogenate used for the experiment illustrated, which gave typical results, was derived from the pooled brains of unchilled pupae. The same homogenate exhibited a rate of 24 (relative units as in Fig. 3) when examined with AThCh at 0.75 m*M*. Thus, the reaction rate decreases in the order: AThCh > PrThCh > BuThCh. Although only a limited range of concentrations was studied with the substrate homologues, it is clear that the substrate-activity curve for PrThCh resembles that for AThCh in showing a marked optimum substrate concentration, above which the reaction rate progressively decreases. Moreover, the position of the substrate optimum for PrThCh is rather close to that described (Fig. 2) for AThCh. In the range studied, reaction rate increased slightly with increase in BuThCh concentration, but showed no clear optimum. It was not possible to carry the studies with BuThCh to higher concentrations than are shown in Figure 3, inasmuch as the non-enzymatic breakdown of BuThCh became excessive when its concentration was increased further. The reactions with PrThCh and BuThCh were found to be fully sensitive to eserine at 10^{-5} M.

3. *ChE activity in Cecropia brain at successive stages in metamorphosis and during diapause*

In order to permit observations on as homogeneous a sample of insects as possible, ChE activity was determined in individuals of the same batch of Michigan-

reared Cecropia. Our 1964 crop, used for this purpose, was especially suitable since the proportion of animals emerging precociously from pupal diapause was unusually low; only two individuals out of more than 800 initiated development and emerged as moths during the first 6 months after pupation, without prior chilling at 6° C.

Table I summarizes measurements of ChE activity in 46 individual brains from animals at successive stages in the life history, ranging from late in larval life through and beyond pupal diapause to the initial phase of adult development. All measurements were made under the routine conditions of assay described under Methods. As shown in the Table, ChE was readily detectable at all stages examined. Of special interest is the finding that ChE activity undergoes no decline

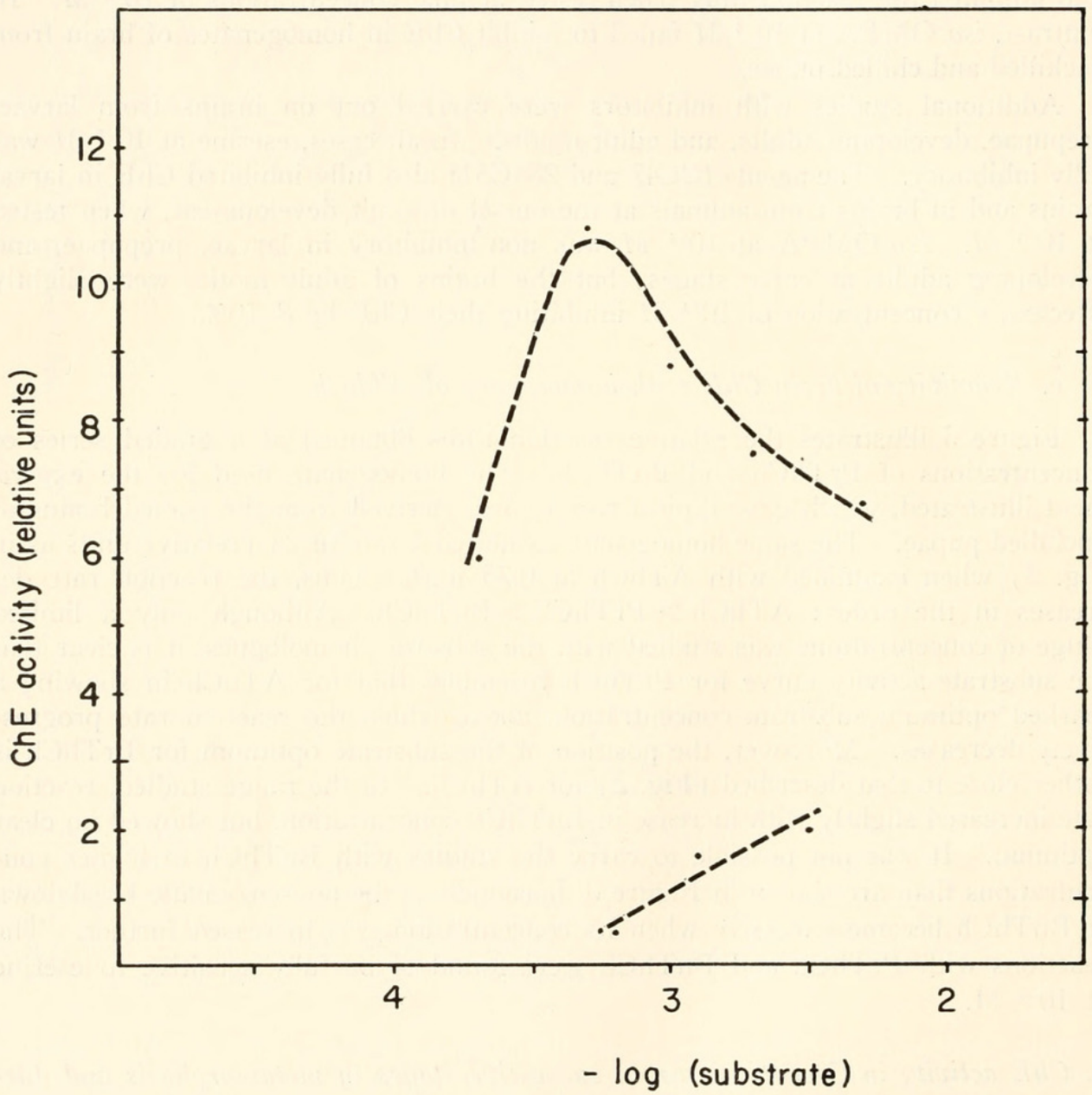

FIGURE 3. ChE activity as a function of PrThCh and BuThCh concentrations. The upper line describes activity for PrThCh; the lower line relates to BuThCh. The activities have been corrected for spontaneous hydrolysis of substrates. Enzyme activity is shown in relative units: one unit corresponds to a change in absorbance of 0.001 per minute.

TABLE I

ChE activity in brain homogenates of Cecropia

Source of brain	Number studied	Enzyme activity* (μmoles AThCh/ brain-hr.)	Range	Remarks**
Larvae	4	0.24 ± 0.05	0.20–0.35	Late 5th instar, feeding
Prepupae	4	0.24 ± 0.02	0.22–0.28	Several hours before pupation
Fresh pupae	4	0.29 ± 0.03	0.24–0.33	2–22 hours after pupation
Unchilled pupae	6	0.32 ± 0.05	0.22–0.38	1–9 days after pupation
Unchilled pupae	8	0.33 ± 0.08	0.20–0.42	4–12 weeks after pupation
Chilled pupae	5	0.25 ± 0.03	0.22–0.31	8 weeks after pupation; then 10 weeks at 6° C.
Chilled pupae	8	0.58 ± 0.04	0.51–0.62	8 weeks after pupation; then 13–20 weeks at 6° C.
Developing adults	7	0.60 ± 0.05	0.51–0.69	2nd day of development

* Mean activity ± average deviation.
** Except where shown, all animals maintained at 25° C.

around the time of pupation, when the neurosecretory system becomes inactive, and when ChE was previously reported to undergo precipitous disappearance (Van der Kloot, 1955). It seems clear from Table I that except for possible minor fluctuations, an essentially unchanging level of ChE activity persists in the newly pupated animal, in unchilled pupae for at least 12 weeks after pupation, and in pupae chilled up to 10 weeks at 6° C. In several of the groups of unchilled pupae, one or two of the animals studied exhibited activity substantially higher or lower than the mean recorded in Table I, but no upward or downward trend in activity was noted during the indicated time intervals after pupation.

Table I also provides evidence for a rise in ChE activity during more prolonged chilling at 6° C. In each of eight pupae, examined at intervals after periods of chilling ranging from 13 to 20 weeks, the level of activity was at least double the mean activity recorded after ten weeks' chilling. Evidently the rise in activity occurred between the 10th and 13th weeks in this batch of animals. No trend of change in activity was noted between the 13th and 20th week. The elevated level was found to persist when chilled pupae were returned to 25° C. and allowed to initiate adult development (Table I). At all stages shown in Table I, the enzymatic activity was fully inhibited by eserine at 10^{-5} *M*. An AThCh concentration of 0.75 m*M* was found to be optimal in assays on larval and developing adult brains, as had earlier been established for pupae.

A total of 50 Cecropia pupae from the same batch as those just considered was returned to 25° C. after 16 weeks of chilling, and was used to provide animals at successive stages during the maturation of the adult moth after termination of diapause. As shown in Figure 4, ChE activity undergoes a six-fold increase during this period, when expressed on a "per brain" basis. In the first two weeks of adult development, activity rises progressively, but the high level thus attained persists without large change until the time of adult emergence. For unexplained reasons, a large variation in activity was observed in the later phases of adult development and in adults.

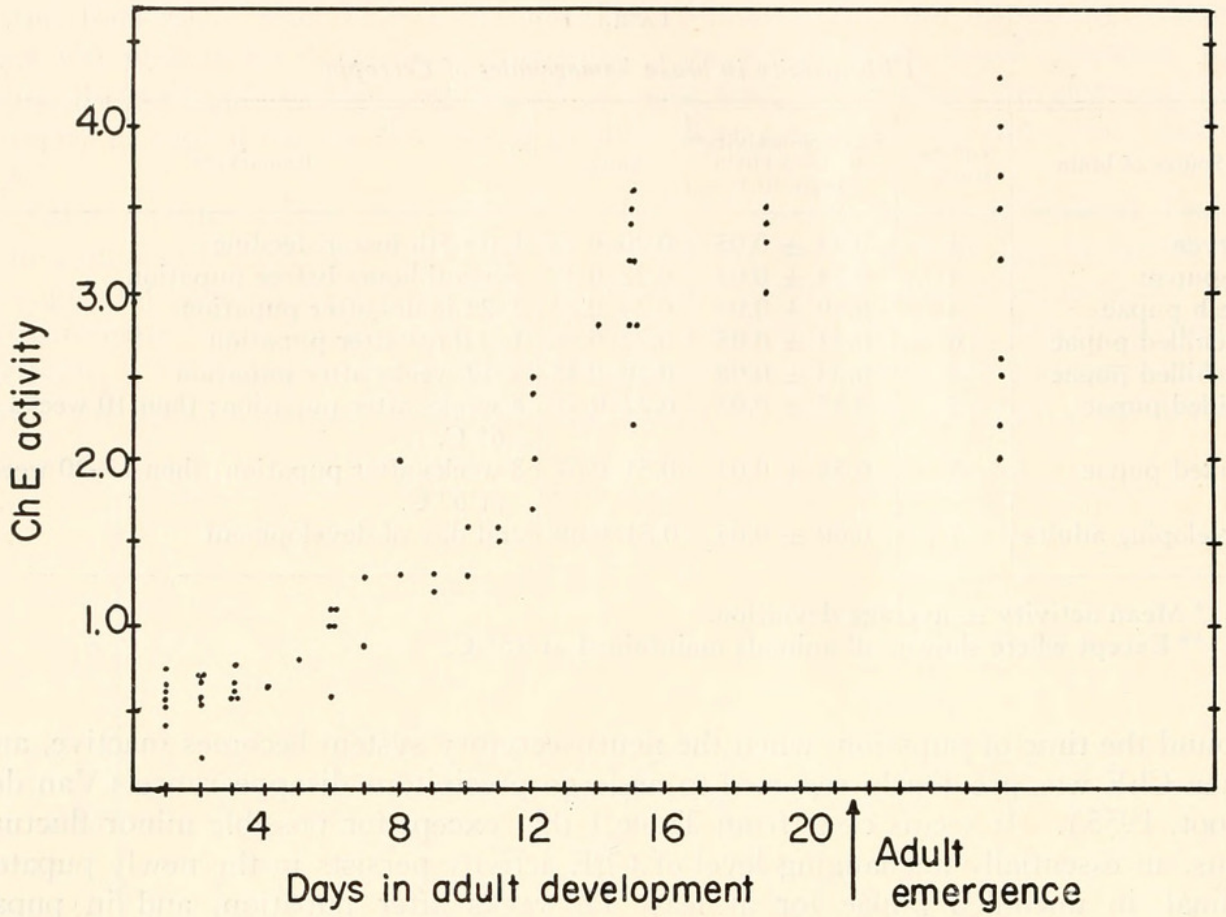

FIGURE 4. ChE activity in individual brains from developing adult and adult Cecropia. Each point represents the average of duplicate determinations on one brain. Enzyme activity is shown as micromoles AThCh per brain-hour. These units of activity are equivalent to those summarized in Table I.

4. *ChE activity in brains from various species of silkmoths*

The preceding assays were carried out on brains from the same batch of Michigan-reared Cecropia. To minimize the possibility that these data were exceptional, additional measurements of ChE activity were carried out, using several species of silkmoths. In more than three dozen additional unchilled and chilled Cecropia from different sources, ChE activity was encountered at approximately the same levels recorded in Table I. Activity was also detected in unchilled Cynthia pupae at about the same levels as in Table I. In unchilled Polyphemus pupae, the activity was somewhat lower, but still detectable. Measurements on Cynthia and Polyphemus were made using a final AThCh concentration of 0.75 m*M;* all activities were fully inhibited by eserine sulfate at 10^{-5} *M*. These observations provide additional evidence for essentially unchanging ChE activity in pupae stored at 25° C. or for at least two months at 6° C.

DISCUSSION

1. *Characteristics of ChE in silkmoth brain*

a. *Identification as AChE*

Throughout most of the life history, the enzymatic activity detected by histochemical and quantitative methods exhibits properties tentatively attributable to

AChE. This conclusion follows from the sensitivity displayed to certain inhibitors, from the reactivity toward thioester homologues, and from the substrate-activity curve obtained using AThCh. The Burroughs Wellcome agents, 62C47 and 284C51j, are strongly inhibitory and selective for vertebrate AChE's (Augustinsson, 1963), and also preferentially inhibit several ChE components of arthropod central nervous systems that have properties similar to vertebrate AChE's (Wigglesworth, 1958; Maynard, 1964). The lack of sensitivity to *iso*-OMPA reinforces this finding, since *iso*-OMPA is known to be relatively selective for vertebrate BuChE's including the pseudocholinesterase of mammalian brain (Aldridge, 1953; Austin and Berry, 1953; Pepler and Pearse, 1957). Further evidence for AChE in the case of Cecropia derives from the finding that activity is much lower toward BuThCh than toward AThCh. Finally, the pronounced reduction in activity at the higher concentrations of AThCh and PrThCh which we employed (Figs. 2 and 3) is typical of AChE's from vertebrate sources (Augustinsson, 1949, 1963). The combination of properties on the part of the Cecropia brain esterase is similar to that described for brain ChE in several insect groups (Gilmour, 1961). Insect ChE's possess properties that do not always lend themselves to convenient classification using vertebrate-based terminology (Chadwick, 1963). Thus, despite the similarities of Cecropia brain ChE to AChE, further study may reveal differences, and we use the term AChE with reservation.

In section 2.d of Results was mentioned the finding that ChE activity in adult brain includes a small but significant fraction which is sensitive to *iso*-OMPA at 10^{-4} *M*. This contrasts with the insensitivity to *iso*-OMPA at earlier stages in the life history, and suggests that an additional ChE component, perhaps with different specificity characteristics from AChE, may appear in the brain during the later phases of adult development.

b. *Changes in AChE during metamorphosis*

On the basis of the present study, the only major changes in AChE activity from the time of pupation through most of adult development are: (a) the two-fold rise in activity which occurs during chilling at 6° C.; and (b) the larger increase in activity that accompanies the growth and morphogenesis of the adult brain. With regard to the former, evidence is insufficient to determine whether the rise is attributable to fabrication of new elements within neuropile and concomitant synthesis of new ChE, or to enhanced titer of ChE within existing neuropile, or to other factors. In any event, the rise in activity during chilling appears to represent the earliest biochemical signal so far reported of the brain's change in neuroendocrine status, which occurs during storage at the low temperature (Williams, 1946, 1956). With regard to the increase of ChE during adult development, it seems most probable that the enzymatic changes in large part mirror the extensive morphogenetic events occurring at this time. A study of the behavior of brain esterases during adult development may yield useful information on changes at the cellular and subcellular levels underlying the physiological and morphological maturation of the adult brain.

c. *Localization of ChE in pupal brain*

In showing the presence of ChE in neuropile of pupal brain, our histochemical findings agree with those of Wigglesworth (1958) on the bug *Rhodnius prolixus,*

and those of Salkeld (1961) on the milkweed bug, *Oncopeltus fasciatus*. In the former study, Wigglesworth concluded that staining in the neuropile, with AThCh as substrate, was largely confined to the glial elements. As used by us with Cecropia pupae, resolution of the histochemical method was insufficient to permit critical assessment of this important point. Wigglesworth also observed staining outside the neuropile when other esterase substrates were used, and his overall results supported the view that several esterases were present in the brain as a whole. Further information regarding multiple esterases and their potential significance will be presented in a subsequent section of this Discussion.

Wigglesworth (1958) noted in the first detailed histochemical study on localization of ChE in the brain of an insect that the restriction or near-restriction of AChE activity to neuropile shows certain kinship with the histochemical picture derived from studies on amphibians. Thus, in many regions of frog brain (*Rana pipiens*), heaviest staining due to AChE is found in areas rich in synaptic terminations and poor in cell bodies. However, in other areas of brain, neuron cell bodies also possess high ChE activity (Shen, Greenfield and Boell, 1955; Koelle, 1963). This situation in the frog differs markedly from that encountered in mammalian brains, as typified by rat or cat, in which AChE is abundant in neuron perikarya as well as in axonal and dendritic processes (Koelle, 1954; Pepler and Pearse, 1957). The localization of ChE in regions of synaptic contact has repeatedly prompted the suggestion that it is functionally involved in transmission; one can speculate similarly regarding the AChE which we detect in neuropile of Cecropia brain, but meaningful judgment on this point awaits further ultrastructural and physiological evidence.

2. *Analysis of present findings in relation to previous studies on Cecropia*

The results of the present study clearly show that substantial ChE activity persists in silkmoth brain throughout metamorphosis and diapause. Our findings therefore contrast with those of Van der Kloot (1955) in which ChE was not detected during diapause, but provide detailed evidence in favor of the conclusions suggested by Schoonhoven (1963) and Tyshtchenko and Mandelstam (1965) to the effect that ChE persists during the pupal diapause of lepidopterous insects.

Some of the discrepancy between our findings and those of Van der Kloot can be resolved if one considers certain properties of ChE in pupal brains as revealed in the course of the present study. It is worth noting that our spectrophotometric method has about the same sensitivity as the manometric technique used by Van der Kloot, since the minimum activity detectable by our method is close to that stated by Van der Kloot (0.05 micromoles substrate hydrolyzed per brain-hour). Although we have not compared the velocities of the ChE reaction in Cecropia with respect to AThCh and ACh, it would not be surprising if the rate were higher with AThCh, as this has been reported from time to time in the literature. Thus choice of substrate may have contributed to our success in detecting enzymatic activity during diapause. However, we believe that other factors are more significant, as stated below.

We find that ChE activity in homogenates of Cecropia brain declines by as much as 15% per hour in 100 m*M* phosphate buffer at pH 8.0 and 0° C., and more

rapidly at 25° C. The activity also declines slowly when homogenates are stored in the frozen state at −15° C.; about 20% is lost after one week's time. The loss in activity would be significant in the case of manometric assays, which ordinarily require one-half to one hour, but much less significant in our spectrophotometric assays, which were completed within ten minutes. Thus, in the earlier study, it appears likely that much activity would have been lost during the 1–40-day periods of frozen storage prior to analysis, and during the manometric assays themselves.

There is ample reason to believe that our studies with AThCh yield information with regard to substrate preferences, substrate-activity relationships, and inhibitor sensitivities, that would also apply for ACh, as used in Van der Kloot's (1955) study. Our confidence derives from a number of studies in which the behavior of AThCh and ACh was compared, using the same ChE preparations (Heilbronn, 1959; Bergmann, Rimon and Segal, 1958; Ellman *et al.*, 1961). Thus we suspect that the 15 m*M* concentration of ACh used by Van der Kloot was supraoptimal for AChE and inhibitory in effect. This follows from our finding that the optimal AThCh concentration for pupal brain homogenates is only 0.75 m*M*, and that activity is markedly reduced at higher concentrations in the manner typical of AChE's (Fig. 2). Literature comparing substrate optima for AThCh and ACh (Heilbronn, 1959; Bergmann, Rimon and Segal, 1958; Ellman *et al.*, 1961), though based on vertebrate preparations, shows the optima to be much closer than the 20-fold difference separating our routine value for AThCh from that used by Van der Kloot (15 m*M*) for ACh. Moreover, in our experience the 0.5 *M* sodium chloride concentration incorporated in Van der Kloot's assay media yields a reaction rate lower than with buffer alone, when rates at substrate optima are compared. This conclusion concerning the effect of added salt conforms in large measure to that of Wolfe and Smallman (1956) on brain ChE from flies, with ACh as substrate. In the light of these arguments, and those of the preceding paragraph, we conclude that the potential activity of ChE in brains from diapausing pupae was not attained in earlier studies on Cecropia.

3. *Neurophysiological status of the brain during pupal diapause*

The present findings clearly show that at least one form of ChE persists in neuropile throughout diapause. It now becomes important to know its localization more precisely. In recent studies on the terminal abdominal ganglion of the cockroach, *Periplaneta americana*, Smith and Treherne (1965), using cytochemical techniques at the electron microscope level, have defined several sites of esterase activity. Eserine-sensitive esterase, presumably ChE, was found in association with axonal membranes at apparent synaptic sites in neuropile. Other sites of esterase activity were found both in and outside neuropile. If the neuropile-associated esterase which we detect in brains of diapausing Cecropia occurs at synaptic sites, and functions in transmission, then at least this element of neuronal interaction would appear to remain patent throughout pupal diapause.

In view of this possibility, and in the light of the still-uncertain electrophysiological status of the brain during diapause, a detailed reinvestigation by contemporary methods is clearly required. In studies of this type commenced recently, Walcott (personal communication, 1965) has confirmed certain electrical activity

in the brains of diapausing silkmoth pupae; this supports the observations of Schoonhoven (1963) and argues that any electrical "silence" of the diapausing brain, as reported by Van der Kloot (1955) and Tyshtchenko and Mandelstam (1965), must be restricted to certain brain regions if it occurs at all. It also now becomes important to reassess the status of ACh itself following pupation, since this (assayed as cholinergic substance effective upon clam heart in bioassays) was reported to undergo precipitous disappearance at the outset of diapause, followed by more gradual reaccumulation in unchilled and chilled pupae (Van der Kloot, 1955).

4. *Multiple forms of esterase in relation to diapause and development*

The arguments in Section 2 of this Discussion do not explain our failure to detect large changes in esterase activity at the onset and termination of diapause, as reported by Van der Kloot (1955). It is possible that our findings are compatible with those of Van der Kloot, but reflect the behavior of different esterases. It has long been known that insect brains or heads contain a variety of esterases (see reviews by Gilmour, 1961; Chadwick, 1963), though few data are available on Lepidoptera. Recently, Maynard (1964) has characterized multiple esterases in the nervous systems of crayfish and lobster. In the light of these seemingly general attributes of arthropod central nervous systems, it is most probable that silkmoth brain likewise contains esterases beyond the single ChE detected in the present study. It is also worth noting that our use of AThCh at the rather low optimal concentration of 0.75 m*M* may preclude detection of esterases other than ChE's, or of ChE's having low activity toward the acetyl ester. Moreover, at higher concentrations, ACh may be hydrolyzed *via* enzymes other than ChE's; in Van der Kloot's study ACh was used at 15 m*M*. Thus the prospect merits attention that the enzymatic changes described in his study are meaningful, and apply to an esterase not detected in the present study. We look upon this prospect with favor, since exploratory spectrophotometric and electrophoretic studies on Cecropia brain, with various substrates, reveal several esterases including a component that undergoes changes at the onset and termination of diapause.

We therefore conjecture that an esterase other than the AChE described in this report, and perhaps already manifested in Van der Kloot's (1955) study, will be found to undergo changes that correlate with the neuroendocrine inactivation and reactivation of the brain. Such a correlation between enzymatic and physiological events would, of course, not in itself assure a causal role for the enzyme in the control of neurosecretion and diapause. Nonetheless, attention will surely center on its localization and properties in efforts to gain further insight into this control at the molecular and subcellular levels.

Meanwhile, the results of the present study clearly oblige us to abandon the attractive view that generalized disappearance and reappearance of ChE can account for neuroendocrine changes in the brain, that in turn bring about the onset and termination of pupal diapause. The history of biology is punctuated with occasions where investigators, confronted with the need to revise an earlier theory, have proceeded beyond their own data and sought to devalue the theory as a whole. Slater (1958) discusses instances of this type in the history of the study of cellular respiration. In present circumstances, we believe it prudent to continue to direct

attention toward enzymatic events as part of the effort to understand the control of neurosecretion and diapause.

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Summary

1. The localization and properties of cholinesterase in the brain of the Cecropia silkmoth were investigated by histochemical and quantitative spectrophotometric methods utilizing acetylthiocholine as substrate.

2. During pupal diapause, substantial activity was visualized in neuropile. At the outset of adult development, activity was also detected in adjacent regions occupied by neuronal or glial cell bodies.

3. Only one form of cholinesterase was detected with certainty. On the basis of substrate-activity relationships for acetylthiocholine, propionylthiocholine and butyrylthiocholine, and on the basis of its sensitivity to certain selective esterase inhibitors, the enzyme has properties of an acetylcholinesterase.

4. Substantial and essentially unchanging enzymatic activity was detected during pupation and most of pupal diapause, when the brain becomes endocrinologically inactive. However, an approximate doubling in activity was detected during storage of diapausing pupae at 6° C., apparently signalling the recovery of neuroendocrine competency by the brain. Subsequent growth and morphogenesis of adult brain were found to be accompanied by a six-fold further increase in activity.

5. Cholinesterase activity also persists during diapause in the Cynthia and Polyphemus silkmoths.

6. Consideration of the properties and optimal assay conditions for this enzyme in pupal brain assists in explaining previous reports that it was undetectable.

7. The presence of substantial cholinesterase activity throughout metamorphosis shows that a generalized disappearance and reappearance of the enzyme cannot be responsible for inactivation and reactivation of the neurosecretory mechanism that controls the onset and termination of diapause.

8. In the light of evidence for multiple forms of esterase in silkmoth brain, the present findings do not preclude a possible role for one or more esterases as part of the physiological mechanism controlling neurosecretion and diapause.

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