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Postembryonic Brain Development in the Monarch Butterfly, *Danaus plexippus plexippus*, L.

I. Cellular Events during Brain Morphogenesis*

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Summary. 1. Cellular morphogenesis during postembryonic brain development in *Danaus plexippus plexippus* L. was examined using histological techniques including radioautography.

2. The production of new neurones is continuous throughout larval and pupal stages and shows no fluctuations corresponding to ecdysis. Glial cell production, on the other hand, occurs at the time of molting.

3. New ganglion cells are formed by the division of neuroblasts found in aggregates or isolated among larval ganglion cells. Asymmetrical neuroblast divisions yield one neuroblast and one ganglion-mother cell which then divides at least once to form the new ganglion cells. Such divisions begin earlier in *Danaus* than in other investigated Lepidoptera. Symmetrical divisions yielding two neuroblasts also occur, but only among aggregated neuroblasts.

4. Radioautographs of brains fixed at progressive intervals after Tritiated Thymidine (H^3TdR) injection have permitted description of the basic pattern by which cells of the adult brain cortex are laid out and progressive changes in the relationship of new ganglion cells derived from a single neuroblast. Ganglion-mother cells are deposited between the neuroblast and the neuropile, thus forming a row of cells which move the neuroblast progressively farther from the neuropile. New ganglion cells produced by ganglion-mother cell mitoses, which usually are oriented at 45° angles to the neuropile, expand the cell cluster. Differentiating fibers of these cells are apparent within a few days of their production and seem to enter the neuropile in one bundle. Later with increased neuropile volume and further cell differentiation the cells are no longer clumped and thus are not recognizable as offspring of a single neuroblast.

5. Neuroblasts found scattered among the larval ganglion cells arise from cells near the neuropile. These cells, at first indistinguishable from their neighbors, gradually assume the size and ready stainability of neuroblasts and subsequently divide according to the pattern described above.

6. Scattered neuroblasts degenerate beginning shortly after pupation and have completely disappeared by the end of the fourth day.

7. Except in the developing optic lobe, glial cell numbers increase through the proliferation of already existing glial cells. All glial cells show H^3TdR uptake during a 12 hour period surrounding each larval-larval molt and for a somewhat longer period after pupation. However, in the larval stages mitotic figures were seen only among glial I, II, and IV. Glial I cells divide through the entire last larval stage and for two days following pupation. Large irregular mitoses seen among glial III cells at pupation indicate that these cells are probably polyploid.

8. In the newly forming adult optic lobe glial II, III, and IV cells appear to develop from preganglion cells or cells indistinguishable from them. These cells gradually stain more and more darkly, segregate into the normal glial positions, and subsequently divide in accord with other glial cells.

9. At the end of the fifth instar the perineurium (glial I cells), which begins to thicken during the third larval instar, is multilayered and contains many vacuolar cells. Just prior to pupation the neurilemma begins to disintegrate and during the next five days all but the

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cells closest to the brain disappear. Hemocytes are seen to engulf portions of the disintegrating neurilemma and already degenerating perineurial cells, but do not seem to engulf live cells. The glial I cells remaining adjacent to the brain secrete a new neurilemma.

10. There is no evidence for mass destruction of larval ganglion cells by either autolysis or phagocytosis, and only in the antennal center is there evidence of degeneration of larval cells (NORDLANDER and EDWARDS, in press).

Introduction

The transformation of the endopterygote brain from larval to adult has attracted interest for many years (e.g., WEISMANN, 1864; BAUER, 1904; SCHRADER, 1938; PANOV, 1957, 1959, 1960a, 1963). It is clear from these studies that growth in volume and increase in cell numbers and complexity occur, and that there is wide variation both in the extent of these changes and in their time course in different insects.

Examination of the mechanisms of brain transformation has been largely focused on the origin of adult neurones and the fate of larval elements. Still many questions remain regarding patterns of cell and fibermass formation and their morphogenesis.

BAUER (1904) first demonstrated the presence in the larval brain of neuroblasts, cells which serve no function other than to divide at the appropriate time in order to produce adult ganglion cells. Though HANSTRÖM (1925) and others denied the existence of such neuroblasts, their presence was subsequently confirmed by HERTWECK (1931) and SCHRADER (1938) in *Drosophila* and *Ephestia* respectively. More recently, however, PANOV (1960b, 1963) has observed neuroblasts in brains of a large number of immature insects and has firmly established these cells as the source of adult brain neurones.

Differences of interpretation obscure the question of the fate of larval neurones at metamorphosis. Some, BAUER (1904) and TIEGS (1922) among others, have claimed that all larval neurones are destroyed and that the adult brain is, in effect, an entirely new structure. Others, SANCHEZ (1925), HANSTRÖM (1925), and PANOV (1963), consider that at least some larval neurones, possibly in a modified form, serve the adult. The mechanism of larval cell destruction where it does occur is also an open question.

With the object of resolving some of these questions, particularly those regarding cell origins and modifications and their time course, the behavior of the brain cells of the monarch butterfly, *Danaus plexippus plexippus* L., during its postembryonic development has been examined. The anatomical background for this study, including detailed descriptions of cell types, has appeared elsewhere (NORDLANDER and EDWARDS, 1968b). Further papers will consider the transformation of specific brain centers (NORDLANDER and EDWARDS, in press).

Methods

Animals used in this study were reared and staged in the laboratory at 21 to 22° C. Under these conditions, the larval stages of *Danaus* span 13 days and the pupal period 11 days.

Sample individuals taken at 12 hour intervals from newly hatched larvae through to adults were dissected under modified Sera's (MADHAVAN, 1963) or Helley's fixative. Brains were sketched for purposes of comparison and then prepared for sectioning at 6 to 8 μ. Sections were stained with Feulgen and toluidine blue or Mayer's hemalum and eosin.

Two series of animals were prepared for radioautographic studies. In series I three individuals selected at each 12 hour interval during development were injected with thymidine-methyl-H^3 (H^3TdR) (1 millicurie/ml., specific activity 17.4 curies per millimole) (New England Nuclear Corporation) in doses of 10 microcuries/gram weight of the insect. Brains of this series were fixed and sectioned as above.

A second series (Radioautographic series II) was prepared from four groups of animals injected with 20 microcuries H^3TdR/gram six hours after each of the last four molts and fixed at increasing intervals after injection.

Radioautographic slides were dipped in diluted (1:1) Eastman Kodak NTB_3 emulsion, dried and packed in black plastic boxes to be stored at −70° C for two to sixteen weeks. Non-radioactive and DNAse digested controls were included. Slides were developed in Kodak Dektol for 3 minutes at 16—18° C, dipped quickly in distilled water, and fixed for 5 minutes in Kodak fixer. After a 30 minute rinse in tap water slides were stained with Mayer's hemalum and eosin or with toluidine blue.

Key to Abbreviations

AC	Antennal Center	Nbl	Neuroblast
CB	Central Body	Nl	Neurilemma
CP	Corpora Pedunculata	Np	Neuropile
GC	Ganglion Cell	OA	Optic lobe Anlage
Gl I	Glial type one cell, perineurial cell	OL	Optic Lobe
Gl II	Glial type two cell, sheath cell	PBr	Protocerebral Bridge
Gl III	Glial type three cell, giant cell	PGC	Pre-ganglion cell
Gl IV	Glial type four cell, neuropile sheath cell	Ph	Phagocytic Cell
Gl V	Glial type five cell, subjacent cell	Pn	Perineurium
GMC	Ganglion-mother Cell	PP	Posterior Protrusion
La	Lamina, outermost optic neuropile	SN	Stemmatal Nerve
Lo	Lobula, innermost optic neuropile	SNbl	Scattered Neuroblast
Me	Medulla, middle optic neuropile		

Observations

The Pattern of Brain Growth

In this work no attempt has been made to quantify changes in brain volume through the course of the brain's complex growth and its coalescense with the subesophageal ganglion. Such has been done for Dipteran and Hymenopteran brain growth (e.g., HINKE, 1961; and LUCHT-BERTRAM, 1962) and for the terminal ganglion of the cricket (GYMER and EDWARDS, 1968). However, changes in one linear dimension, the horizontal width, are presented here as an index of the extent of volume changes and demonstrate that brain growth in *Danaus* follows the general pattern of holometabolan brain growth so far established. As Figs. 1 and 2 show, most of the increased width is due to the development of the optic lobes which increase from 3% of the earliest larval brain width to 65% in the adult brain. Meanwhile, the width of the protocerebrum increases only 8 times. Slight stepped increases in width were noted with each molt.

The Temporal Pattern of Cellular Multiplication

The temporal distribution of cellular proliferation events among glial elements and neurones of the various brain regions is illustrated in Fig. 3. The general chronology of developmental events occurring in the brain of *Danaus* during its postembryonic life is also shown. It should be noted that the production of new

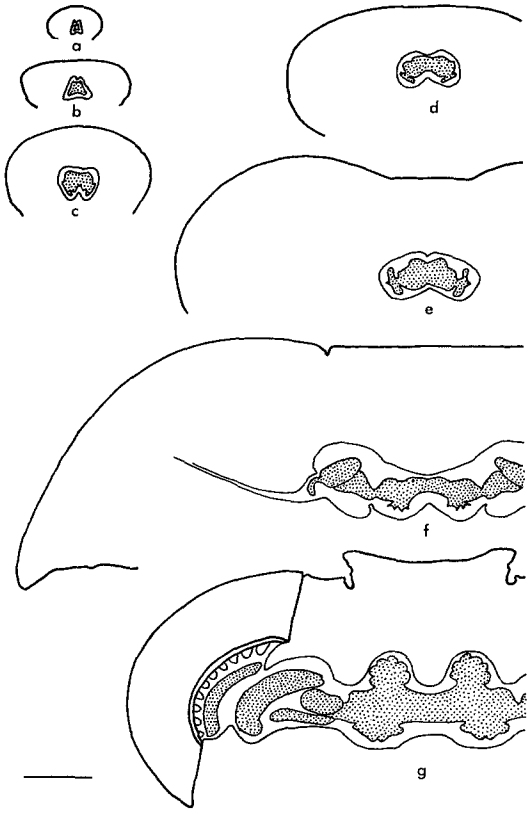


Fig. 1 a—g. The head and brain at successive developmental stages. Tracings from representative frontal sections. Neuropile areas shaded. a Newly hatched larva. b Early second instar. c Early third instar. d Early fourth instar. e Early fifth instar. f New pupa. g Adult. Scale, 400 μ

ganglion cells occurs continuously during the first two thirds of the animal's immature life, and that mitotic events show no perceptible correlation with the molting cycle. The production of glial cells, on the other hand, takes place during a short period on either side of each larval ecdysis and for a somewhat longer period at pupation.

The Formation of Adult Neurones

New neurones arise as a result of the proliferation of neuroblasts, which occur either as aggregates or as scattered single cells among larval neurones from which they are easily distinguished by their relatively large size and ready stainability (Fig. 4). Much of the increase in cell number and diversity in the brain is due to growth of the optic lobes and other centers which are absent or reduced in the larva; activity of the neuroblasts involved in the elaboration of these regions will be considered in subsequent papers (NORDLANDER and EDWARDS, in press). Cells of cortical areas not directly associated with specific brain centers grow in number

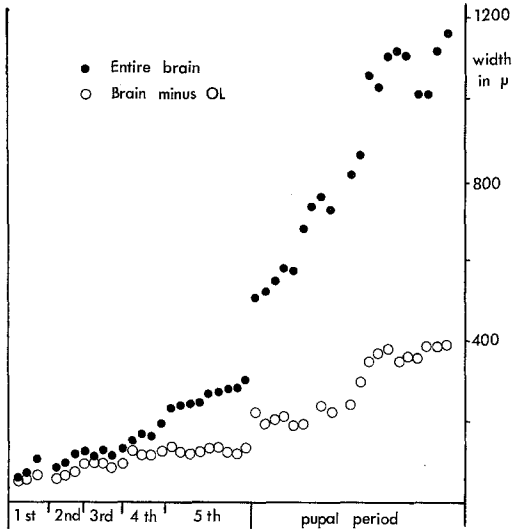


Fig. 2. A plot of brain width during the larval and pupal periods. Each point represents the average of the widest sections of two or three brains of the developmental stage. Note the sudden increase in width at pupation and the increasing proportion of brain width contributed by the optic lobe

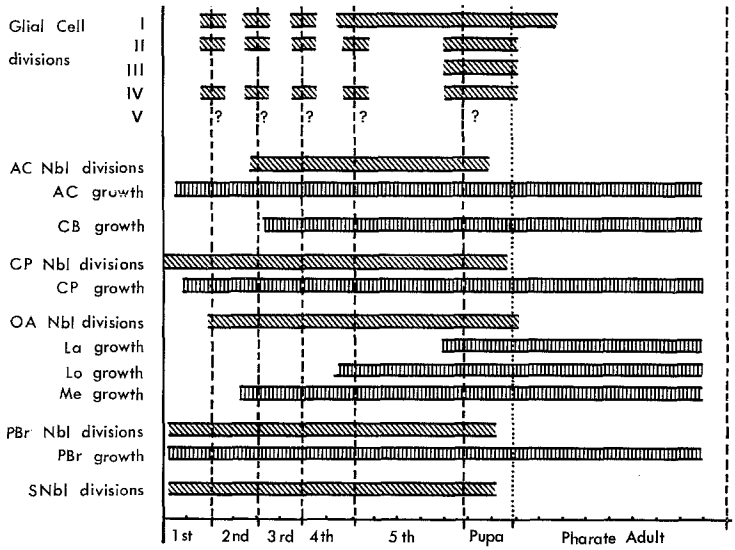


Fig. 3. The temporal distribution of proliferation and growth in the principle centers of the brain. See key to abbreviations

as a result of the activity of numerous scattered neuroblasts (Fig. 4). The processes by which these neuroblasts arise and proliferate are described below.

The Origin of Scattered Neuroblasts. In the brain of the newly hatched first instar larva no neuroblasts are distinguishable except those associated with the

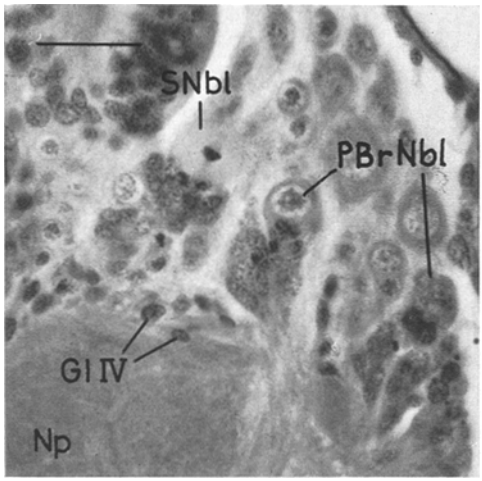


Fig. 4

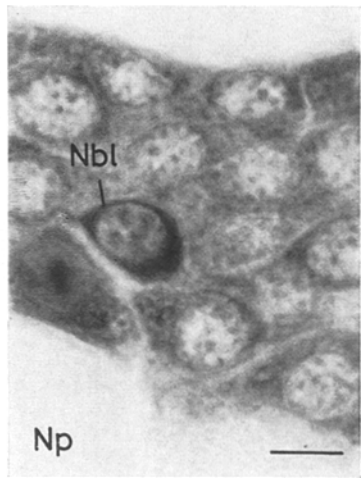


Fig. 5

Fig. 4. Scattered neuroblast (SNbl) undergoing mitosis. Next to it are two protocerebral bridge neuroblasts (PBr Nbl). Small cells directly below the SNbl are ganglion-mother cells. Frontal section of a third instar brain. Scale, 20 μ

Fig. 5. A recently formed protocerebral neuroblast (Nbl). Newly hatched first stage larva. Scale, 5 μ

antennal center, the corpora pedunculata, and the optic lobe anlage (NORDLANDER and EDWARDS, in press). During the first three larval stages, however, a few scattered neuroblasts are seen to arise from cells near the neuropile. These cells, which are at first indistinguishable from their neighbors, gradually enlarge until they assume the dimensions typical of neuroblasts (6 to 10 μ) (Figs. 5 and 6) and at the same time acquire the characteristically basophilic cytoplasm. Subsequently, they are seen to divide in the manner typical of scattered neuroblasts, as described below.

The Origin of New Ganglion Cells. A neuroblast normally divides asymmetrically (Fig. 7) to form another neuroblast and a smaller cell, the ganglion-mother cell. This cell is quite small (2—3 μ) and contains very little cytoplasm. The ganglion-mother cell goes on to divide at least once more forming two cells which later differentiate into neurones. The incidence of neuroblast and ganglion-mother cell divisions has no evident correlation with the molting cycle.

The spindle axis of a dividing scattered neuroblast is always perpendicular to the neuropile (Fig. 7) and at the completion of mitosis, the newly formed ganglion-mother cell lies between the neuroblast and the neuropile. As a result, the distance between the neuroblast and neuropile increases with each neuroblast division. The ganglion-mother cell divides soon after it is formed and its spindle axis is characteristically at a 45° angle to the neuropile surface or, less frequently, perpendicular to it (Fig. 7). New ganglion cell differentiation[1] as evidenced by the

1. "Differentiation" is used throughout to mean the acquisition of characteristic adult traits so far as can be seen with the light microscope using the techniques of this investigation.

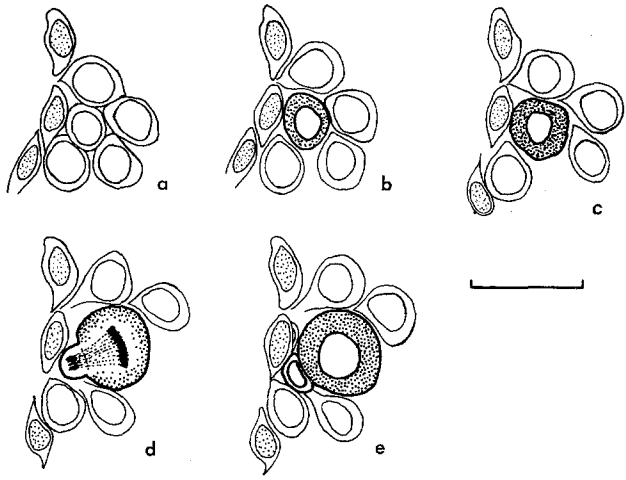


Fig. 6a—e. Diagram showing the intermediate stages in the origin of a scattered neuroblast. a Ganglion cells are indistinguishable from one another. b A cell adjacent to the neuropile appears slightly enlarged and basophillic. c The cell has achieved neuroblast characteristics. d The new neuroblast divides. e Division is complete and a new ganglion-mother cell can be seen between the neuroblast and the neuropile. Scale, 10 μ

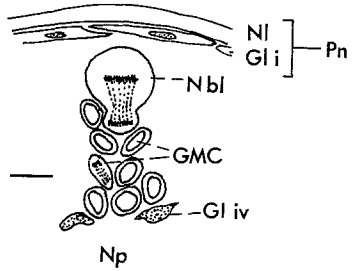


Fig. 7. Drawing of a typical scattered neuroblast group found in a young larva. The neuroblast (Nbl) divides assymetrically with its spindle axis perpendicular to the neuropile (Np) surface. The ganglion-mother cells (GMC) divide with their axes at approximately 45° to the neuropile surface. Scale, 5 μ

appearance of fibers and axon hillocks, begins within a few days of cell formation. Fibers of differentiating ganglion cells which are the progeny of a single neuroblast usually enter the neuropile together in a small bundle. In the early larva these cells are arranged in a row extending between the neuroblast and the neuropile (Fig. 8). Later as the neuropile volume increases, the row expands in width but remains a discrete cluster of cells. However, these clusters are no longer apparent when adult development is complete.

Experiments using H^3TdR pulse labelling and progressively delayed fixation (Radioautographic series II) provide further evidence for the observed pattern of scattered neuroblast proliferation (Fig. 8). In brains fixed within two hours after injection, the neuroblast nucleus and a few ganglion-mother cell nuclei are labelled (Fig. 8a). As brains are fixed after successively longer intervals, the

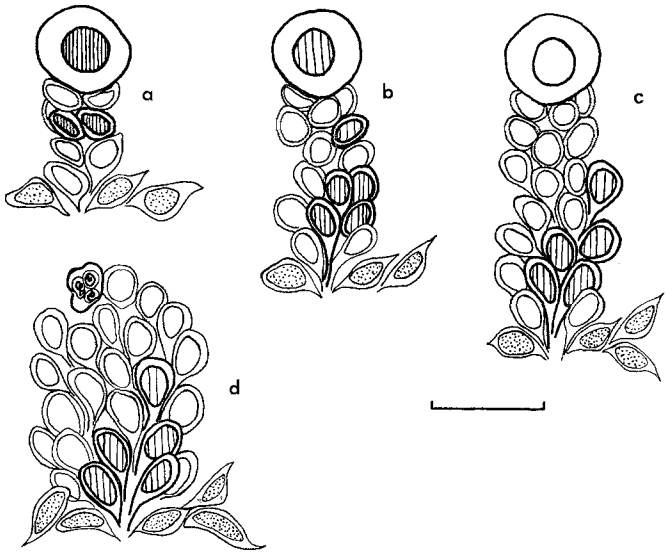


Fig. 8a—d. Diagram showing the occurrence of labeled cells (shaded) among a cell cluster including a scattered neuroblast and its progeny in brains fixed at successively longer intervals after injection of H^3TdR. All samples were injected at the beginning of the fourth instar. a Fixed two hours after injection. The neuroblast and a few ganglion-mother cells are labelled. b Fixed at the beginning of the fifth stage. The neuroblast is less intensely labelled and another ganglion-mother cell shows label. The original two ganglion-mother cells have divided and there are now four less intensely labelled ganglion cells. c Fixed late in the fifth instar. Label no longer is apparent in the neuroblast; the distance between the labelled ganglion cells and neuroblast has increased while they remain approximately the same distance from the neuropile. d Fixed two days after pupation. The neuroblast is degenerating. Note that the cell group which in earlier stages formed two rows perpendicular to the neuropile is now a clump of cells. Scale, 10 μ

labelled cells are found increasingly farther from the neuroblast but remain at approximately the same distance from the neuropile (Fig. 8b, c, d).

The Fate of Neuroblasts. A day or so after the last neuroblast divisions occur, "chromatin droplets" characteristic of degenerating cells (WIGGLESWORTH, 1942) are seen in all neuroblasts. During the first day and a half after pupation, these dense, deeply staining droplets, which are particularly prominent in Feulgen preparations, appear among scattered neuroblasts. Degeneration of corpora pedunculata and optic neuroblasts, however, occurs over a longer period of time. Those in the corpora pedunculata gradually shrink in size during the first days after pupation and degenerate during the next two or three days. All have disappeared by the end of the fourth day. Throughout the period of cell proliferation, degenerating neuroblasts are seen in the optic lobe anlagen along the proximal edges of both the outer and inner neuroblast aggregates (NORDLANDER and EDWARDS, in press). Later, after neuroblast divisions have ceased, during the third, fourth, and fifth days after pupation, the entire layer undergoes degeneration and the aggregates completely disappear (Fig. 12c).

The Differentiation of New Ganglion Cells. A new pre-ganglion cell measures 2 to 3 μ in diameter. Visible features of its differentiation as observed with the

light microscope are cell enlargement and the acquisition of axon hillocks. The latter are apparent within a few days of the cell's formation, well before there are perceptible differences between adjacent cells. That differentiation of at least one population of cells, that of the optic lobes, is accompanied by histogenetic cell death[2] is evidenced by the appearance of pycnotic nuclei among recently produced differentiating cells (NORDLANDER and EDWARDS, 1968a).

The Origin and Multiplication of Glial Cells

Glial elements increase in number throughout postembryonic development. Their pattern of proliferation differs from that of neuronal elements in showing a cyclic correlation with molting (Fig. 3) and exhibits few regional peculiarities.

The glial cell types discussed below have been described in a previous paper (NORDLANDER and EDWARDS, 1968b), and with the exception of glial type V their classification follows that outlined by WIGGLESWORTH (1959). Glial nuclei are readily distinguished from neurones by their dense finely granular chromatin, their sometimes irregular outlines, and topographic positions.

Glial I Cells. Apart from a period of four or five days beginning just prior to pupation, the brain is continuously invested by the neurilemma, and the underlying perineurinum (glial I cells) which secretes it. During the first four larval stages the glial I cells follow very much the same reproductive pattern as do other glial elements (Fig. 3), dividing during a 12 hour period on either side of ecdysis. However, beginning with the fifth instar, mitotic figures are seen continuously until the fourth day after pupation. The mitotic level falls off gradually from the second to the fourth days of the pupal period and finally falls to an imperceptible level by the end of the fourth day.

During the third larval instar the perineurium begins to thicken, particularly at the lateral border of the developing optic areas and in the posterior medial regions. At first the distance between nuclei decreases and the cells become cuboidal or columnar (Fig. 9a). Then as more and more cells are formed, the perineurium is gradually transformed into a multilayered structure (Fig. 9b) which continues to increase in thickness until early in the pupal period when cell divisions cease.

At the end of the fifth instar the perineurium posterior to the brain is particularly thick and spongy in appearance (Fig. 9c), and among its cells there appear numerous tracheae and vacuolar cells, probably hemocytes. During the last day of larval life the neurilemma begins to disintegrate, and over the course of the next five days it and all but three or four layers of cells proximal to the brain gradually disappear. The remaining perineurial cells secrete the adult neurilemma, and subsequently form a single layer around the developing brain.

In the late larva the perineurium is especially thick at the point where the stemmatal nerve enters the brain, and here the thickening extends the entire length of the nerve. As the stemmata migrate to the brain just prior to pupal ecdysis, the thickened perineurium migrates with them (Fig. 10), and finally together they form a sac-like protrusion behind the developing adult optic lobes (Fig. 11). This protrusion shows the same internal composition as does the

2. That which is involved in the differentiation of organs and tissues (GLÜCKSMANN, 1951).

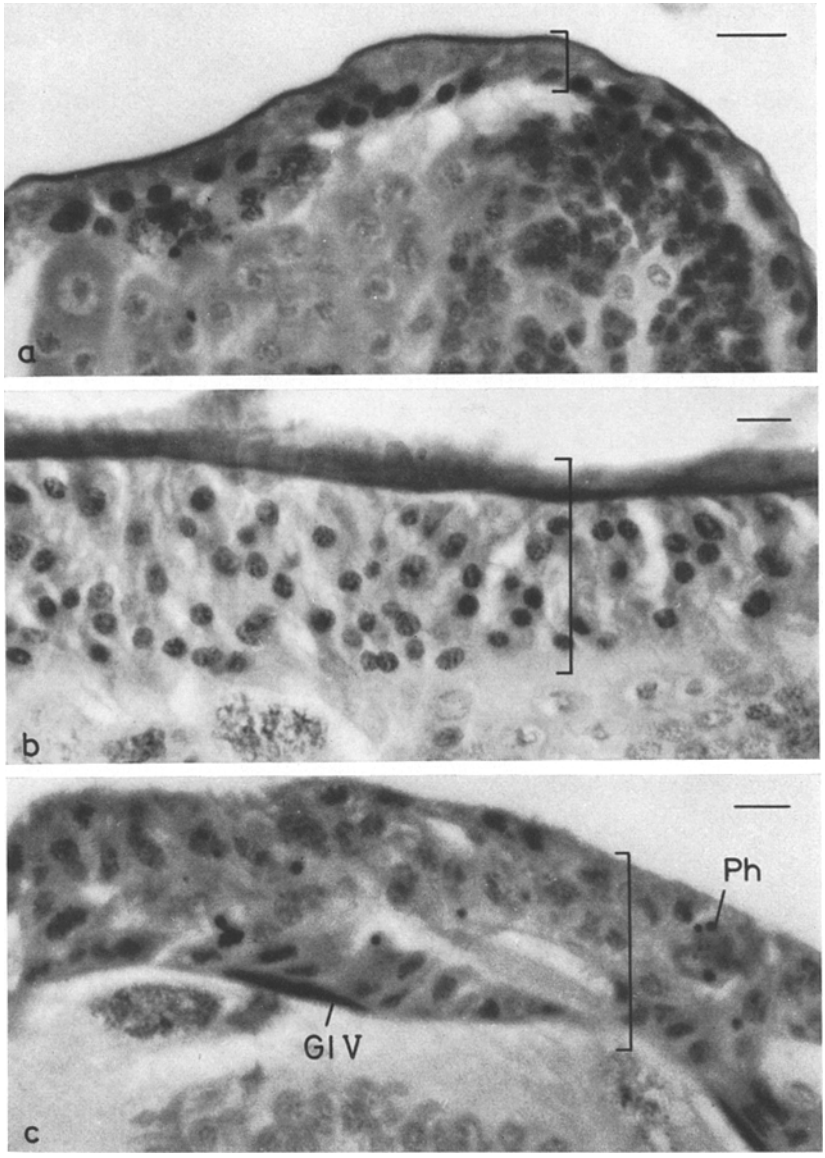
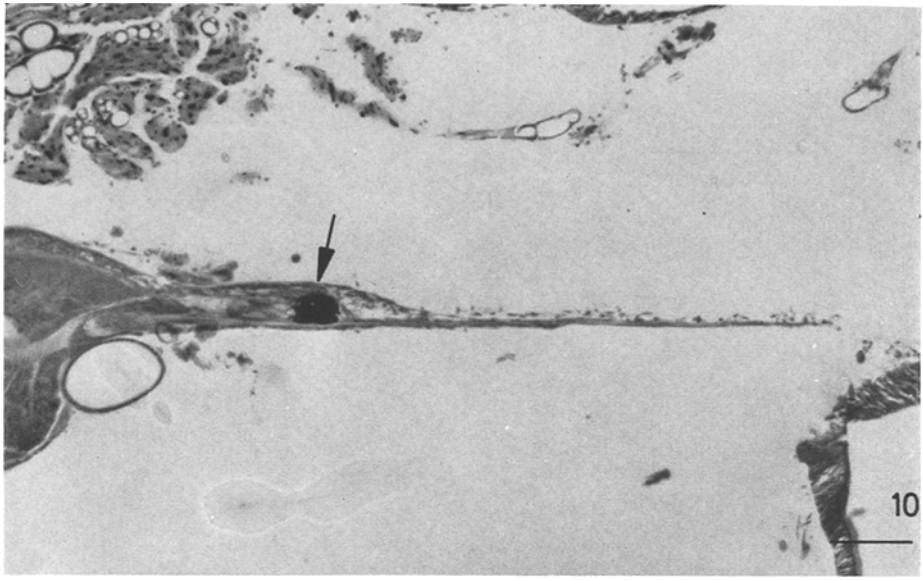


Fig. 9a—c. Progressive changes in the perineurium and neurilemma (brackets). a The early third instar. b The late fifth stage. c The three day pupa. Note the phagocytic cell (Ph) among the cells and debris of the perineurium. Scale, 10 μ

thickened perineurium except that it contains the degenerating parts of the stemmata, as well. At the same time that the neurilemma disintegrates, this sac-like protrusion disappears and only the pigment of the stemmata remains here in the adult.

Glial II and IV Cells. In the earliest larval brain glial cells are seen forming a sheath surrounding the central neuropile area (type IV) and in the nerves



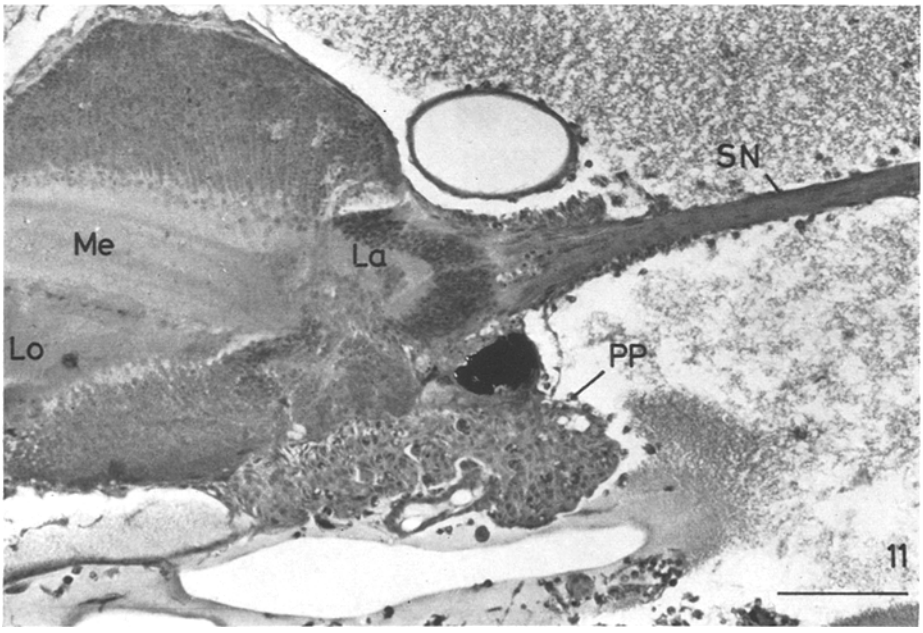


Fig. 10. Migrating remnant of the stemmata and thickened perineurium just before it reaches the brain. Stemmatal pigment indicated by arrow. Frontal section, one hour pupa. Scale, 100 μ

Fig. 11. Posterior sac-like protrusion (PP) containing remnants of the larval stemmata and characteristic thickened perineurium. Frontal section, second day pupa. Note phagocytic-like cells and debris. Scale, 100 μ

leaving the brain (type II). Throughout larval life these cells multiply only at the time of molting and show H^3TdR uptake for 12 hours on either side of larval-larval ecdysis (Fig. 3). At the larval-pupal molt, however, many more mitoses are

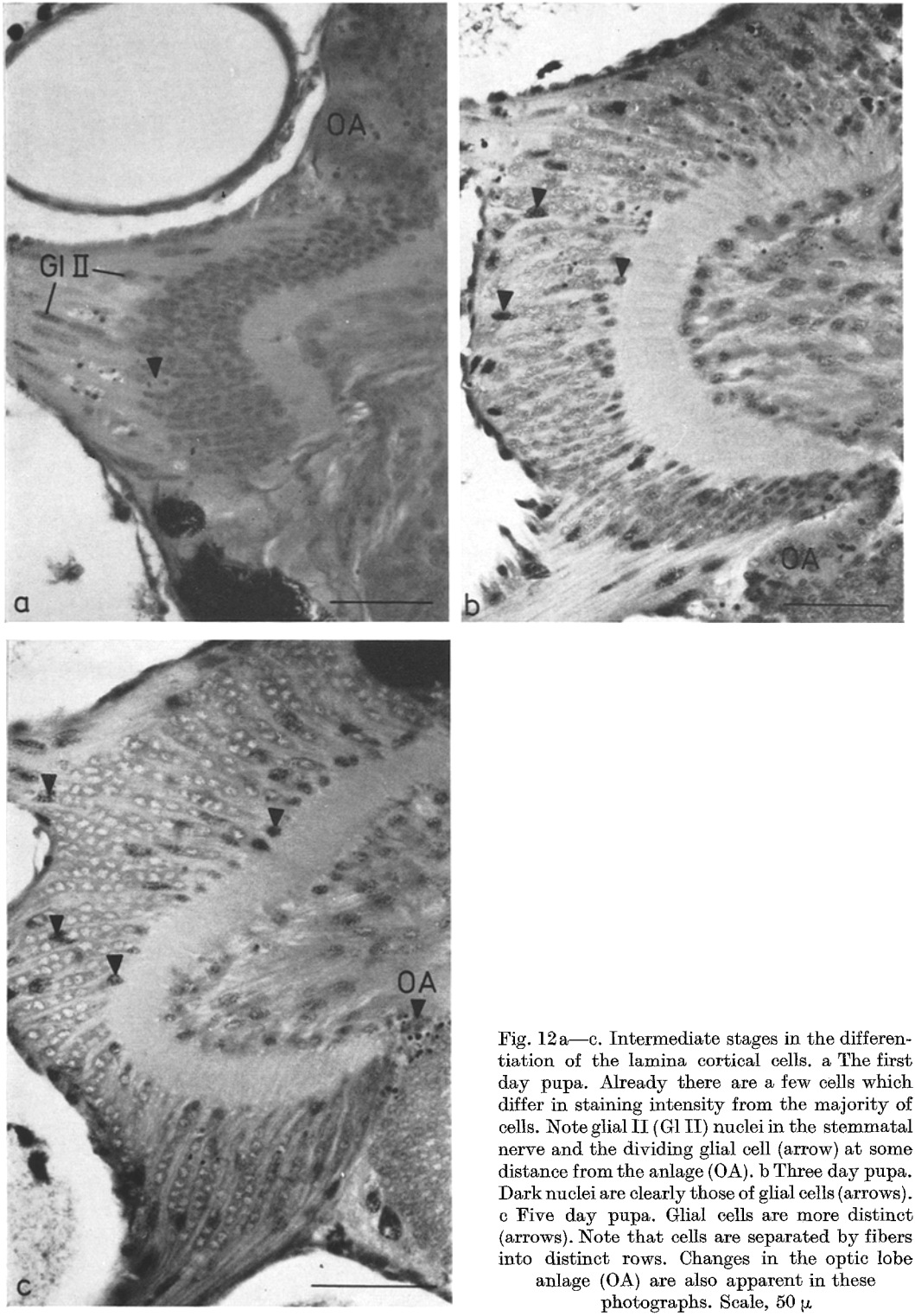


Fig. 12a—c. Intermediate stages in the differentiation of the lamina cortical cells. a The first day pupa. Already there are a few cells which differ in staining intensity from the majority of cells. Note glial II (Gl II) nuclei in the stemmatal nerve and the dividing glial cell (arrow) at some distance from the anlage (OA). b Three day pupa. Dark nuclei are clearly those of glial cells (arrows). c Five day pupa. Glial cells are more distinct (arrows). Note that cells are separated by fibers into distinct rows. Changes in the optic lobe anlage (OA) are also apparent in these photographs. Scale, 50 μ

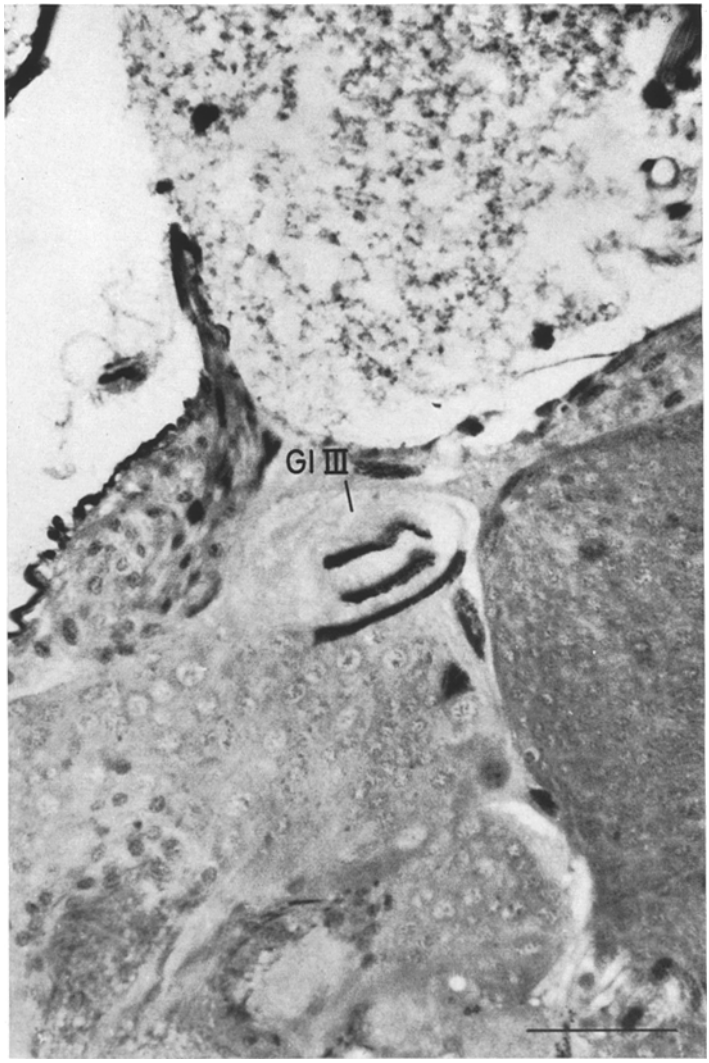


Fig. 13. A dividing giant glial cell (Gl III). Second day pupa. Scale, 50 μ

seen and cells show uptake for two full days following ecdysis. Type IV cells surrounding rapidly growing centers, such as the antennal centers and the corpora pedunculata, divide more intensely at this time. Occasional mitoses can be seen among type IV cells as late as five days after pupation. No degeneration is seen among them.

In the rapidly growing optic lobes, glial II and IV cells appear to be produced by a different method than are those of other parts of the brain. Cells dispersed among differentiating ganglion cells of the optic cortex, and at first indistinguishable from them, gradually appear to stain more darkly than their neighbors (Fig. 12). As the optic cortex approaches maturity, these cells, which stemmed from the same cells as the optic ganglion cells, now have the appearance of glial

cells, and seem to sort themselves from them. During subsequent development the new glial cells divide in phase with those elsewhere in the brain.

Glial III Cells. During the early part of brain development the giant glial cells (glia III), dispersed among and wrapped around neurones in the cellular cortex, show increases in size but not in number. Before the end of the fifth larval instar their nuclei have enlarged and become more irregular in contour. The characteristically dispersed chromatin and fibrous cytoplasm seem to increase, and additional nucleoli appear. H^3TdR experiments (radioautographic series I) show that the pattern of uptake is similar to that of type II or IV cells, indicating that DNA is actively synthetized by these cells for twelve hours on either side of each larval-larval ecdysis. However, no mitoses are seen until a short period beginning just before pupation and extending for two days (Fig. 3) when giant and sometimes irregular metaphase plates (Fig. 13) appear.

Glial V Cells. In early larval brains glial V cells, the flat cells just beneath the perineurium, are not distinguishable. In third stage brains they are first discernable as a layer of very thin nuclei. In subsequent stages the nuclei appear to enlarge and their number to increase. Though no mitosis was observed among them, their pattern of thymidine uptake is like that of other glial cells.

Discussion

Volume Growth

In most endopterygotes thus far studied, the pattern of postembryonic increase in central nervous system volume follows a smooth curve without fluctuations corresponding to ecdysis (HINKE, 1961; LUCHT-BERTRAM, 1962; POWER, 1952). In the brain, mesothoracic ganglion, and fourth abdominal ganglion of *Antheraea pernyi*, on the other hand, PANOV (1961) finds slight increases in growth rate immediately following each molt. He attributes these increases to the cyclic production of glial cells which undergo division just prior to ecdysis. The stepped increase in brain width observed in *Danaus* (Fig. 2) may likewise be due to rhythmic glial cell production; it may instead represent a form change in response to increased head capsule width at the molt and need not reflect a change in growth rate.

Qualitative observation of the relative contribution of cortex and neuropile to total brain volume in *Danaus*, as in *Antheraea* (PANOV, 1961), indicates that the proportion of neuropile increases throughout the larval period, decreases during the last larval and early pupal stages, and then shows a strong increase for the remaining two thirds of the pupal period. The temporary decline in neuropile proportion apparently reflects the rapid accumulation during the late larval and early pupal stages of pre-ganglion cells which, though occupying a large portion of the cortex, lack fully differentiated fibers. Later, despite considerable perikaryon enlargement, this relationship reverses due to the combined effects of the cessation of cell production, the elimination of neuroblasts from the cortex, and abundant fiber growth.

The relative contributions of glial and neuronal cytoplasm to increases in total brain volume cannot be measured with this material. However, a comparison of progressive numerical increases in glial and neuronal nuclei during brain develop-

ment is possible but was not attempted in this study. In their investigation of postembryonic growth of the cricket terminal ganglion, GYMER and EDWARDS (1968) observed that the neuronal population remains stationary while there is a 17-fold increase in glial numbers. The resulting ratio is 8 glial cells for each neurone in the adult. Unlike the cricket terminal ganglion, the brain of *Danaus* acquires an enormous number of neurones during its development. Qualitative observations suggest, nevertheless, that the proportion of glial II and IV cells increases at least in parts of the brain. Whether increases in glial cell numbers are related to the growth in volume of individual neurones or to increased numbers of neurones is not known. It would be useful to compare the proportions of glia and neurones in brain regions as different as the corpora pedunculata, with its large population of small cells, and the pars intercerebralis, with its varied population including some large neurones. Further comparisons might be made with the ventral ganglia.

The Temporal Pattern of Morphogenetic Events

The brain of *Danaus* appears to differ from that of most previously studied Lepidoptera in that its transformation begins early in the first larval stage and extends over a larger portion of the animal's life. In *Ephestia* (SCHRADER, 1938) and a number of other lepidopteran species (PYLE, 1941) changes begin toward the end of the last larval stage. In *Antheraea pernyi*, on the other hand, some neuroblasts are active during the last days of the third larval instar (PANOV, 1957, 1960a). PANOV has also shown that brain metamorphosis begins early in a number of other endopterygotes. It is possible that the brains of the Lepidoptera studied by SCHRADER and PYLE similarly begin metamorphosis in earlier larval stages but that these authors failed to recognize early brain changes because they did not expect them.

The possibility that cell proliferation in nervous tissue, apart from the demonstrated cyclic division pattern of glial cells, occurs continuously is of interest in relation to the humoral regulation of synthetic activity in other tissue, e.g., chitogenous epithelium and Malphighian tubules (KRISHNAKUMARAN, et al., 1967; RISLER, 1954). The restriction of mitotic activity in these tissues to certain periods is undoubtedly related to cyclic hormone activity. Yet the production of neurones in the brain appears to be independent of the cyclic aspects of hormonal environment. It may be that cells of imaginal anlagen are not susceptible to such regulation of their mitotic activity while those of other tissues, functional in the larva, are susceptible.

That brain morphogenesis is influenced by hormones was demonstrated by SEHNAL (1965). Brains of *Galleria* larvae to which the juvenile hormone was administered still exhibit growth but lack internal differentiation beyond that typical of the developmental stage of hormone introduction. The actual cellular events affected by the administration of the hormone are yet to be determined.

Neuroblasts

The formation of neuroblasts from cells near the neuropile, the occurrence of unequal divisions among them, and their subsequent degeneration in *Danaus* conforms to patterns described by previous authors (e.g., BAUER, 1904; PANOV,

1963). PANOV distinguishes prospective neuroblasts from cells surrounding them by their possession of finer granular chromatin. However, no such distinction was possible in *Danaus*. The appearance of new neuroblasts by this means in the brain of *Antheraea pernyi* (PANOV, 1963) during the third instar occurs much earlier in the monarch, whose neuroblasts have begun to differentiate at the time of hatching.

The pattern of unequal division of scattered neuroblasts in *Danaus* differs from other Lepidoptera described (BAUER, 1904; SCHRADER, 1938; PYLE, 1941, and PANOV, 1960b, 1963) only with regard to the time course of these events; scattered neuroblast divisions in *Danaus* appear at least two larval stages earlier than those of other investigated Lepidoptera.

As in other Lepidoptera (BAUER, 1904; SCHRADER, 1938), all neuroblasts in the brain of *Danaus* have degenerated by midway through the pupal period. There is no evidence of their persistence in the corpora pedunculata of the adult as in the bee, *Apis* (PANOV, 1957).

The Formation and Differentiation of New Ganglion Cells

The role of neuroblasts in brain morphogenesis, summarized below (Fig. 14) occurs in two phases: 1. the proliferation of new cells and 2. their subsequent differentiation (see footnote, p. 202).

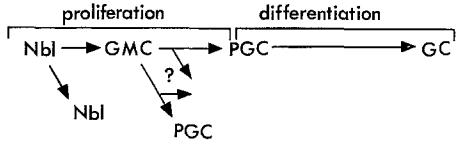


Fig. 14. Summary of the pattern of new brain ganglion cell formation. See text

Events of the first phase of this process have been worked out by previous authors (BAUER, 1904; UMBACH, 1934; SCHRADER, 1938, PYLE, 1941; and PANOV, 1957, 1959, 1960a, b, 1961, 1963), and this investigation further demonstrates that the proliferation pattern is uniform among endopterygotes. Evidence for this scheme is so abundant and conclusive that it is difficult to see how HANSTRÖM (1925, 1928) and SANCHEZ (1925) failed to note its occurrence. SANCHEZ observed a few equal divisions of small cells in *Pieris* and *Bombyx* but reported neither large embryonic cells (neuroblasts) nor large numbers of mitoses among brain cells. It seems likely that the small dividing cells which he described are actually ganglion-mother cells. Though HANSTRÖM described no divisions of any sort within the brain of *Pieris* and supposed that all adult elements are present in undifferentiated form in the larva, PANOV has established beyond a doubt that new elements are formed in *Pieris* according to the same pattern found in other insects and described here in *Danaus*.

The only aspect of the proliferation scheme in question is the number of cell divisions between the formation of the ganglion-mother cells and the formation of new ganglion cells. Most authors report only one division, but, as BAUER (1904) and PANOV (1963) point out, it is difficult to determine decisively how many times the ganglion-mother cells divide. It was expected that a comparison of grain

counts in radioautographs would indicate the number of generations as a result of successive dilution of labeled nucleotide, but the small size of the brain cell nuclei and their compact arrangement did not permit adequate resolution and the question thus remains unresolved.

The subsequent maturation of brain neurones and the formation of the neuropile masses has received relatively little attention. The various patterns by which the cell cortices and fibermasses of different brain regions are laid out all are modifications of the basic pattern described here for scattered neuroblasts. Morphogenesis of these cortical areas and their associated neuropile masses will be discussed in subsequent papers (NORDLANDER and EDWARDS, in press).

Morphological cell death (among recently produced cells in the process of differentiation) which occurs in the developing vertebrate nervous system (HAMBURGER and LEVI-MONTALCINI, 1949; LEVI-MONTALCINI, 1963; and HUGHES, 1959) and in the embryonic nervous systems of some insects (ULLMAN, 1967; and COUNCE, personal communication) also occurs during postembryonic development in *Danaus*, and is discussed in another paper (NORDLANDER and EDWARDS, 1968a).

Except for the central migration of the larval stemmata at the time of pupation there is no evidence that cell migration plays any major role in brain morphogenesis in the monarch. No counterpart is found for the striking cell migration observed in the chick embryo (LEVI-MONTALCINI, 1963). In *Danaus* any apparent migration can be explained by the continuous production of cells and fibers between neuroblasts and their earliest progeny and, later, by changes in cortex surface area caused by expansion of neuropile volume.

The Fate of Larval Neurones

The destruction of larval neurones at metamorphosis has been variously regarded as an autolytic or a phagocytic process (e.g. WEISSMAN, 1864; BAUER, 1904). Among Lepidoptera phagocytic activity has been implicated in the destruction of larval elements in *Pieris* and *Bombyx* (CAJAL and SANCHEZ, 1921; and SANCHEZ, 1924). In the ventral ganglia of *Pieris*, however, HEYWOOD (1965) observed no such activity. Similarly, the destruction of larval neurones in *Ephestia* (SCHRADER, 1938) and *Antheraea pernyi* (PANOV, 1963) without the aid of phagocytes has been described, and PYLE (1941), though noting phagocytic-like cells in the brains of several Lepidoptera, found no evidence for phagocytosis. In the light microscopic preparations used for this study there is no evidence of phagocytic elimination of larval elements in the brain of *Danaus* and only in the antennal centers is there evidence of any degeneration of larval neurones (NORDLANDER and EDWARDS, in press). On the basis of this negative evidence it is inferred that the majority of larval brain cells are incorporated into the adult brain.

Glia

The fate of the perineurium and neurilemma at metamorphosis, first examined by VIALLANES (1882), was described by SCHRADER (1938) in terms of the phagocytic action of blood cells which gain entry through breaks in the posterior neurilemma. Subsequent work with Lepidoptera (PANOV, 1963; ASHHURST and RICHARDS, 1964; HEYWOOD, 1965; PIPA and WOOLEVER, 1965) has shown that

amoebocytes do engulf already degenerating cells and parts of the neurilemma. Hemocytic cells with vacuolar cytoplasm appear in the perineurium at the time of neurilemma breakdown in *Danaus*, but they appear to engulf only already degenerating perineurial cells and are thus presumably not responsible for their death.

A further long standing question regards the source of new glial cells which appear in the brain during postembryonic development. Several early workers sought their origin outside the nervous system. These authors described the migration along degenerating larval nerves, of extrinsic cells, either of blood (JOHANSEN, 1892; BAUER, 1904) or epidermal (UMBACH, 1934) origin, which after reaching the brain settle into the role of glial cells. From the work of SCHRADER (1938), PANOV (1963), and this work with *Danaus*, however, it is clear that new glial cells are formed by divisions of already differentiated glial elements. The sudden degeneration of these cells at the time of metamorphosis, reported by SCHRADER, was not seen by PANOV in *Antheraea pernyi* nor in *Danaus*.

The origin of glial II and IV cells from undifferentiated ganglion cells of the optic lobes, not previously reported, parallels the embryonic origin of glial II and IV cells in the brain of *Antheraea pernyi* (PANOV, 1963) from ganglion cells which lie adjacent to the neuropile mass and which are distinguishable initially by staining slightly more darkly than neighboring cells. Differences in the method of glial cell production in the optic lobes and the rest of the brain may be attributed to the fact that the adult optic lobes are entirely new structures whereas other centers are elaborations of larval centers which undergo less spectacular volume changes and which are already provided with glia.

The behavior of glial III cells during postembryonic development deserves comment, since their pattern of proliferation differs from that of other glial cells. These cells were observed to appear first in mid-instar brains of a large number of insects (BAUER, 1904) and in *Ephestia* (SCHRADER, 1938), but in *Danaus* and in *Antheraea* (PANOV, 1963) they are clearly present from hatching. In all three species these cells undergo mitosis only at the time of metamorphosis when they produce giant metaphase plates. As the cells grow in volume through postembryonic development, the number of nucleoli and the quantity of chromatin increases. DNA synthesis, as demonstrated by incorporation of H^3TdR, occurs in schedule with that of other glial cells during larval stages, but mitoses do not follow except at the larval-pupal molt. Like the "Zwischenzellen" of the bee central nervous system (RISLER, 1954), glial III cells appear to become increasingly polyploid during larval stages. Such a phenomenon seems to occur in functional larval cells which produce substances for storage or secretion.

Zusammenfassung. 1. Postembryonale Gehirn-Morphogenese wurde mit histologischen und radioautographischen Methoden untersucht.

2. Neue Neuronen werden kontinuierlich produziert während Larven- und Puppenstadien, ohne eine mit der Häutung zusammenhängende Fluktuation zu zeigen. Gliazellen hingegen werden nur während der Häutung gebildet.

3. Neue Ganglionzellen entstehen durch Teilung von Neuroblasten, die einzeln oder in Aggregaten unter larvalen Ganglionzellen gefunden werden. Asymmetrische Teilungen von Neuroblasten führen zur Entstehung eines Neuroblasten und einer Ganglion-Mutterzelle, die dann mindestens eine weitere Teilung durchmacht und dabei neue Ganglionzellen bildet.

Solche Teilungen beginnen in Danaus früher als bei anderen untersuchten Lepidopteren. Manchmal werden auch symmetrische Teilungen beobachtet, die zwei Neuroblasten bilden; solche Teilungen erfolgen aber nur bei den Neuroblasten, die in Aggregaten vorkommen.

4. In verschiedenen Intervallen nach ^{3}H-Thymidin-Injektion wurden Radioautogramme von Hirnschnitten hergestellt. Dadurch war es möglich, das Muster der Zellen im Cortex zu beschreiben und die Beziehungen von neuen Ganglionzellen zu einzelnen Neuroblasten zu verfolgen. Ganglion-Mutterzellen werden zwischen Neuroblasten und Neuropyle angelegt und bilden dabei eine Zellreihe, die die Neuroblasten zunehmend von der Neuropyle trennt. Neue Ganglionzellen entstehen durch Mitosen der Ganglion-Mutterzellen; dabei sind die Mitosen in einem Winkel von 45° zur Neuropyle angelegt, und das Zellaggregat wird ausgedehnt. Die neuentstandenen Zellen entwickeln innert wenigen Tagen Fortsätze, und diese treten in Bündeln durch die Neuropyle ein. Später ist das Neuropylenvolumen größer, die Zellen sind nicht mehr deutlich gruppiert, und sind deshalb nicht mehr eindeutig als Abkommen eines einzelnen Neuroblasten zu erkennen.

5. Vereinzelt unter larvalen Ganglionzellen vorkommende Neuroblasten entstehen aus Zellen in der Nähe der Neuropyle. Diese Zellen sind zunächst von Nachbarzellen nicht unterscheidbar, werden aber allmählich größer, können dann wie Neuroblasten angefärbt werden und teilen sich dann wie oben beschrieben.

6. Die vereinzelt vorkommenden Neuroblasten degenerieren kurz nach der Verpuppung und sind am Ende des 4. Tages völlig verschwunden.

7. Gliazellen vermehren sich durch Teilung schon bestehender Gliazellen, außer im optischen Lappen. Alle Gliazellen inkorporieren ^{3}H-Thymidin während 12 Std um Larven-Larven-Häutungen, und während einer etwas längeren Zeitdauer nach der Verpuppung. In den Larvenstadien wurden indessen Mitosen nur in Glia I, II und IV festgestellt. Glia I-Zellen teilen sich durch das gesamte letzte Larvenstadium und noch während 2 Tagen nach der Verpuppung. Große, unregelmäßigen Mitosen in den Glia III-Zellen deuten auf Polyploidie dieser Zellen hin.

8. Im neu entstehenden optischen Lappen der Imago scheinen sich Glia II, III und IV aus Präganglionzellen zu entwickeln, oder aus Zellen, die sich von diesen nicht unterscheiden lassen. Diese Zellen werden mehr und mehr färbbar, sgregieren in die für Glia typische Position, und teilen sich dann wie andere Gliazellen.

9. Am Ende des 5. Stadiums ist das Perineurium (Glia I-Zellen), das während des 3. Stadiums an Dicke zuzunehmen begann, vielschichtig und enthält viele vakuolenhaltige Zellen. Unmittelbar vor der Verpuppung beginnt das Neurilemma zu desintegrieren, und während der folgenden 5 Tage verschwinden alle Zellen mit Ausnahme derjenigen, die unmittelbar am Gehirn liegen. Teile des Neurilemmas und bereits degenerierende Perineuriumzellen werden von Hemozyten phagozytiert. Die auf dem Gehirn gebliebenen Glia I-Zellen sezernieren ein neues Neurilemma.

10. Keine Anzeichen von Zerstörung, in großem Ausmaß, von larvalen Ganglionzellen durch Autolyse oder Phagozytose wurde beobachtet. Lediglich im Antennzentrum wurde Degeneration von larvalen Zellen beobachtet (Nordlander und Edwards, im Druck).

References

Ashhurst, D. E., and A. G. Richards: A study of the changes occuring in the connective tissue associated with the central nervous system during the pupal stage of the wax moth. J. Morph. **114**, 225—256 (1964).

Bauer, V.: Zur Innern Metamorphose des Zentralnervensystems der Insecten. Zool. Jb., Abt. Anat. u. Ontog. **20**, 123—152 (1904).

Cajal, S. R., y D. Sanchez: Sobre la estructura de los centios opticos de los insectos. Rev. Chil. Hist. Nat. (Santiago) **25**, 1—18 (1921).

Glücksmann, A.: Cell deaths in normal vertebrate ontogeny. Biol. Rev. **26**, 59—86 (1951).

Gymer, A. L., and J. S. Edwards: The development of the insect nervous system. I. An analysis of postembryonic growth in the terminal ganglion of *Aceta domesticus*. J. Morph. **123**, 191—197 (1968).

Hamburger, V., and R. Levi-Mantalcini: Proliferation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J. exp. Zool. **111**, 457—502 (1949).

Hanström, B.: Comparison between the brains of the newly hatched larva and the imago of *Pieris brassicae*. Ent. Tidsskr. **46**, 43—52 (1925).

— Vergleichende Anatomie des Nervensystems der wirbellosen Tiere. Berlin: Springer 1928.

Hertweck, H.: Anatomie und Variabilität des Nervensystems und der Sinnesorgane von *Drosophila melanogaster* (Meigen). Z. wiss. Zool. **139**, 559—663 (1931).

Heywood, R. B.: Changes occuring in the central nervous system of *Pieris brassicae* L. (Lepidopteran) during metamorphosis. J. Insect. Physiol. **11**, 413—430 (1965).

Hinke, W.: Das relative postembryonale Wachstum der Hirnteile von *Culex pipiens*, *Drosophila melanogaster* und *Drosophila*-Mutanten. Z. Morph. Ökol. Tiere **50**, 81—118 (1961).

Hughes, A.: Studies in embryonic and larval development in amphibia II. The spinal motor root. J. Embryol. exp. Morph. **7**, 128—145 (1959).

Johansen, H.: Die Entwicklung des Imagoauges von *Vanessa urticae* L. Zool. Jb., Abt. Anat. u. Ontog. **6**, 445—480 (1892).

Krishnakumaran, A., S. Berry, H. Oberlander, and H. A. Schneiderman: Nucleic acid synthesis during insect development II. Control of DNA synthesis in the Cecropia silkworm and other Saturniid moths. J. Insect. Physiol. **13**, 1—57 (1967).

Levi-Montalcini, R.: Growth and differentiation in the nervous system. In: The nature of biological diversity (ed. J. M. Allen), p. 261—297. New York: McGraw Hill 1963.

Lucht-Bertram, E.: Das postembryonale Wachstum von Hirnteilen bei *Apis mellifica* L. und *Myrmeleon europaeus* L. Z. Morph. Ökol. Tiere **50**, 543—575 (1962).

Madhavan Nair, K.: Occurrence of anucleolate oocytes in *Chrysocaris purpureus* (Westw.). Exp. Cell Res. **30**, 431—432 (1963).

Nordlander, R. H., and J. S. Edwards: Morphological cell death in the postembryonic development of the insect optic lobes. Nature (Lond.) **218**, 780—781 (1968a).

— Morphology of the larval and adult brains of the monarch butterfly, *Danaus plexippus plexippus*, L. J. Morph., in press (1968b).

— Postembryonic brain development in the monarch butterfly, *Danaus plexippus plexippus* L. II. Development of the optic lobes. III. Development of brain centers other than the optic lobes. (In press.)

Panov, A. A.: The structure of the brain in insects in successive stages of postembryonic development. Rev. Entomol. URSS **36**, 269—284 (1957).

— Structure of the insect brain at successive stages of postembryonic development. II. The central body. Rev. Entomol. URSS **38**, 276—284 (1959).

— The structure of the insect brain during successive stages of postembryonic development. III. The optic lobes. Ent. Obozrenie **39**, 86—105 (1960a).

— The character of reproduction of the neuroblasts, neurilemma, and neuroglial cells in the brain of the Chinese oak silkworm larvae. Dokl. Akad. Nauk SSSR **132**, 689—692 (1960b).

— Ontogenetische Entwicklung des Zentralnervensystems von *Antheraea pernyi* Guer. (Lepid.) Zool. Anz. **167**, 241—245 (1961).

— The origin and fate of neuroblasts, neurons, and neuroglial cells in the central nervous system of the Chinese silkworm, *Antheraea pernyi* Guer. (Lepid., Attacidae). Rev. Entomol. URSS **42**, 337—350 (1963).

Pipa, R. L., and P. S. Woolever: Insect neurometamorphosis II. The fine structure of perineurial connective tissue, adipohemocytes, and the shortening ventral nerve cord of a moth, *Galleria mellonella* (L.) Z. Zellforsch. **68**, 80—101 (1965).

Power, M. E.: A quantitative study of the growth of the central nervous system of a Holometabolous insect, *Drosophila melanogaster*. J. Morph. **91**, 389—411 (1952).

Pyle, R. W.: Changes in the nervous system of Lepidoptera during metamorphosis. Thesis, Harvard University (1941).

Risler, H.: Die somatische Polyploidie in der Entwicklung der Honigbiene (*Apis mellifica* L.) und die Wiederherstellung der Diploidie bei den Drohnen. Z. Zellforsch. **41**, 1—78 (1954).

Sanchez, D.: Influence de l'histolyse des centres nerveux des insectes sur les metamorphoses. Trab. Lab. Invest. Biol. Madrid **21**, 385—422 (1924).

— L'histogenèse dans les centres nerveux des insectes pendant les metamorphoses. Trab. Lab. Invest. Biol. Madrid **23**, 29—52 (1925).

SCHRADER, K.: Untersuchungen über die Normalentwicklung des Gehirns und Gehirntransplantationen bei der Mehlmotte *Ephestia Kühniella* ZELLER nebst einigen Bemerkungen über das Corpus allatum. Biol. Zbl. **58**, 52—90 (1938).

SEHNAL, F.: Einfluß des Juvenilhormons auf Metamorphose des Oberschlundganglions bei *Galleria mellonella* L. Zool. Jb., Abt. allg. Zool. u. Physiol. **71**, 659—664 (1965).

TIEGS, O. W.: Researches on the insect metamorphosis. Roy. Soc. S. Austr. **46**, 222—224 (1922).

ULLMAN, S.: The developing nervous system in *Tenebrio*. Phil. Trans. B **252**, 1—25 (1967).

UMBACH, W.: Entwicklung und Bau des Komplexauges der Mehlmotte, *Ephestia Kühniella* Zeller, nebst einigen Bemerkungen über die Entstehung der optischen Ganglien. Z. Morph. Ökol. Tiere **28**, 561—594 (1934).

VIALLANES, M. H.: Recherches sur l'histologie des insects et sur les phénomènes histologiques qui accompagnent le dévelopment postembryonnaire de ces animaux. Ann. Sci. nat. (Zool.) **14**, 1—348 (1882).

WEISMANN, A.: Die nachembryonale Entwicklung der Musciden nach Beobachtungen an *Musca vomitoria* und *Sarcophaga carnaria*. Z. wiss. Zool. **14**, 187—336 (1864).

WIGGLESWORTH, V. B.: The significance of "chromatin droplets" in the growth of insects. Quart. J. micr. Sci. **83**, 141—152 (1942).

— The histology of the nervous system of an insect, *Rhodnius prolixus*. II. The central ganglia. Quart. J. micr. Sci. **100**, 299—313 (1959).

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