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STUDIES ON THE NEUROSECRETORY CELLS OF THE BRAIN IN LEPIDOPTERA, WITH SPECIAL REFERENCE TO THE CIRCADIAN RHYTHM OF THE ADULT INSECTS.

by

CHRISTOPHER F. HINKS, B.Sc. (Lond.), A.R.C.S.

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Department of Zoology and Applied Entomology,
Imperial College of Science and Technology,
Field Station,
Silwood Park,
Sunninghill, Ascot,
Berkshire.

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ABSTRACT

Detailed histological studies of the brains of 23 species of Lepidoptera reveal a remarkable uniformity in the disposition, multiplicity of neurosecretory cells, and in the number of most types of cells. The recognition of A-, B-, C- and D-cells is based upon the paraldehyde fuchsin staining technique. These major categories are further divided, to give a total of 10 types of cells. In all species neurosecretory cells are confined to medial and lateral groups of the pars intercerebralis. With the exception of medial B-cells, all of the neurosecretory cells that occur in the adult become differentiated during the second larval instar. The cycles of secretion and increases in volume of the cytoplasm are estimated in 6 types of cells and correlated with physiological processes.

In adults, activity of the neurosecretory cells was studied in the short-living saturniid moth, Philosamia cynthia ricini, and compared with the comparatively long-living noctuid, Triphaena pronuba. Circadian cycles of secretion in the medial A_1- and A_2-cells are correlated with diuresis and flight activity respectively.

A detailed histochemical analysis provides evidence in support of the assumption that inclusions in the various types of neurosecretory cells differ in chemical composition. It is suggested that the inclusions of A- and C-cells are glycoproteins and that the B-cell inclusions are proteins. There is histochemical evidence that A_2-cells also contain 5-hydroxytryptamine.

Factors affecting activity were analysed, using an actograph

designed to record the insects in flight. An endogenous flight rhythm was demonstrated in nocturnal moths. The effect of ablating neurosecretory cells and areas of the brain was studied, and a hypothesis of hormonal control of flight activity is put forward. The role of 5-hydroxytryptamine in the initiation and maintenance of the circadian flight rhythm is discussed.

CONTENTS

I. INTRODUCTION

Advances in our understainding of neurosecretion in insects have developed from experimental and histological studies. To a large extent these two approaches have progressed independantly.

Kopec (1922) first demonstrated the humeral influence of the brain, upon pupation of the lepidopteran, Lymantria dispar. Wigglesworth (1934, 1940) showed that implantation of neurosecretory cells from the pars intercerebralis of the brain initiated moulting in Rhodnius prolixus. Since then, the role of these neurosecretory cells in growth and moulting has been confirmed in several other species, including: Hyalophora cecropia (Williams, 1946); Calliphora erythrocephala (Possompes, 1953); Pieris limbata (Nayar, 1956). In a series of transplantation and ligature experiments Fukuda (1940a, b, 1941) demonstrated the importance of the prothoracic glands. Williams (1946, 1947, 1952) proved that the brain hormone functioned in stimulating the prothoracic glands.

Although the histological studies of Weyer (1935), Scharrer (1939) and Hanstrom (1938) suggested secretory activity of certain large neurones in the pars intercerebralis, further developments were hampered by lack of specific staining techniques. This situation persisted until Bargmann (1949) discovered the specificity of Gomori's chromehaematoxylin towards neurosecretory materials in mammals. This method was subsequently found to be suitable for insect neurosecretion (Stutinsky, 1952). In the same year Halmi modified Gomori's paraldehyde-fuchsin technique for differentially staining the pancreas,

and Gabe (1953), modified and adapted it for insect neurosecretion.

The full paraldehyde fuchsin staining sequence includes a triple counterstain, and this has facilitated differentiation of neurosecretory cells which are classified according to their tinctorial affinities. Nayar (1955) recognised two types of cells which be designated as A- and B-. Since then further categories have been recognised, eg. A-, B-, C-, and D-cells in the brain of Oncopeltus fasciatus (Johansson, 1958). Some authors have found it necessary to further divide A- and B- categories, to accommodate all of the cells having a distinct appearance (Panov and Kind, 1963; Chalaye, 1965; Delphin, 1965). Others, however, believe that the various categories merely represent different phases in a secretory cycle of a single type of cell (Brandenburg, 1956; de Lerma, 1942; Herlant-Meewis and Pacquot, 1956; Thomsen, 1952). Nevertheless there are numerous histological studies providing substantial evidence that thdre are several types of neurosecretory cells (eg. Kobayashi, 1957; Johansson, 1958; Goldiay, 1959; Highnam, 1961; Ladduwahetty, 1962; McLeod and Beck, 1963; Herman and Gilbert, 1964; Delphin, 1965; Raabe, 1965; Hsiao and Fraenkel, 1966; Dogra, 1967).

In addition to moulting and metamorphosis, neurosecretory cell products have been shown to influence a diversity of physiological processes. These include:- diuresis (Maddrell, 1963; Highnam, Hill and Gingell, 1965; Berridge, 1966), carbohydrate and lipid metabolism (Steele, 1961; Van Handel and Lea, 1965), protein metabolism (Hill, 1962; Thomsen and Moller, 1963), migration of epidermal pigment during colour change (Mothes,

1960), rate of heart beat (Gersch, Fischer, Unger and Koch, 1960), tanning of adult cuticle (Fraenkel and Hsiao, 1963, 1965) and locomotor activity rhythms (Eidman, 1956; Harker, 1960c; Roberts, 1965). Despite repeated efforts to establish a system of classifying insect neurosecretory cells, in no instance in the list above can any single function be attributed to the product of defined type of cell.

Partly as a result of confusion in classifying neurosecretory cells, and partly as a result of the failure to recognise more than one or two types of cells, many of the earlier histophysiological studies are not particularly meaningful. As an example, Rohm (1951, 1955) described in detail the histological changes that occur in the brain neurosecretory cells of Ephestia kuhniella and Pieris brassicae during moulting and metamorphis, based upon the assumption that she was considering a single type of cell. In contrast, Herman and Gilbert (1964) recognised 8 distinct types of cells in the brain of Hyalophora cecropia, all of which are active during at least one stage in development. Their report is so brief that it only mentions that activity of one type of medial - and the latural A-cells can be correlated with moulting.

Hanstrom (1940) showed that neurosecretory material is transported from the cell bodies in the dorsum of the brain to the corpora cardiaca, and that these organs function in storage and release of the hormone(s). This transport of neurosecretory material was proved to occur in Leucophaea maderae by Scharrer (1952), and in Calliphora erythrocephala by Thomsen (1954).

In the latter, the movement of neurosecretory granules was observed directly, in the nerves passing from the brain to the corpora cardiaca. While this is representative of many insects, the neurosecretory materials are not always conducted to the corpora cardiaca; Scharn and Ittycheriah (1966) have demonstrated that in Ephita limbata one type of secretion passes to the corpora allata and another goes to the aorta which serves as a storage and release organ. Furthermore, Johansson (1963) has shown that in aphids neurosecretory material is conducted directly to target tissues by the nervous system.

The role of neurosecretion in circadian locomotor rhythms has been subject to considerable controversy. Harker (1956, 1958, 1960a, b, c,) in a series of elegant experiments demonstrated a complex endocrine mechanism in Periplaneta americana, involving the corpora cardiaca and certain neurosecretory cells in the suboesophageal ganglion. Roberts (1959, 1965) was unable to duplicate Harker's results in the same species and found, contrary to her results, that the controlling endocrine centre lay in the pars intercerebralis. In a more recent study of this species, Brady (1967b, c) obtain results conflicting with both Harker's and Robert's, he postulates that rhythmicity is primary neural and not hormonal. However the experimental evidence of Eidmann (1956) using Carausius morosus, and Nishiitsutsuji-Uwo (1964) using P. americana and L. maderae implicates the medial neurosecretory cells as the controlling centre. Brady (1967a) showed that the neurosecretory cells in the sub-oesophageal ganglion did not undergo any circadian

cycles of secretion that could be revealed after staining with paraldehyde fuchsin. Klug (1958) showed that neurosecretory cells in the pars intercerebralis of Carabus nemoralis become almost depleted of inclusions at night, when the beetles are most active. Bunsing (1964, 1965a, b) and Bunsing, Bruce and Thach (1965) demonstrated similiar but less marked bimodal cycles of secretion in medial neurosecretory cells in the brain of Drosophila melanogaster, which could also be correlated with locomotor activity. Unfortunately in these species in which a circadian cycle of secretion has been identified in the neurosecretory cells there is no experimental evidence to support the view that they are intimately connected with locomotor rhythms.

Although experimental studies made on various Lepidoptera have contributed much to our knowledge of endocrine participation in moulting and metamorphosis, histological confirmation of the part played by brain neurosecretory cells has lagged far behind, and even now are incomplete. Beyond this, very little is known of the activity of these cells, and of their function in the adult.

Various Lepidoptera, especially Bombyx mori have been used for histochemical studies of the neurosecretory cells, and as a source of the brain hormone for chemical analysis. With respect to the latter extracted substances have been shown to have prothoracotropic activity. Kirimura, Saito and Kobayashi (1962) claimed that the activating hormone was cholesterol, whereas Ichikawa and Ishizaki (1963) found that their "active" material

was a protein. Based upon histochemical studies, Kobayashi (1957) concluded that A-cell secretion is rich in lipid and phospholipid; Rohm (1955), that it is phospholipoprotein; Ganguly and Basu (1962) that it is mucopolysaccharide or mucoprotein; Arvy and Gabe (1962) that it is glycoprotein. With the exception of Rohm, these workers studied B. mori, therefore, the varying conclusions cannot be attributed to specific differences.

The paucity of studies upon the functions of neurosecretion in adult Lepidoptera, and in adult male insects, prompted this investigation. After considering the literature, this work was undertaken with the following objectives:- (I) to provide a detailed histological account of neurosecretory cells occurring in adults in a selected number of species. (II) to trace the development of the different types of cells, and determine their temporal sequence of activity in each stage. (III) an evaluation of their activity in adult males, in a species that feeds (Triphaena pronuba) and one that does not feed (Philosamia cynthia ricini), with particular reference to circadian rhythms. (IV) to establish the chemical basis of selective- and differential histological stains, by using histochemical techniques. (V) to elucidate the factors controlling flight activity and experimentally determine the role of the neurosecretory cells in this process.

This study, therefore, falls into three main sections: a. histology, b. histochemistry, c. experimentation.

III MATERIALS AND METHODS.

(1) Materials

Captures in light traps fitted with 150 watt mercury vapour lamps, situated on Imperial College Field Station, Silwood Park, provided most of the British moths used in this study. These include the following: Deilephila porcellus Linn, Stauropus fagi Linn, Notodonta zic-zac Linn, Pheosia tremula Cl, Phalera bucephala Linn, Spilosoma lubricipeda Linn, Bena prasinana Linn, Agrotisclavis Hufn, Agrotis ipsilon Hufn, Rusina umbratica Goeze, Triphaena pronuba Linn, Autographa gamma Linn, Diataraxia oleracea Linn, Biston betularia Linn, Colotois pennaria Linn, Tethea ocularis Linn, Habrosyne derasa Linn, Zeuzera pyrina Linn, and Hepialus humuli Linn. Pupae of Herse convolvuli Linn, Samia gloveri Strock and Celerio euphorbiae Linn were purchased from Worldwide Butterflies or the Butterfly Farm. Adults of Diaparopsis castanea Hmps of known age, were provided by Tropical Products Institute.

Cultures of Philosamia cynthia ricini Jones were maintained in a CT room at 20°C; larvae, pupae or adults were available throughout the year. Numerous attempts were made to establish a culture of T. pronuba, but no more than 2 generations were ever successfully reared, before a virus infection which was always present, killed all remaining larvae. Some virus resistant larvae do occur, but the adults from these are always very small and fail to breed. By giving the larvae a varied diet, changing the food frequently and minimising crowding the incidence of infection was reduced, but at best an ultimate survival rate of 40% was obtained in the first generation. Mortality always rose appreciably in the second generation.

(2) Histological Techniques.

Preparation of section.

In this study emphasis has been placed upon a critical analysis of the neurosecretory cells, which has depended to a large extent on obtaining sections in which all cells are well preserved and optimally stained. Numerous fixing and embedding schedules were tested in an effort to find a single method that would give reproducible results when applied to any of the 23 species detailed in this section of the work.

At an early stage it became apparent that Bouin fixed tissues, dehydrated with dioxan, transferred to chloroform and embedded in ester wax (Steedman, 1947) gave superior results. Unfortunately this wax is extremely difficult to section and is quite unsuitable for any routine histological work where serial sections are needed.

After testing paraffin waxes with melting points ranging from 39-63°C, in conjunction with various dehydrating agents and antemedia the following schedule was adopted for brains of adult insects.

1. Brain, thoracic ganglia and aorta were dissected out under Hoyles (1953) Ringer solution.

2. Transferred to a drop of Ringer in a solid water glass, and Bouin added. After 1 hour tissues transferred to fresh Bouin and fixed for 16-18 hours.

3. Excess picric acid removed by treating with 5% ammonium acetate in 70% alcohol for 10 minutes.

4. Dehydrated in graded solutions of dioxan for 24 hours, followed by 3 changes of pure dioxan over 24 hours.

5. Dioxan replaced by chloroform with several changes until tissues cleared.

6. Tissues placed in 1:1 chloroform, wax mixture (52°C M.P.) in the embedding oven.

7. After 20 minutes tissues paried through 3 changes of pure filtered wax, allowing 15 minutes in each.

For larval and pupul brains a similiar procedure was used except that dioxan was replaced by ethyl alcohol.

In some species adult brains were poorly preserved in Bouin and the fixative of Halmi (1952) was used (9 parts of Susa to 1 part saturated picric acid solution). No appreciable differences were noticed between either fixative on brains that were well preserved in Bouin.

Blocks were cut on a Cambridge rocking microtome, in frontal, transverse and sagital planes at a standard thickness of 6

Staining procedures.

Four methods were used for differentiating the neurosecretory cells; Heidenhain's Azan, Gomori's Chrome-haematoxylin /phloxine, Alcian blue/phloxine (After Dolphin, 1963), and Gomori's Paraldehyde-fuchsin; henceforth referred to as Agan, CHP, ABP and PAF respectively.

The use of PAF and Agar permits a more precise differentiation of neurosecretory cells than either CHP or ABP, and for this reason were chosen for routine use. CHP or ABP were only used to demonstrate phloxinophil cells.

The original formula for PAF staining as given by Gomori (1950) has since been modified several times; Halmi (1952), Gabe (1953),

Dawson (1953), Cameron and Steele (1959), Ewen (1962) and Jennings (1965).

The method that I found to give the best results involves the use of paraldehyde-fuchsin prepared and applied according to Halmi (1952). Each preparation of paraldehyde-fuchsin was ripened for 24-36 hours at about 22°C, and discarded after 72 hours. Used in this limited time the stain has a high degree of specificity. Sections thus stained, were then stained for 10 min. in Ehrlich's haematoxylin, blued in tap water, rinsed in distilled water then counterstained for 20 minutes. The recipe of the counterstain found to give the best results is that of Delphin (1963). This has the same proportions of the dyes as Ewen's (1962) formula, but has in addition, 0.5 gm of phosphotungstic acid added. No mordant is necessary.

The criteria for optimal staining with PAF are; the purple dye from paraldehyde fuchsin confined to the neurosecretory cells, tracheae and neurilemma, and, after counterstaining, neuropile green, nuclei orange, nucleoli bright red.

All subsequent reference to the abbreviation PAF are to the complete staining sequence, and PAF-positive is used to describe structures staining purple (fuchsinophil).

II MORPHOLOGY OF THE NEURO-ENDOCRINE COMPLEX

Cazal (1948) has given an account of the nerves connecting the brain, corpora-cardiaca and allata and stomatogastric chain in 8 species of Lepidoptera. Of the twenty three species were dissected in this study the innervation of the corpora-cardiaca and allata were considered in detail in 3 of them; Deilephila porcellus, Philosamia cynthia ricini and Triphaena pronuba. All 3 species differ only in the shape and disposition of the corpora allata. That in D. porcellus and P. cynthia ricini is the most common; in the former these glands consist of ovoid bodies lying directly behind the corpora cardiaca, but in the latter, they are larger, broadly lobed, lying above the corpora cardiaca. In T. pronuba they comprise an elongate cluster of 20-40 ovoid cells each measuring 120 x 135 - 135 x 210. These cells are connected by a separate nerve to the corpora cardiaca. The cardiac-allatal nerve (NCA) divides on contact with the corpora allata in the other species, and ramifies through the tissues, supplying each cell.

The corpora cardiaca lie on either side of the aorta to which they are loosely connected in T. pronuba and P. cynthia ricini, but intimately connected in D. porcellus. Two pairs of nerves connect the corpora cardiaca directly with the brain, the nervi corporis cardiaci of Hanstrein (1940). The inner pair, designated NCC I and the outer pair NCC II, receiving axons from the medial, and lateral neurosecretory cells respectively. Between NCC I and NCC II a third nerve passes laterally to descend over the posterior tentorial arms int. the head capsule. It corresponds to

nerve "x" of Cazal (1948), and nerve 3 of McLeod and Beck (1963), who traced the distal connection to the suboesophageal ganglion in the larva of Ostrinia nubilalis. This nerve, which I have designated nervi cardiacis lateralis (NCL) divides after passing over the tentorial arms; one branch extending dorsally to connect directly with the sub-oesophageal ganglion in P. cynthia ricini and D. porcellus, but with the labial nerve in T. pronuba. The main branch of NCL continues anteriorly to a point above the maxillary palp where it divides into several fine nerves lying on the surface of the maxillary muscle. Preparations stained with methylene blue reveal a distinct dilatation in NCL below the tentorial arms. The dilated portion terminates in a bulbous structure near the point where the nerve to the suboesophageal ganglion arises. Whole mount preparations show quite clearly that the bulbous structure consists of 2 intrinsic cells. Dissected nerves stained with Heidenhains iron haemotoxylin, Acid fuchsin and PAF clearly demonstrate that these cells are enclosed within the neural sheath and bear axon-like processes extending dorsally between the neurites of NCL. Whether these cells have an endocrine or purely nervous function could not be decided; while they stain very similiarly to the intrinsic cells of the corpora cardiaca, no fuchsinophil inclusions were evident.

It is likely that NCL is homologous to the allatal-suboesophyeal nerve described by Hanstrom (1940) in Plecoptera and Ephemeroptera, and more recently in various orthopteroid insects, Engelman (1957), Harker (1960), Willey (1961) and Dogra (1964).

The neuro-endocrine complex of T. pronuba and P. cynthia ricini is illustrated in figures 1 and 2

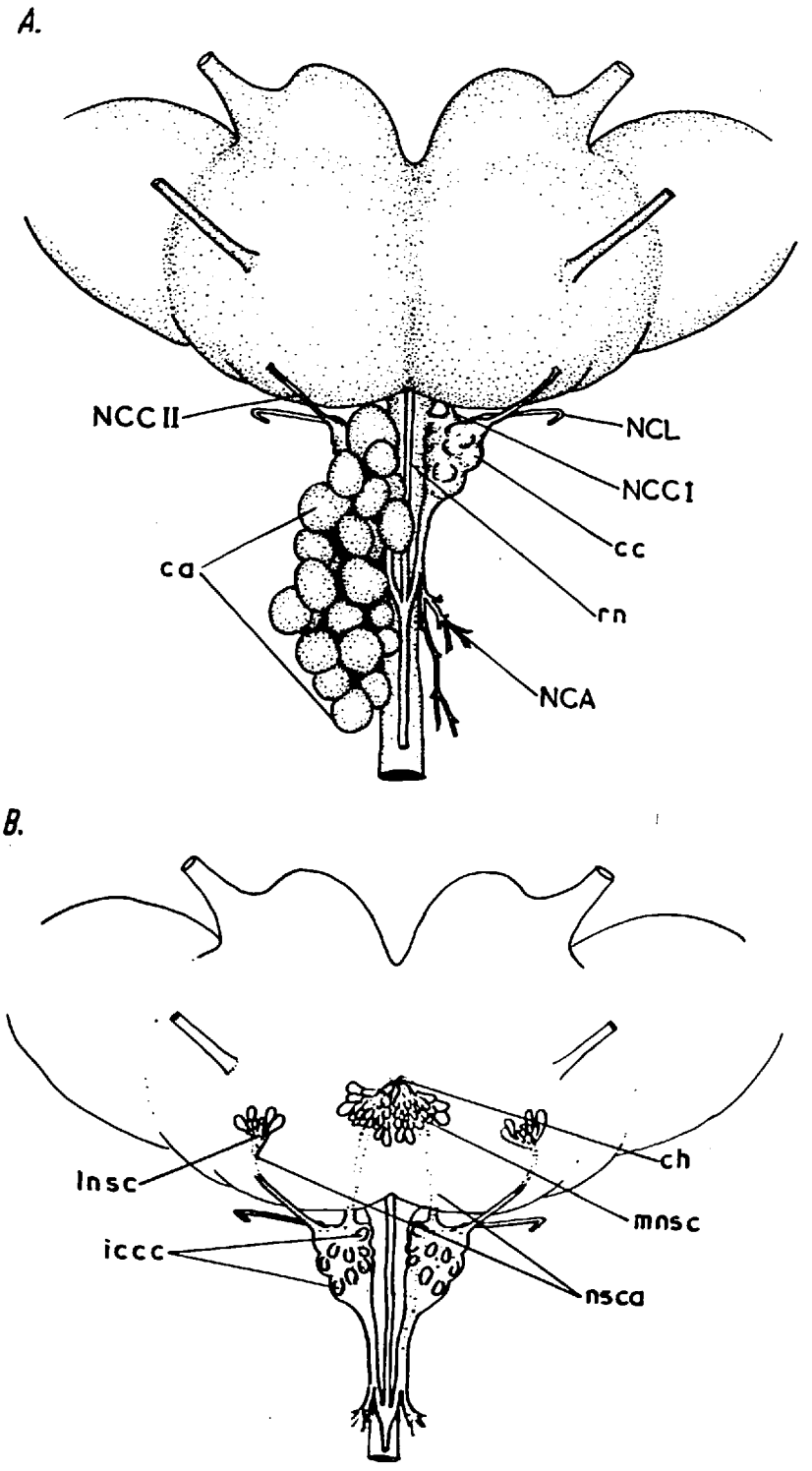

Fig. 1. The neuroendocrine complex of T. pronuba A. Dorsal aspect. B. Distribution of neurosecretory cells and the axonal pathways. ca, corpus allatum; cc, corpus cardiacum, ch, chiasma; iccc, intrinsic cells of corpus cardiacum lnsc, mnsc, lateral- and medial neurosecretory cells; nsca, neurosecretory cell axons.

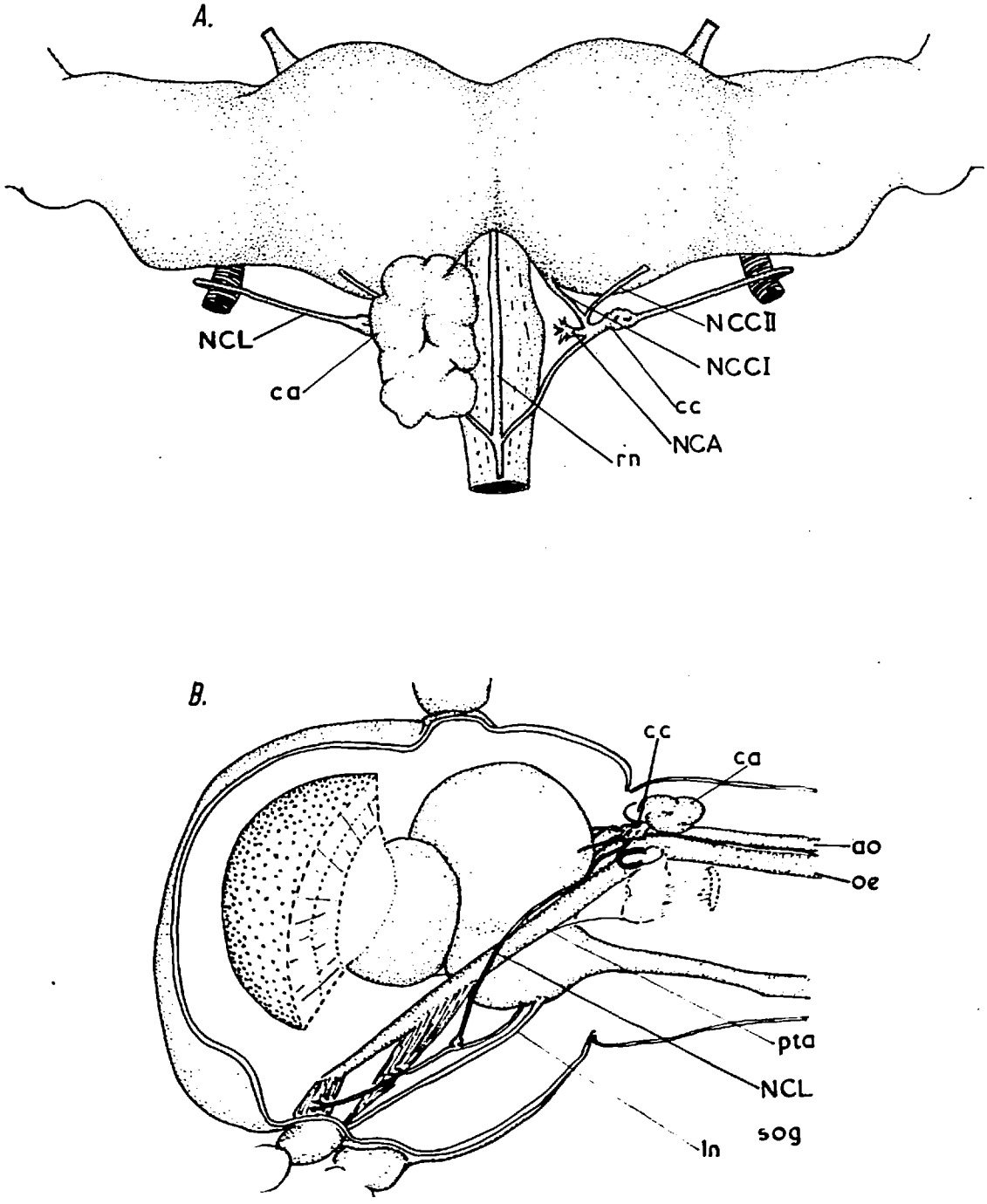

Fig. 2. The neuroendocrine complex of P. cynthia ricini. A. Dorsal aspect. B. Lateral aspect, ao, aorta; ln, labial nerve; oe, oesophagous; pta, posterior tentorial arm, other lettering as in fig. 1.

III HISTOLOGY.

(1) Classification of Neurosecretory Cells.

In reviewing the published research on neurosecretion in insects up to 1959, van der Kloot (1960) drew attention to the need to differentiate between neurosecretory cells as defined by Scharrer (1956) and "neuro-endocrine" cells. Sharrer's criterion, "nerve cells which show cytological evidence of secretory activity" undoubtedly includes neurones that have no endocrine function. Bern (1962) and Bern and Hagadorn (1962) have dealt extensively with the problem of defining neurosecretion. Bern (1962) states "a modern conception of neurosecretion includes the attachment of functional significance to neurones possessing morphologic indicators of secretory activity." He later points out, that, "acceptance at present is based on little more than the confidence that functional activities and specific hormonal agents with eventually be found." This is indeed the case for every accepted neurosecretory cell in insects. While there is a great deal of experimental evidence in support of the view that the products of neurosecretory cells are hormones, in no case is the chemical nature of the hormone known. Histological methods therefore, remain as the principal means of studying these cells.

The development and refinement of the PAF sequence has permitted differentiation of at least 4 major types of neurosecretory cell. As yet no standard procedure for fixation and staining has been adopted. Variations in technique that are encountered are aimed at specificity and balance of staining to impart some col-

oration by each of the 5 dyes comprising the PAF method.

The alphabetical symbols that are now widely used to denote different neurosecretory cells, follow several systems of classification. Consequently the literature is quite confusing; it is often difficult to relate the system of one author to that of another. Delphin (1963, 1965) made a detailed study of the neurosecretory cells in the ventral cord of Schistocerca gregaria, he describes 4 major types of cells, A, B, C, and D, further subdivided into A_1, A_2, and A_3 and B_1 and B_2. As part of this study he has brought together much of the literature up to 1962 and related the types of cells described in 23 papers to his system of classification. All attempts at classifying neurosecretory cells derive from the work of Mayer (1955) who recognised A and B cells in the brain of Inlita limbata, based upon CHP and Azan staining, but failed to obtain selective staining with PAF. In a successful application of the PAF technique, Johansson (1958) extended the classification to include C and D cells, although the endocrine function of these additional types of cells seemed uncertain. Highman (1961) adopted Johansson's classification, and applying it to PAF-stained sections of S. gregaria brain found A, B, C and D cells; their staining characteristics together with dimensions are given. These 2 papers form the basis of Delphin's more comprehensive system. Highman (1961) describes C and D cells as staining pink with sparse reddish-purple inclusions; the larger diameter of the D-cells distinguishes them from C-cells. By altering the ratio of the dyes in the trichrome counterstain Delphin (1963) was able to impart a green background colour in C- and D- cells, which

revealed a difference in the cytoplasmic morphology of the 2 cells. In D-cells the reddish-purple inclusions lie in clear areas of cytoplasm, appearing as vacuoles, whereas C-cells cytoplasm stains uniformly green.

Delphin based the differentiation of A cells upon; (i) background staining, greenish-orange in A_2-, but absent in A_1- and A_3- cells. (ii) stainability with Agan; A_2- cell inclusions bright red, A_1- and A_3- cells uncoloured. (iii) dimensions; A_2- and A_3- constant (22.0 and 45.0 u respectively), whereas A_1- cells vary from 11.0-18.0 u in diameter.

Panov and Kind (1963) have described the neurosecretory cells occurring in the brain of 20 species of Lepidoptera, but their system of classification is only in partial agreement with Delphins. While recognising A_1-, A_2- and B- cells they make no reference to C- or D- cells, and where further classification is necessary give groups of cells a topographic designation; dividing the medial cluster into, M_1, M_2 and M_3, and lateral cells into L_1, L_2 and L_3. Panov and Kind (1963) found a remarkable constancy in numbers of cells in each hemisphere of every species; A_1 (4), A_2 (4) in M_1 group, 2 A cells in M_2 and M_3, 5 or 6 A- cells in L_1 and 2 B- cells in L_2 and L_3 groups.

The only other comparable study of this nature, again in Lepidoptera, is that of Mitsuhashi (1963). After the application of PAF, he describes medial, lateral, posterior and optic groups of cells. However, his results are at variance with those of Panov and Kind with respect to numbers of cells, types of cell, and their distribution. Of the 8 species he studied, the number

of medial A- cells varies from 3-9 in each hemispheres and from 5 to 9 B- cells. He reports a similiar variation among the lateral cells, with some species having no B cells. His system of classifying the cells is identical to their which Mitsuhashi and Fukaya (1960) used for the larva of Chilo suppressalis; in which A- cells are recognised as PAF-positive and B- cells as less positive. The neurosecretory cells of the brain of Ostrinia nubilalis have been described by McLeod and Beck (1963) who report the presence of 9 cells bearing PAF-positive inclusions in each medial group, regarded as 1 type of A- cell, and in addition 2 B- cells recognised as staining green with no PAF-positive inclusions. Herman and Gilbert (1965) have given a detailed description of the neurosecretory cells in the adult Hyalophora cecropia. They recognise 4 categories of A- cells in the medial cluster, designated; 'small-', 'large-', 'deep-', and 'posterior- A cells'. B- cells occur medially and laterally, with a fifth category of lateral-A cells. Their system of classification is essentially similiar to that of Panov and Kind (1963) and McLeod and Beck (1963) in which A- cells are PAF-positive and B- cells PAF-negative. While this fundamental differentiation of A- and B- cells has been most extensively used, a different but quite comprehensive system has been proposed by Raabe (1965) following a detailed study of the neurosecretory cells occurring in the ventral cord of the phasmid, Clitumnus extradentatus. Four categories of cells are recognised; A, B_1, B_2 and C. The first three are PAF-positive; A-, B_2- and C- cells stain with Azan (but only after Helly fixation in the case of the C- cell),

and B_1- and B_2- cells give a positive reaction with RSR reagent (indicating sulphydryl and disulphide groups). This system has been adopted by Chalaye (1965) in a description of the neurosecretory cells in the ventral cord of Locusta migratoria, and added a further category, A_1- cells; as being PAF-positive, but uncoloured by Azan or RSR. From the tinctorial affinities of the cells classified as B_1, B_2 and C it is apparent that they do not correspond to the B- and C- cells of Johansson and others.

It is recognised that the classification of neurosecretory cells is quite arbitrary, however adherance to one author's system is becoming increasingly necessary. While the system of Raabe and Chalaye offers some chemical rationale, individing fuchsinophil cells into B- and A- cells according to the presence or absence of -SH groups, it necessitates the use of 2 fixatives and 3 staining procedures to differentiate 5 types of cells. Care in the application of PAF, as Delphin (1965) has demonstrated, permits recognition of 7 types of cell in S. gregaria. Furthermore, the fundamental division of cells into those that are PAF-positive (A cells), and those not so (B- cells) has been more widely adopted. Consequently the classification of Delphin (1963, 1965) is used in this study, with the exception that B- cells are recognised by the complete absence of PAF-positive inclusions. The staining reactions of these 4 basic cell types has been clearly enunciated by Siew (1964).

(2) Staining Reactions of the Neurosecretory Cells.

The following scheme of classification, based upon PAF-staining

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recognises the 4 major classes of cells described by Siew (1965a). and Dolphin (1965). Further division of 3 of these has been necessary to embrace all of the types of cells which occur in the brain of Lepidoptera, but they do not necessarily correspond to Dolphin's sub-divisions. In sub-dividing A- cells I have adhered to the A_1/A_2 division of Panov and Kind (1963) and created further categories for cells appearing consistently distinct.

A- Cells.

Inclusions strongly PAF-positive, often coalesce to form aggregates of varying sizes. Cytoplasm pale greyish-green, pink or colourless; always colourless when inclusions are sparse.

A_1- Cells: Inclusions bright purple or reddish-purple usually form very distinct granular aggregates. Occur only in the medial group. Neurosecretory material often present in the axons.

A_2- Cells: Inclusions either bluish-purple or purple-brown, large accumulations form dense aggregates appearing as flakes. Occur only in the medial group, interspersed among A_1- cells. Nsm often present in the axons.

A_3- Cells: Inclusions purple, consisting of very small granules evenly distributed in some species; forming a fine reticulum in others. Cytoplasm usually dull greyish green, occasionally colourless. Occur only in the medial group, lying posterior to the A_1- and A_2- cells in most species, both posterior and anterior in others. Neurosecretory material only occasionally visible in the axons, which have a characteristically large diameter. When depleted of secretions they appear as 'giant' neurones.

A4- Cells: Inclusions greenish-purple, have either a coarse granular form or appear as flakes. Cytoplasm colourless or pale grey-green. Comprise the lateral A- cells. Always considerably smaller than any of the medial A- cells. Nsm occasionally visible in the axons, which are invariably fine.

B- Cells.

Inclusions always PAF-negative, but having a strong affinity for one or more of the counterstain; green, orange-green, or orange; occasionally staining additionally with chromotrope 2 R, then colouring orange-brown or brown.

B_1-Cells: Inclusions fine and evenly distributed in most species, but appearing as small aggregates or flakes in others. Bright green when inclusions are plentiful, brownish-green to orange when few or no inclusions are present, at which stage the cells are usually smaller. Form a discrete block of cells in the medial group, lying between and beneath the A-cells. Neurosecretory material frequently visible in the axon hillocks, but less frequently in the axons.

B_2- Cells: Inclusions bright green, blue-green or brown, depending upon the species. Occuring in most species as very fine evenly distributed granules, less commonly as aggregates, which often have a whorled appearance. Comprise the lateral B-cells. Their axons can be traced for only a short distance, as neurosecretory material is rarely evident in them.

C-Cells.

Sparse finely granular PAF-positive inclusions, colouring

reddish-purple when separate, but purple in some species where they coalesce to form small streaks of irregular shape or a disrupted reticulum. Background cytoplasm bright green, or, bright orange in which case it has a distinct granular form.

C_1-Cells: Inclusions variable in shape, sometimes absent. Cytoplasm intense green or exceptionally orange. One cell occurs in the medial group of each brain hemisphere, lying between A_1- and A_2- cells. Larger than B- cells. Axons usually fine with PAF-positive inclusions together with inclusions staining the same colour as the cytoplasm occasionally visible.

C_2-Cells: PAF-positive inclusions always present, but individually beyond resolution, when abundant form darker staining patches of ill-defined outline. Cytoplasm stains similiarly to the B_2-cells. One or two cells occur laterally, associated with B_2-cells. Axons fine with PAF-positive neurosecretory material occasionally evident.

D-Cells.

Small PAF-positive inclusions distributed in bright green cytoplasm, each inclusion surrounded by an area of clear cytoplasm having the appearance of vacuoles. Neurosecretory material occasionally visible in the axon hillocks, axons very fine and can only be traced a short distance.

The staining reactions, after PAF, CHP, ABP and Azan, of the neurosecretory cells and other structures of the brain are summarised in table 1, and cells of each type are illustrated in Fig. 3.

Table 1.

	PAF	CHP	Azan	ABP
A_1	Bright purple	Blue	Red	Pale Blue
A_2	Purple-blue	Blue-black	Orange or orange-red	Blue
A3	Pale purple	-	Grey	Pale Blue
A_4	Dull purple	Blue	Blue	Pale Blue or Pink
B_1	Green or orange	Pink	Blue, red inclusions	Red
B_2	Green	Pink	Red	Pink
C	Green or orange, purple red inclusions	-	Orange, red inclusions	Red
D_1	Green, purple-red inclusions	-	Pale blue	Blue-green
D_2	"	-	Pale blue	Pale Blue
Neurilemma	Dull purple	Purple-blue	Purple-blue	Blue
Neuropile	Green	Pink	Pale blue	Pale blue
Other neurones	Orange or pale green	Pink or blue	Orange-red	Pale blue
Trachea	Purple-red	Blue	Grey-brown	Blue

(3) Survey of the Neurosecretory Cells Occurring in the Brain of various Lepidoptera.

The recognition of A_3-, C- and D- cells depends, to a large extent upon satisfactory preparation of the tissues. While Bouin, or Picro-Susa is suitable for most species, these cells are not always apparent. In the following table the numbers of each cell type are given for 23 species of Lepidoptera, representing 10 families. Instances where a particular type of cell could not be identified with any degree of confidence are left blank. The numbers represent the count for each cell type in one hemisphere of the brain, except the B_1-cells, which occur as one discrete group in the midline; for this cell, the figure is the total number.

Table 2.

SPECIES	Medial Neurosecretory cells.						Lateral Neurosecretory cells.			
Saturniidae:	A_1	A_2	A_3	B_1	C	D_1	A_4	B_2	C_2	"D_2"
Philosamia cynthia ricini	4	4	2	28	1	2	6	4		2
Samia gloveri	4	4		120	1		5	2	1	1
Sphingidae:										
Herse convolvuli	4	4	2	180	1		5	3	1	
Celerio euphorbiae	4	4	2	50	1	2	5	3	1	2
Deilephila porcellus	4	4	2	60	1	2	5	3	1	
Notodontidae:										
Stauropus fagi	4	4		24	1		5	3	1	
Notodonta zic-zac	4	4	2	34	1		5	3	1	
Pheosia tremula	4	4		40	1	2	5	2	2	
Phalera bucephala	4	4		45	1	2	6	3	1	
Arctiidae:										
Spilosoma lubricipeda	4	4			1		5	3	1	
Noctuidae:										
Bena prasinana	4	4	2	52	1	2	5	3	1	
Agrotis clavis	4	4		190	1		5	2	2	
Agrotis ipsilon	4	4	2	82	1		5	3	1	
Diaparopsis castanea	4	4		36	1		5	3	1	
Rusina umbratica	4	4		30	1		5	3	1	
Triphaena pronuba	4	4	2	84	1	2	5	3	1	
Geometridae:										
Biston betularia	4	4		25	1		5	3	1	
Colotois pennaria	4	4	2	25	1		5	2	2	

SPECIES	Medial Neurosecretory cells.						Lateral Neurosecretory cells.			
	A_1	A_2	A_3	B_1	C	D_1	A_4	B_2	C_2	"D_2"
Thyatiridae:										
Tethea ocularis	4	4	2	40	1		5	3	1	
Habrosyne derasa	4	4	2	40	1		5	3	1	
Zygaenidae:										
Zygaena filipendulae	4	4			1		5	3	1	
Cossidae:										
Zeuzera pyrina	4	4			1	2	5	3	1	
Hepialidae:										
Hepialus humuli	4	4			1		5	3	1	

The A_1-cells described and enumerated here correspond to the A-cells of Kobayashi (1957), Arvy and Gabe (1962), Mitsuhashi (1963) and to the 'large medial' A-cells of Herman & Gilbert (1965), the A_1-cells of Panov and Kind (1963) and probably to the larger of the 2 cells described in the brain of Mimas tiliae by Highnam (1958) A_2-cells correspond to A_{11}-cells of Panov & Kind, the "small medial" A-cells of Herman and Gilbert and B-cells of Mitsuhashi, and some of the smaller cells in M. tiliae.

A constant number of both A_1- and A_2-cells are readily differentiated, furthermore in most species there is a noticeable difference in both cell and nuclear dimensions, and the appearance of the inclusions (Table 3, and fig. 3).

A_3-cells were identified in 13 species, the characteristic appearance of the cytoplasm is particularly clear in T. pronuba, C. euphorbiae, A. ipsilon and D. castanea. From their location and staining reactions these cells undoubtedly correspond to the M_3 group of Panov and Kind, and the 'posterior' A-cells of Herman and Gilbert. When present just 2 cells occur in each hemisphere. (Fig. 3).

Five A_4-cells comprise the lateral A-cells in most species, exceptions are P. cynthia ricini and P. bucephala which have 6. Panov and Kind similiarly found 5 cells in 18 species, but 6 in Bombyx mori and the larva of P. bucephala. (Fig. 3).

The B_1-cells described here have not previously been recognised as neurosecretory cells in Lepidoptera. In many species, it is only with difficulty that they can be differentiated from

Table 3. Differentiation of A_1- and A_2-cells.

Mean maximum diameters (μ).

	cell	nucleus	cell	nucleus
	A_1		A_2	
P. cynthia ricini	38.0	11.0	24.0	9.0
C. euphorbiae	40.0	15.0	37.5 x 31	12.5
B. prasinana	26 x 23	10.5	21 x 15.5	8.5
T. pronuba	39 x 29	13.5 x 10.5	38 x 29	11 x 8.5
D. castanea	22.5 x 17.5	8.0	21 x 17	7.0
P. bucephala	26 x 21	10.0	18.5 x 15.5	8.5

Appearance of neurosecretory material.

	Colour of inclusions		Form of inclusions.	
	A_1	A_2	A_1	A_2
P. cynthia ricini	purple	purple-blue	granules	coarse granules.
C. euphorbiae	violet	purple-brown	coarse granules	coarse granules.
B. prasinana	purple	purple	sparse aggregates	dense aggregates
T. pronuba	purple	purple-blue	coarse granules	flakes
D. castanea	purple	purple-blue	flakes	flakes
P. bucephala	purple	dull violet	large granules	flakes

adjacent neurones. However in all of the noctuids examined they have characteristic appearance and staining reaction which is in accordance with the description of B cells given by Kopf (1957b), Johansson (1958), Highnam (1961), Siew (1965a), Thomsen (1965) and Hsiao & Fraenkel (1967). These cells appear to undergo cycles of secretion in T. pronuba and A. ipsilon and their axons can be traced through the brain (together with A-cell axons) bearing the green-staining colloid. However, in many species the cytoplasm is sparse and has little affinity for light green, phloxine or Azan. Identification is further complicated by their relatively small size; in some species, notably saturniids and geometrids, the demarcation between B-cells and other neurones is indistinct. This probably accounts for the fact that these B-cells were overlooked by Herman & Gilbert (1965), Ichikawa (1962) and Yokoyashi (1957) who studied neurosecretory cells in saturniids only (Fig. 4).

B_2-cells vary from one species to another in both configuration of inclusions in the cytoplasm and stain affinity. In all species stained with ABP or CHP, they are phloxinophil; with PAF they usually stain a shade of green but are orange-brown in C. euphorbiae and dull brown in P. cynthia ricini. With Azan B_2-cells stain bright red, thus resembling A_1- and A_2-cells. Panov and Kind (1963) found 4 lateral B-cells in every species they studied, whereas Herman and Gilbert (1965) could find only 2 cells in the saturniids; Hyalophora cecropia, Antheraea polyphemus and Philosamia cynthia. In the 23 species comprising this study 3 B_2 cells

were identified in most species, 4 in 1 species and 2 in the remainder. A total of 4-cells were found in each hemisphere having similiar dimensions and staining reactions, but the one cell and occasionally 2 cells contained PAF-positive inclusions. To be consistent with the classification adopted here, these have been put in a separate category (Fig. 4).

The single C_1-cell present in the medial group of each hemisphere was located in every species studied. This cell is most conspicuous, is intensely phloxinophil, and has previously been regarded as a B-cell; the "medial" B-cell of Herman and Gilbert (1965), M_1-B-cell of Panov and Kind (1963) and the single large phloxinophil cell of Ichikawa (1962). Mitsuhashi (1963) describes a single C-cell in each hemisphere in larvae of Euproctis flava and Dendrolimus spectabilis. Following PAF-staining, the appearance of the C-cell conforms to the C-cells of Siew (1964) and Delphin (1965), in all species except P. cynthia ricini, in which the cytoplasm is granular and stains bright orange. The size, shape and number of PAF-positive granules or aggregates varies from one species to another; they are particularly clear in C. euphorbiae and A. clavis (Fig. 4). The inclusions are frequently masked by dense background staining with light green or orange G., and they are best examined before counterstaining.

C_2-cells occur laterally with B_2 cells and differ in several ways from C_1-cells. Some PAF-positive material is always present and imparts a faint purple-pink colour to these cells before counterstaining, but whether this is due to the presence of PAF-

positive inclusions could not be decided; resolution of individual inclusions is not possible with the light microscope. After counterstaining C_2-cells have a bluish-green colouration, and where the PAF-positive material is most abundant, bluish purple patches of ill-defined outline can be observed. Occasionally this stainable material can be seen in the axon hillocks. It seems unlikely that these cells represent a phase in the secretory cycle of B_2-cells; in all specimens of each species examined a constant number of these cells is apparent, furthermore the dimensions of both cell and nucleus differentiates them from B_2-cells (table 4). In some species they are larger, in others smaller, but the size ratio of B_2/C_2 cells remains fairly constant in every species. (Fig. 4).

Table 4. Major diameters (u) of B_2/C_2-cells and their nuclei.

	Inclusions present (C_2)			Inclusions absent (B_2)		
	Number in each hemisphere	Cell	Nucleus	Number in each hemisphere	Cell	Nucleus
H. convolvuli	2	32.5 x 22.5	10.0	2	41.0 x 25.0	11.0
D. porcellus	1	12.5	10.0	3	13.5	5.0
P. tremula	2	16.0	8.0	2	19.0 x 12.5	8.0
N. zic-zac	1	25.0 x 15.0	7.0	3	14.5 x 12.0	8.5
T. ocularis	1	13.0	6.5	3	13.5	6.5
H. derasa	2	13.5	7.0	2	16.0	7.5
T. pronuba	1	16.0	12.0 x 7.5	3	24.0 x 17.5	8.0
B. prasinana	1	15.0 x 10.0	7.5	3	12.0	8.5

D_1-cells were identified in 8 species, and were located posterior to the A_1- and A_2- clusters in each hemisphere. In older moths the cytoplasm has less affinity for light green and superficially they resemble A_3-cells which lie postero-ventral to them. From their staining reaction and situation D_1-cells correspond to the "posterior" A-cells of Herman and Gilbert (1965) and to the M_2 group of Panov and Kind (19..3). However, the arrangement of the cytoplasm forming 'vacuoles' in which lie PAF-positive granules clearly differentiates them from A_3-cells, and places them in the class D-cells as described by Delphin (1965). The axons are often very large in diameter and can be traced far into the neuropile together with other neurosecretory cell axons. D_1-cells which appear to be actively secreting show fine strands of cytoplasm extending into the axons, but identification of inclusions was not possible; the vacuolar structure becomes disrupted near the axon hillock and individual inclusions are at the limit of optical resolution. In the brain of Oncopeltus fasciatus (Johannson, 1958) and in the brain of S. gregaria (Highton, 1961) the D-cells are the largest of the neurosecretory cells; in Lepidoptera they are always smaller than A_1-cells. (Fig. 4).

The lateral, D_2-cells were found only in P. cynthia ricini and C. euphorbiae, careful searching of sections of this area of the brain failed to reveal them in any other species. Their staining reaction clearly indicates a secretory function; PAF-positive granules are comparatively large (larger than D_1-cells) and are discharged at the time of emergence of the adult.

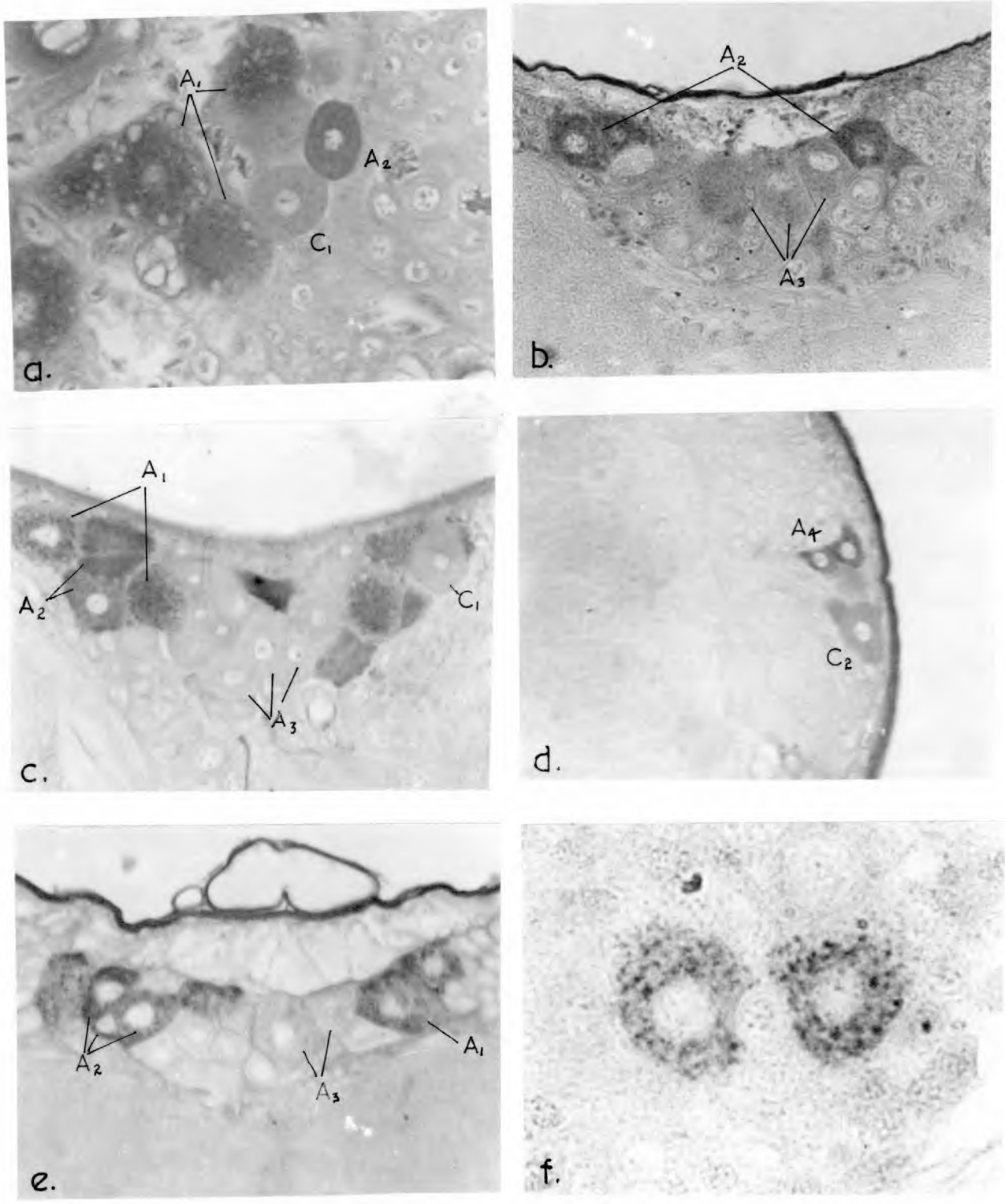

Fig. 3. The types of A-cells occurring in the protocerebrum of adult Lepidoptera. a.A_1- and A_2-cells in P.cynthia ricini b. A_2- and A_3-cells in D.castanea. c.A_1-, A_2- and A_3-cells in T. pronuba. d.A_4- and C_2-cells in T.pronuba. e.A_1-, A_2-, and A_3-cells in P.tremula. f.A_4-cells in P.cynthia ricini.

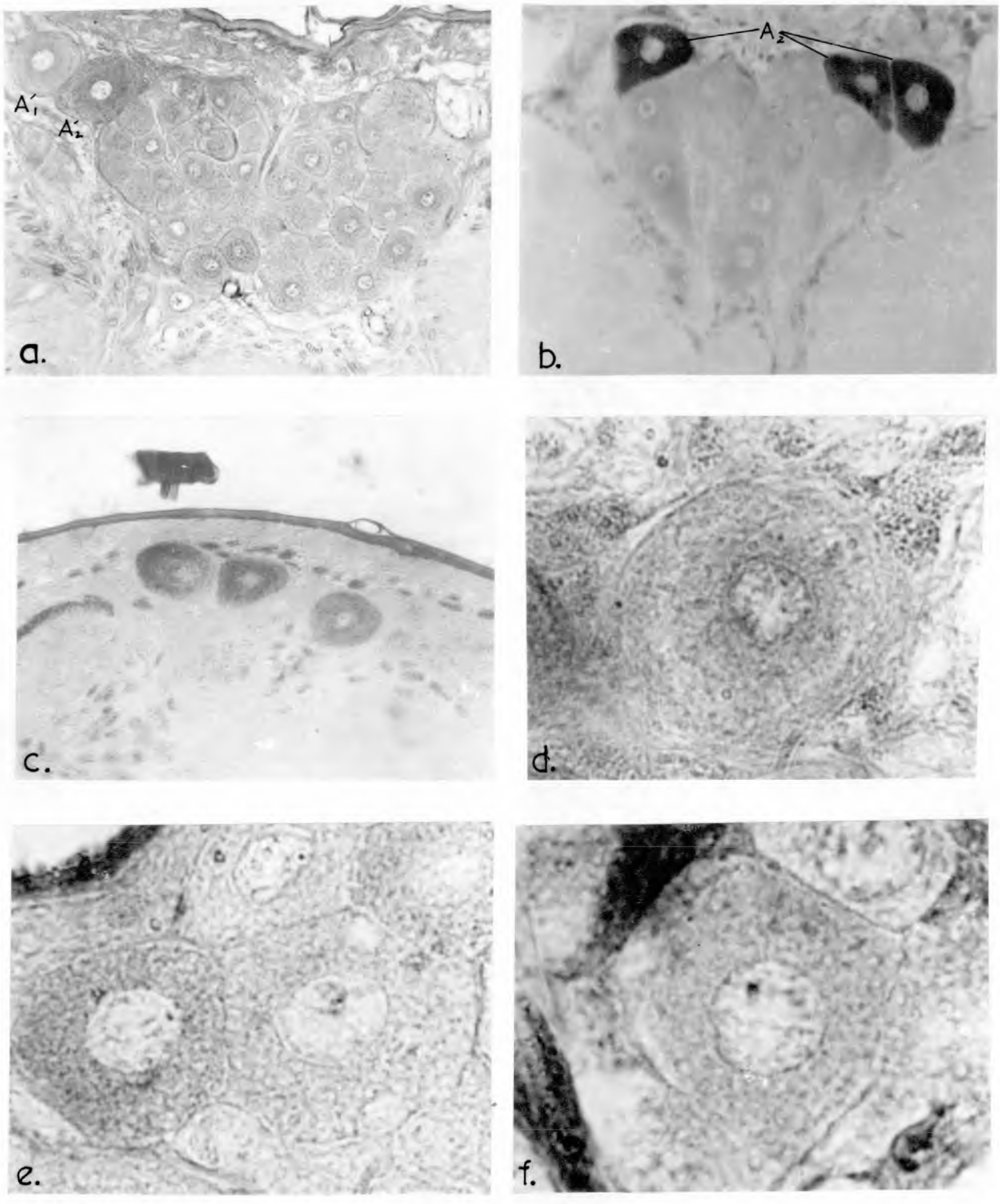

Fig. 4. B-,C- and D-cells in the protocerebrum of Lepidoptera. a.B_1-cells H.convolvuli. b.B_1-cells in T.pronuba. c.B_2-cells in P.cynthia ricini (stained with Azan). d.C_1-cell C.euphorbiae. e.C_1-cell A.clavis. f.D-cell in P.bucephala.

(4) Transport and Release of Neurosecretory Material

The axonal pathways of both medial and lateral neurosecretory cells are illustrated in figure 1. In all species the axons extend anteriorly for a short distance, then descend through the neuropile forming distinct tracts of fibres. The 2 main tracts cross over at a point just below the dorso-ventral deflection, forming a chiasma similiar to that which has been described in all insects so far examined.

Most frequently sections of the brain reveal only short lengths of the neurosecretory cell axons but occasionally favourable sections cut in the plane of the dorso-ventral course show tracts from the pre-chiasmatic region to the floor of the brain, at which point they recurve back and extend antero-posteriorly to their exit from the brain in NCC I. When the axons bear numerous PAF-positive inclusions in T. pronuba, it can be seen that, some axons cross over directly, whereas others divide into 2 branches. One branch crosses over to supply the contralateral corpus cardiacum, the others remain in the hemisphere of origin and supply the ipsilateral corpus cardiacum (fig. 5). Using a silver-staining technique, van der Kloot (1960, 1961) observed a similiar division and decussation in the brain of Hyalophora cecropia. Although the Golgi-dichromate method that he employed is not specific to neurosecretory material, it seems fairly certain that the axons described are derived from medial n eurosecretory cells.

Neurosecretory granules become more difficult to discern in the distal extensions of the axons (in NCC I and II), and only occasionally are PAF-positive inclusions visible in the tracts within the corpora cardiaca. In no instance was any accumulation of neurosecretory material evident in the corpora cardiaca; it therefore, appears that these glands do not have the same storage function, or capacity in adult Lepidoptera, that is well documented in some other insect orders. Of the nerves arising from the corpora cardiaca small quantities of neurosecretory material were sometimes visible in the proximal portions of NCL and NCA, but never observed in the finer nerves extending to, or into the corpora allata.

Axonal transport of neurosecretory cell products can be clearly studied in PAF-positive material only, secretions from B- and C-cells stain so similiarly to the neuropile that lack of contrast precludes clear definition at any distance from the respective axon hillocks. Nevertheless, sections cut in the plane of the chiasma occasionally reveal the presence of green-staining droplets in axons intermingled with those bearing PAF-positive inclusions. It seems likely that B- and C-cell secretions are transported to NCC I. No division of B-cell axons was ever observed; they form a simple chiasma.

(5) Post-embryonic Development of Neurosecretory Cells.

The histology of the neurosecretory cells during development in Lepidoptera, has been studied in detail in Ephestia kuhniella (Rohm, 1951) and Pieris brassicae (Rohm, 1955). Using Azan and Heidenhains haematoxylin she recognised but a single type of cell and enumerated 8 medial and 3 lateral one in each hemisphere. Arvy, Bounhiol and Gabe (1953) provide a short account of the post-embryonic changes occurring in Bombyx mori, although they used CHP and Azan, only one type of cell is mentioned. More recently, in a very brief account of the developmental changes in the neurosecretory cells of H. cecropia, Herman and Gilbert (1964) describe 8 types of cell and find that each type exhibits secretory activity during at least one stage of development. Other studies on neurosecretion in lepidopteran larvae have been confined to changes associated with diapause (Mitsuhashi & Fukaya, 1960, Mitsuhashi, 1961, Waku, 1960, McLeod & Beck 1963).

In the present study the distribution and numbers of A-cells occurring in larvae are in agreement with those of Panov & Kind (1963) and Herman & Gilbert (1964). However, the discovery of medial B-cells in adults raises the question of their derivation and, if present, their activity in immature stages. The present study together with previously published accounts show that the medial A-cell complex comprises 3 distinct types of cell; while Herman & Gilbert (1964) state that activity of one type of medial A-cell (and the lateral A-cells) is correlated with moulting they give no indication whether it is the A_1- or A_2-cell that is concerned.

The incidence of a virus infection in T. pronuba larvae produced many individuals with a sub-lethal infection and a staggered rate of development. Consequently, analysis of neurosecretion during development was confined to larvae and pupae of P. cynthia ricini.

A single group of 1st instar larvae were dissected, 2 days after eclosion. During the 2nd and 3rd instars, 3 groups were dissected i.e., freshly moulted larvae (post-moult), mature larvae and moulting larvae. During 4th and 5th instars and 'pre-pupae' larvae were dissected at daily intervals. The commencement of spinning was taken as the onset of the pre-pupal stage. After pupation dissections were made at daily intervals for the first 5 days and at 3 or 4 day intervals thereafter.

1st instar larvae. Using the complete PAF sequence neurosecretory cells are barely discernible as the cytoplasm of practically all neurones stains intensely with haematoxylin and Orange G, probably signifying an abundance of RNA and acidophil proteins. However, sections stained with PAF, but with the counter-stains omitted, reveal 4 A_1-, 2 A_2-cells medially and 2 posterior A_3-cells in each hemisphere (fig. 5).

2nd instar larvae. Freshly moulted larvae have 4 A_1-, 2 large and 2 small A_2-, 1 C and 2 D-cells medially, 2 A_3-cells posteriorly but lateral cells are not apparent. Twelve hours later 5 A_4- and 4 B_2-cells are just visible laterally, and the medial cells stain more intensely. In the mature 2nd instar larvae, the lateral cells are clearly visible and the medial A_2-cells are all of equal

diameter. In neither 1st instar nor 2nd instar larvae were any mitotic figures evident in cells near the lateral and medial neurosecretory cells; the appearance of A_2-, C-, D- and lateral cells is most likely caused by differentiation of existing neurones at these stages.

Subsequent instars. The cells present in the mature 2nd instar larvae are evident in all subsequent instars and no other neurosecretory cells were observed other than 2 D_2-cells in the 4th instar. Apart from B_1-cells the numbers of all other types are the same as in the adult brain.

Pupae. No additional cells were evident up to the 5th day after pupation, but by the 7th day imaginal development of the brain results in a closer fusion of the hemispheres with a reduction in the antero-dorsal cleft. Between the neurilemmal remnants of the cleft and the medial A-cells a compact group of small neurones develops. A few of these cells bear green inclusions which delineate them as the developing B_1 cells. After 14 days after pupation, the B_1 cells are larger, and more cells contain inclusions (fig. 5) at this stage each cluster consists of about 10 cells; increasing to a total of 28 by the 20th day. This is the number found in the adult, and B_1-cells of the 20 day pupae are completely differentiated and meet in the mid line forming a single group. The axons from each group can be clearly seen extending into the neuropile together with those from the A cells. (fig. 5).

While no distinct mitotic figures were observed among the differentiating B_1-cells, larger cells containing 2 nucloi were

occasionally observed. This observation is reminiscent of Fraser's (1959) suggestion that neurosecretory cells are derived by endomitoses; which has received further support by Mitsuhashi (1960).

The posteriorly situated A_3-cells are incorporated into the medial cluster by the time the pupa is 20 dyas old. One pair of cells does so in the 5th instar larva and the remaining pair do so during imaginal differentiation. Figure 5 shows the relative positions of these and some medial cells in the pre-pupa of T. pronuba.

A further 2 pairs of D cells are evident in the female pupae, appearing in the 20-day pupa of P. cynthia ricini (Fig. 5) and at 6 days in T. pronuba. These cells lie ventrally in the tritocerebrum, their axons extend dorsally, towards those of the medial cells; they were never visible in adults or larvae.

(6) Cycles of Secretion in Immature Stages.

Second and 3rd instar post-moult larvae show a marked reduction in the density of inclusions in A_1-, A_4-, B_2- and C-cells. (fig. 6). Immediately after moulting, before the cuticle of the head capsule becomes tanned, A_4- and B_2-cells are virtually devoid of inclusions. However, by the time the cuticle is tanned material is again visible in these cells, in fact quite large aggregates are apparent within them (fig. 6). Accumulation of material occurs in all cells until the larva ceases to feed before ecdysis. Release of neurosecretory material from A_1-, A_4- and B_2-cells commences after the first visible signs of ecdysis which is indicated by a change in colour of the cuticle and an antero-ventral deflection of the head capsule. Before this occurs, in the 2nd and 3rd instars C-cells begin to show a characteristic change in the cytoplasm as a large crescentic vacuole appears which partially encircles the nucleus, indicating a rapid release of material. (fig. 6).

Freshly moulted 4th instar larvae similiarly show a depletion of A_1-, A_4- and B_2-cells. The C-cells too, contain very little material. Two days after moulting A_1-cells contain a few inclusions but A_4- and B_2-cells stain intensely. The mature 4th instar larva (about 7 days after moulting) show signs of abrupt release from A_1-cells, which have meanwhile become packed with inclusions. The discharge of inclusions from A_1-cells at this stage is marked by the appearance of peripheral vacuoles in each cell (fig. 6), and coincides with a considerable deple-

tion of A_4- and B_2-cells, whereas C-cells are still packed with inclusions. In contradistinction to the A_4- and B_2-cells, and to these and A_1-cells in previous instars, the rate of synthesis of neurosecretory material in A_1-cells of the 4th instar proceeds slowly during the first 3 days, then, rapidly for the two following days. An accumulation of PAF-positive granules reveals the lateral D_2-cells at the beginning of the moult to the 5th instar. Later stages of ecdysis reveal a rapid restoration of inclusions in A_1- and C-cells but there is a complete loss of inclusions from D_2-cells and these cells become visible again only in the late pupae.

In the first 4 instars, the patterns of secretion and synthesis, as revealed by fluctuations in stainable inclusions, show a distinct cyclical activity in most cells in each instar. The activity of A_1-, A_4-, B_2- and C-cells is correlated with moulting. There are nevertheless some irregularities; for example the slow rate of synthesis of neurosecretory material in A_1-cells of the 4th instar and the paucity of C-cell material. This is however, likely to be due to the sudden increase in cytoplasmic volume of these cells at the 3rd moult (fig. 7) While there is a flux in the content of A_3-cells, no comparable phases of accumulation and secretion occur; there is usually a peripheral and perinuclear concentration of inclusions. Activity of the D-cells cannot be correlated with moulting as they usually show maximal staining density at all ecdysial stages; difficulties are often encountered in satisfactorily resolving the cytoplasmic constituents of these cells between the moults.

As far as A_2-cells are concerned, they show little fluctuation in content, however, neurosecretory material is evident in the axons after moulting when the greatest concentration of inclusions lies in the abaxonal region of each cell.

Fifth instar post-moult larvae have a relative abundance of inclusions in both A_1- and C-cells, (fig. 6) indeed, the latter are packed. In A_4- and B_2-cells the stainable material is very sparse, but unlike previous instars, at this stage, some material is evident in A_4-cells. During the first 3 days there is a progressive accumulation of inclusions in A_1-cells is much slower, and by the 3rd day these cells are still faint, but subsequently their content increases considerably and by the 7th day they stain densely (fig. 6). Release of secretory material commenced from A_1-cells on the 7th day. On the following day the A_4- and B_2-cells begin to discharge, and in the mature (10 day old) larva much of the densely aggregated inclusions of A_1-cells have been discharged and vacuolation is extensive (fig. 6). By this stage, A_4-cells are totally depleted, B_2-cells inclusions are very sparse, as also are those in C-cells which have the crescentic vacuole much enlarged. This phase of secretion constitutes the 'critical period' and it is interesting that A_2-cells are the only cells that are not active.

Prepupae: At the commencement of the prepupal stage the cuticle changes colour, silk is produced and the 'larvae' become very active. During the first day the phase of secretion which had previously commenced in the maturing larvae continues; A_1-, A_4-, B_2- and C-cells are either completely, or almost completely depleted.

A_2-cells also discharge and show a lower density of inclusions than at any preceding stage.

For the remainder of the prepupal stage (4-5 days) there is a progressive accumulation in A_1- and A_2-cells, although the latter appear to continue secreting for several days, as is evidenced by the lack of inclusions in the axonal poles of the cells and the axon hillocks. Very little material is apparent in A_4- and B_2-cells, but there is such a rapid accumulation in the C-cells, that by the 2nd day they are more or less packed with orange-staining inclusions. The 5th day prepupa shows little change in all cells other that C-cells which show an extensive loss of material.

Pupae. Twelve hours after pupation the histological picture is very similiar to that in the 5th day prepupa; A_1-cells have numerous uniformly distributed inclusions and few peripheral vacuoles. C-cells are again packed with inclusions but A_4-cells are completely depleted. A high rate of secretion appears to persist during the first 4 days, at the end of which A_1-cells are considerably vacuolated, A_2-cells bear few inclusions and the lateral A_4- and B_2-cells are quite devoid of any stainable material. Pupae dissected at 7, 10, 14 and 17 days from pupation show a progressive accumulation of inclusions, periods of secretion still occur which is evident in the presence of inclusions in the axons, but the rate of synthesis obviously exceeds that of release. Without a detailed knowledge of anatomical and histological changes in the other tissues it is impossible to suggest any correlations.

In the 20 day pupa (about 5 days before emergence) both medial, and lateral D-cells and A_3-cells contain numerous inclusions (fig. 8) which are subsequently released. This phase of accumulation and secretion by the B types of cells appears to be associated with emergence or final stages of imaginal development. PAF-positive inclusions are evident in axons of A_1- and A_4-cells, but absent from A_2-cell axons; otherwise the histological picture is similiar to that in the newly emerged adult. The development of B_1-cells has already been outlined; it is noticeable that B_1-cells show a peak development towards the latter part of pupal life and that the degree to which they take up light green stain diminishes in the adult. Furthermore, the presence of green inclusions in both their axon hillocks and distal parts of the axons indicate transport of this secretory product, and its probable release during this period. The secretory activity of B_1-cells appears, therefore, to be maximal in the pupae stage.

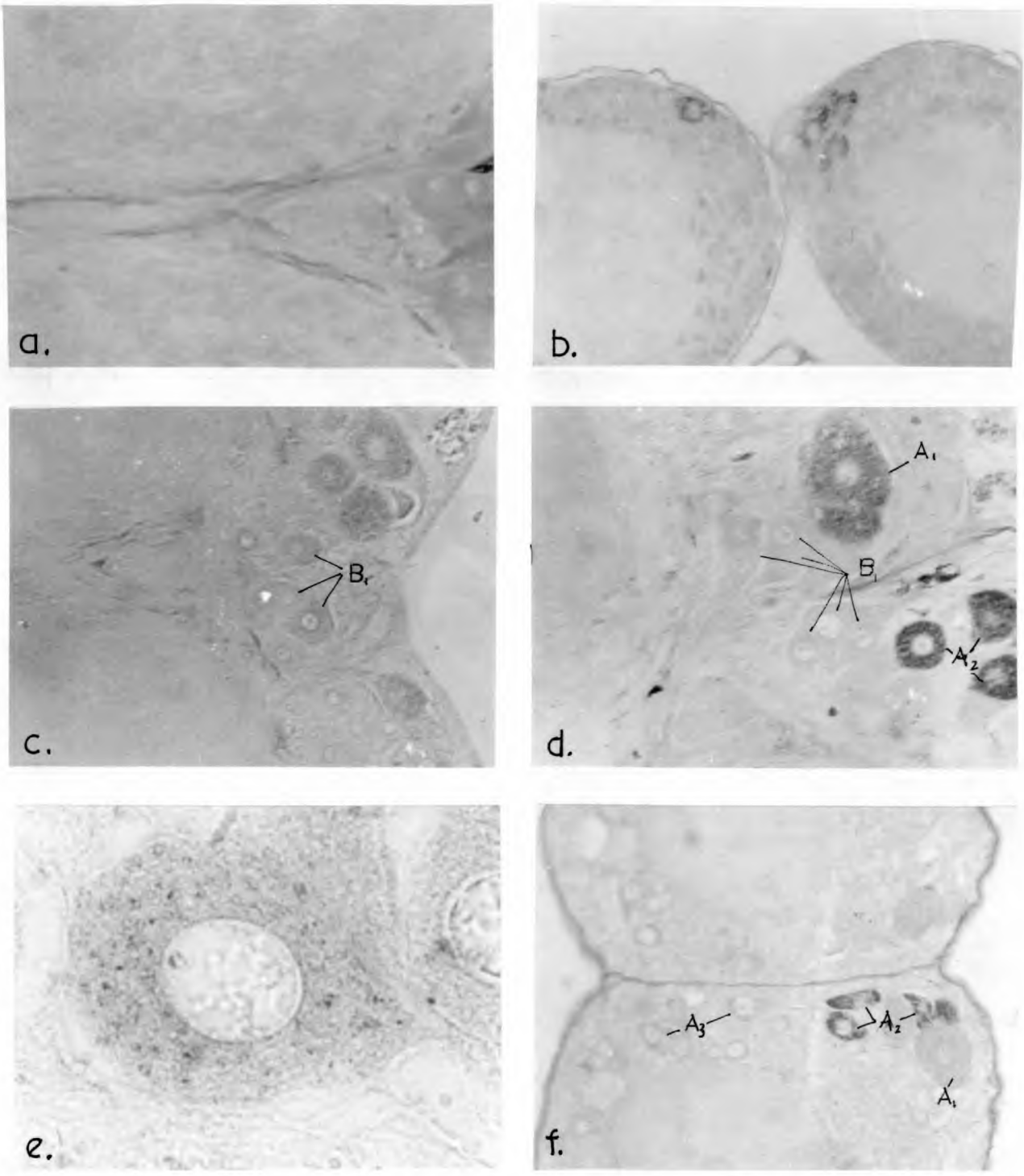

Fig. 5. a.The medial neurosecretory cell axon tracts in T.pronuba. b.A_1-cells in mature 1st instar.c.B_1-cells in 14-day pupa. d. B_1-cells in 20-day pupa. e.Ventral D-cells in 20-day pupa. f. A-cells in prepupa of T.pronuba. b-e P.cynthia ricini.

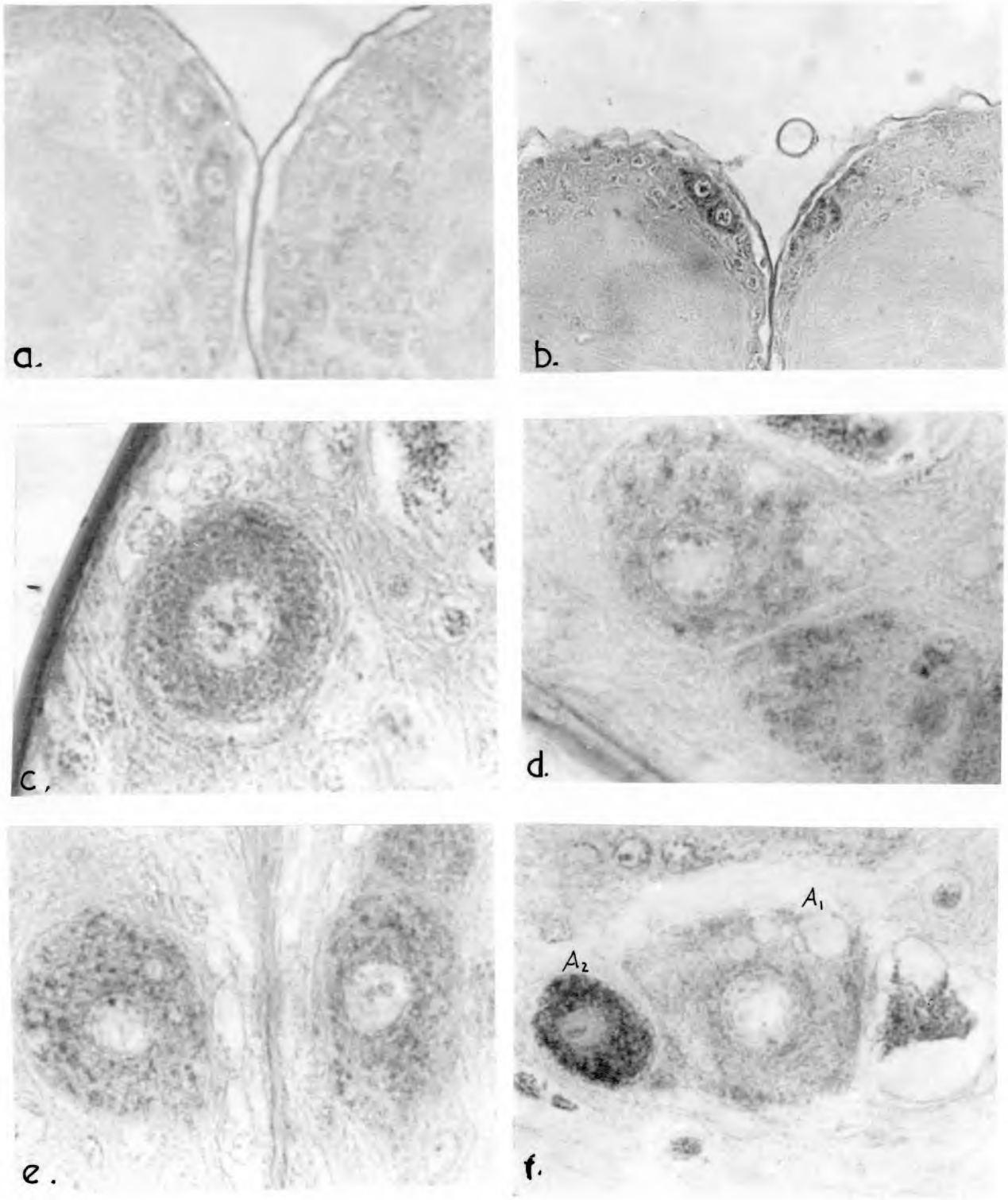

Fig. 6. Neurosecretory cells in larvae of P.cynthia ricini. a. A_1-cells in post-moult 2nd instar, cuticle untanned. b.A_1-cells in post-moult 2nd instar, cuticle tanned. c.C-cell in 4th instat, note peripheral vacuole. d.A_1-cells in 7-day 4th instar. e. A_1-cells in 2-day 5th instar. f.A_1-cell in 10-day 5th instar.

(7) Growth Increments of Neurosecretory Cells.

In table 5 the calculated volume of the cytoplasm of 6-types of neurosecretory cells are given at representative stages. It is apparent that the major increment in growth in each cell occurs during ecdysis. The volume of cytoplasm in A_1-, A_4-, C- and B_2-cells attains a maximum some 20 days after pupation, thereafter they decrease in volume. Initially A_2-cells show, a comparatively slow growth rate (up to the 3rd instar), which increases by a factor of 2.3 during the 3rd-4th instar moult, and by a factor of 2.4 at the following moult. Unlike A_1-, A_4-, C- and B_2-cells, the remainder (i.e. A_2-, A_3- and D-cells) reach their maximum volume in the adult; the growth rate of D_1-cells closely parallels that of A_2-cells.

Table 5. Development of Neurosecretory Cells in P. cynthia ricini volume of cytoplasm (μ^3).

STAGE	A_1	A_2	C	D	A_4	B_2
2nd instar, post moult	892	208	1,037	844	-	-
2nd instar, mature	1,499	268	929	929	334	731
3rd instar, post moult	2,394	268	1,394	1,218	584	1,109
3rd instar, mature	2,628	268	1,966	1,294	749	1,109
4th instar, post moult	5,196	617	6,920	2,823	1,294	1,546
4th instar, mature	5,992	971	6,860	2,673	1,375	1,924
5th instar, post moult	8,162	2,352	6,860	3,922	1,499	6,860
5th instar, mature	10,975	3,371	8,683	3,869	3,371	3,869
4 day old pupa	19,890	3,324	7,735	3,869	3,371	2,352
20 day old pupa	32,373	7,254	22,411	8,183	3,449	15,081
Adult	28,044	11,117	16,140	10,444	1,244	10,370

The development of A_3-cells is not related to any other cell type. From a gradual increase up to the mature 3rd instar larva, these cells rapidly increase in volume (by a factor of 4.3) during the following moults, after which they resume a gradual increase in volume to the adult stage.

Apart from A_3-cells, the most rapid growth occurs in A_4-cells which, in the freshly moulted 4th instar larva have a cytoplasmic volume greater than that in the adult. Expressed as a percentage of the volume of adult cells, the following figures are obtained; A_1-34, A_2-5, C-42, D-27, A_4-104 and B_2-15. Further increases in volume occur in all cells subsequently as indicated by the following percentages in the mature 5th instar larvae; A_1-32, A_2-33, C-53, D-37, A_4-270 and B_2-37; and in the 4 day old pupa:- A_1-70, A_2-30, C-47, (D-37), (A_4-270) and (B_2-22). The B_2-cells are of interest in this context, as they attain a volume of 66% (of the adult B_2-cells) in the post moult 5th instar larvae, but the volume declines to 22% in the 4 day old pupa; thereafter a pronounced growth occurs reaching 145% in the 20 day old pupa.

Each phase of cytoplasmic growth is usually preceded by an increase in nuclear volume which also occurs during ecdysis.

The calculated volumes of neurosecretory cell cytoplasm are of interest for several reasons; an abrupt increase in volume will have a 'diluting' effect upon the neurosecretory material present and may give a false picture of reduced density of stainable material. Increase in volume can be used as an index of present or impending hormonal requirement; and the relative rates of increase

among the various types of cell is likely to reflect their importance in respect to the major physiological events during development (fig. 7).

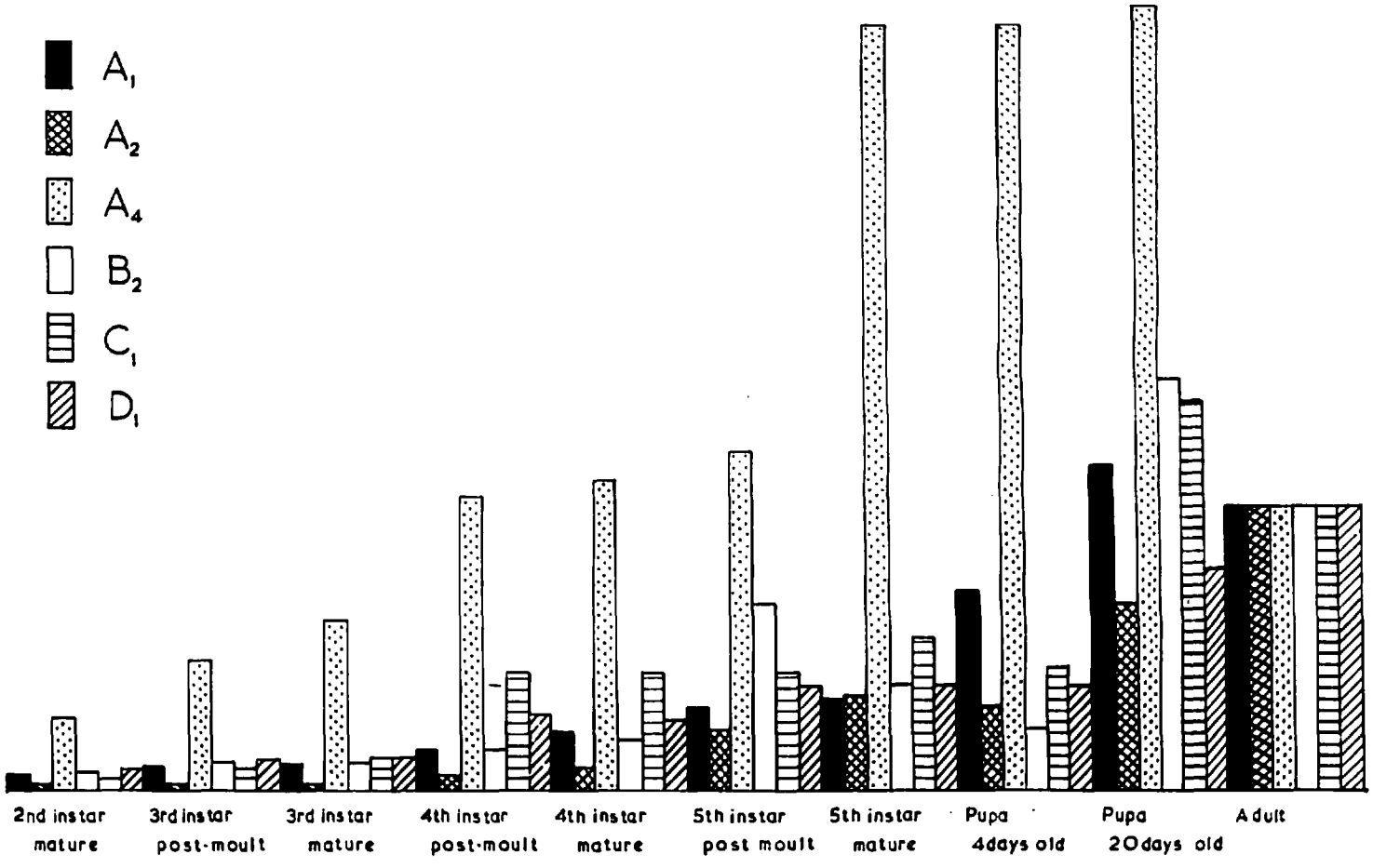

Fig. 7. The cytoplasmic volumes of neurosecretory cells during the development of Philosamia cynthia ricini, in relation to those of the adult.

(8) Neurosecretion in Adults: P. cynthia ricini and T. pronuba.

One major aspect of this study comprises an investigation into the relationship between the circadian flight rhythm and the activity within the neurosecretory cells of the brain. Males were primarily used to avoid any complicating factors that may arise in the females, in which cycles of secretion associated with reproductive cycles may occur. For most species light trap captures of males far exceed those of females, and this has aided this study. Nevertheless neurosecretory cells were examined in females from several species; it was found that precisely the same cells occur in both sexes, in fact the histological picture is remarkably similiar in all respects. However, as the females that are attracted to mercury vapour lamps appear much later than the males in most species, it is likely that this phenomenon occurs at a particular physiological phase. So far as I am aware no definitive study has been made of the neuro-endocrine system in relation to the reproductive cycle in female Lepidoptera. In their brief account of the brain neurosecretory cells during the development of B. mori, Arvy et al (1953) state that in young females a fresh accumulation and transport of the neurosecretory material occurs before oviposition, regardless of whether the moths have mated or not. To attempt such an investigation was considered to be beyond the scope of this study, but the occurrence of an identical neurosecretory cell system in both sexes arises the question of the function of these cells in males, where a distinct reproductive cycle may be lacking.

a. Philosamia cynthia ricini.

Adults are short lived in this species; females and unmated males live 7-8 days at 20°C, but mated males usually live 5-6 days. Like all other saturniids they do not feed, and moths dissected over an 8 day period show a progressive decline in the volume of the fat body. Krishnakumaran & Schneiderman (1964) have shown that in fact, death of saturniids is due to lipid depletion.

Effect of Age.

Neurosecretory cells were examined in both males and females at daily intervals from emergence until the eighth day. In this series all moths were chloroformed and dissected between 2.00 p.m. and 4.00 p.m. on each day. No major cycles of secretory activity were observed during adult life in any of the neurosecretory cells, but some minor changes do occur. The A_1-cells are vacuolated peripherally upon emergence, and this condition persists for the remainder of their life. With increasing age the vacuoles enlarge, tending to become confluent particularly at the axonal and abaxonal poles of each cell. Granule density also changes; in newly emerged moths the granules are largely aggregated, but become dispersed with age. No vacuoles were ever observed in A_2-cells, which, nevertheless show a greater diminution of PAF-positive material. The bulk of the stainable inclusions in A_3-cells are discharged before emergence, becoming almost completely depleted 3 days later. The lateral A_4-cells bear numerous aggregated inclusions shortly after emergence, but their concentration progressively decline during adult life. Nuclear volume and total cell volume does not alter in any of the A-cells, the appearance of the chromatin and

nucleoli remain equally consistent. Inclusions are frequently seen in axon hillocks but only rarely observed in axons traversing the neuropile.

The low affinity that B_1-cells have for any of the stains employed permit no conclusions to be drawn regarding dynamics of secretion in the adult. There are no fluctuations in volumes of cells or their nuclei, all of which are of closely similiar dimensions; they give the impression of having little secretory activity. On the other hand, B_2-cells stain distinctively and reveal a progressive increase in neurosecretory material. In no instance were B_2-cell inclusions observed in their axons.

The pair of medial C-cells were uniformly and densely stained at all ages, and 'favourable' sections reveal the presence of the orange-staining colloid in the axons, suggestive of a steadily maintained level of secretory activity. Inclusions from both D_1- and D_2-cells are discharged before and during emergence, no further accumulation takes place. The volume of the nuclei of D_1-cells decreases and the nuclear membranes become irregular in section. Together with the sparse chromatin this change is interpreted as indicating cessation of synthetic activity.

The loss of inclusions from A_1-, A_2- and A_3-cells, occurs gradually in unmated moths. When recently emerged males and females are put in a cage together, copulation usually commences during the first night. Both sexes are strictly nocturnal and females begin 'calling' from the first night after emergence. Calling, or release of the pheromone is accomplished by eversion of the terminal abdominal segments exposing the scent brushes and thus dispersing the

attractant. Copulation lasts from 24-48 hours during which time both sexes are inactive. Sectional brains of both males and females after 24 hours of copulation revealed little change in content of the neurosecretory cells, other than A_2-cells when compared with unmated moths of the same age. The quantitative changes occurring in any type of cell were small during any 24 hours period, nevertheless, it became evident that in mated moths as secretion is released from A_2-cells, whereas there is a reduction of inclusions in these cells in unmated moths. It seems possible that the lack of secretion in mating moths is associated with their inactivity.

Neurosecretion at Timed intervals during a 24 hour period.

Although a decline in stainable material is clearly apparent, neurosecretory material was rarely detected in the axons. As all these moths had been dissected during the afternoon, it is possible that secretion occurs at some other time in each 24 hour period. Examination of the flight rhythm had demonstrated that even in constant darkness this species adheres to a circadian rhythm of activity. In the male moth, apart from copulation, nocturnal flight activity comprises the major physiological event; participation of one of more types of neurosecretory cell in control of this endogenous rhythm, was therefore anticipated.

For this investigation, groups of moths of the same age were dissected at 12.30 p.m., 8.30 p.m., 12.30 a.m. and 8.30 a.m., only males were used and none had mated. After PAF, no differences were noticed between those dissected at 8.30 or 12.30 p.m., whereas axon hillocks of all A_2-cells showed a marked reduction in inclusions in moths dissected at 12.30 a.m., and to a lesser extent in those dissected at 8.30 a.m. Of 4 moths dissected at 12.30 a.m. one brain had been successfully orientated for sectioning whereby axons in the region of the chiasma were present in 2 sections and revealed numerous PAF positive inclusions. Only short lengths of A-cell axons could be seen in the remainder of this group, these too, unlike all others contained numerous inclusions. Although neurosecretory material can be clearly differentiated in the perikarya of A_1- and A_2-cells by their staining reaction, this is not possible with axonal inclusions. While there is a detectable loss of material from A_2-cell axon hillocks, there is no comparable loss from

A_2-cells at either 12.30 a.m. or 8.30 a.m. The possibility that A_1-cells also secreti at a similiar time cannot be discounted, but it seems more likely that the axonal material observed is derived from A_2-cells.

Induced Changes in Neurosecretory Cells.

Some preliminary experiments were conducted in an attempt to establish which type of cell, if any, control diuresis. Unmated male moths were selected from 3 age groups; 12 hr., 30 hr. and 5 days after emergence. Each moth received 0.2 ml distilled water, injected into the haemocoel of the abdomen. Their brains were dissected out and fixed either 7 hr., or 24 hr., after injection. An additional group of 3 five-day old moths, were each injected with 0.2 ml of 10% dextrose solution. The time at which injections were made was arranged so that dissections were made between 2.00 p.m. and 4.00 p.m. This allowed direct comparison with those previously prepared at this time at daily intervals.

All moths showed a reduction of inclusions in A_1-cells, but no changes were detected in any other cells. The reduction was most marked 24 hr. after injection and was accompanied by increased vacuolation. Twenty four hours after injection in the 5 day old moths a considerable amount of neurosecretory material was evident in the axons, and the hillocks were practically devoid of inclusions. In this group vacuolation had become so extensive that some vacuoles approached the nuclei. (fig. 8).

b. Triphaena proruba.

As many field moths were used it was necessary to assess the effects of feeding and of age upon the neurosecretory cells.

Longevity is very much greater than in P. cynthia ricini, as feeding in adults of T. pronuba is obligatory. Laboratory reared moths lived for as long as 2 months. In the field they are present from early July until late September, but emergence is spread over several weeks. They are strictly nocturnal and copulation occurs at night and is of a very short duration. The differences between the 2 species makes a comparative study of neurosecretion of particular interest.

Effect of Age.

A batch of pupae, from 1st generation laboratory reared stocks were retained for this investigation. The dates at which moths emerged were noted and they were separated into age groups. All dissections were carried out between 2.00 p.m. and 4.00 p.m., commencing from the day of emergence and thereafter at 5 day intervals. The final group were dissected after 45 days when the supply of moths had become exhausted.

Both A_1- and A_2-cells reveal a progressive accumulation of neurosecretion, but no cycles of secretory activity were apparent. Moths over 20 dyas old showed a very considerable density of inclusions, and this was paralleled in individuals captured in the field later in the season. In contrast A_3- and A_4-cells do show fluctuation in the density of inclusions, reflecting phases in cycles of secretion and of accumulation. These occurred at irregular intervals, but the processes to which they were linked did not become apparent. The A_3-cells together with D-cells discharge accumulated material in a period of secretion that commences before adult emergence, and continues for several days afterwards. Thereafter inclu-

sions gradually accumulate in D-cells. The lateral, B_2-cells showed no evidence of activity at any age, whereas B_1-cells appear to undergo marked cycles of secretion. The latter form a wedge-shaped group of cells that lies beneath the A-cells and which is easily distinguished from the latter and the adjacent neurones. Existing in two distinct phases, differential cell counts show a progressive recruitment to produce more cells in which neurosecretory material is present. These bear bright green inclusions without distinct outline, their nuclei are large and spherical and the chromatin is sparse and aggregated. In the distinctive pre-secretory phase the cells are much smaller with basiphibe cytoplasm, staining orange-red; the nuclei are also smaller, ellipsoid with evenly dispersed chromatin. It appears that this stage is followed by rapid synthesis of the neurosecretory material and by increase in volume. The elaborated material is equally rapidly discharged, but only when the cell is packed with inclusions. This cycle occurs independently within each cell, unlike that in all other types of neurosecretory cell.

The total number of cells is always 82; at emergence there are 32 cells bearing inclusions and 50 without. After 5 days there are 28 cells with inclusions and correspondingly, 54 without; thereafter, the ratio alters as follows:- at 10 days, 33 with and 49 without, at 15 days 40 with and 42 without, at 25 days 48 with and 34 without and at 30 days 52 with and 30 without. The ratio at 30 days does not alter at 35, 40 or 45 days. Old moths from field populations almost invariably have this ratio of B_1-cells. It maybe noted that the decrease in the number of cells with inclusions

was found consistently in 6 individuals examined 5 days after emergence. In fact very little variation was found between individuals of the same age. The progressive increase that occurs between 5 and 30 days after emergence could be interpreted as continued differentiation of B_1-cells that commences in the pupa. However, the initial decrease in the number of cells with inclusions supports the view of short term cyclical activity, and of diminution of requirement for this secretory material, since the number of cells bearing inclusions increases as the moths get older. The physiological role of these cells remains obscure, but they are of particular interest as they are the only type that is absent from larvae and are therefore functionally significant to the adult T. pronuba and primarily to the developing adult P. cynthia ricini.

C_1-cells remained constant in appearance throughout all age groups. They are packed with bright green-staining material with small reddish-purple inclusions which are widely but regularly dispersed. The lateral, C_2-cells on the other hand, shows signs of cyclical activity. The secretory product differs from that of C_1-cells in that the purple inclusions are more numerous but much smaller and while they are similiarly associated with green-staining material the proportion of purple to green is much greater. The accumulation of neurosecretory material in these cells becomes apparent with the formation of patches of purple and green material. Even when patches of inclusions are absent the cells stain faintly reddish-purple; this stage, or various stages of accumulation occur in different individuals of the same age. Furthermore the apparent

phases of accumulation and secretion occur irregularly and could not be correlated with any function.

It is immediately apparent that whereas in P. cynthia ricini there is a progressive loss of neurosecretory material in most cells, quite the opposite occurs in T. pronuba. Probably this is related to longevity, and the maintenence of synthetic capacity further indicates participation of most if not all cells in physiological events of the adult.

Neurosecretion during a 24 hr. period.

There is little change in secretory activity of the medial and lateral A-cells, with age. The moths had however, all been dissected within the same 2 hr. period on each day of sampling. Flight activity in this species shows a strong and a persistent endogenous rhythm. When subjected to 16 hr. illumination flight commences shortly after the lights are switched off (11.00 p.m. in the C.T. room). In a preliminary study moths were, therefore, dissected at 4 time intervals, when it was hoped to reveal the maximum fluctuations in granular density within the cells. This was based on the assumption that a circadian cycle of secretion occurred in this species, and was similiar to that in P. cynthia ricini. The times selected were; 9.00 p.m., 2.30 a.m., 8.30 a.m. and 3.30 p.m., 6 moths were dissected on each occasion.

By the use of the complete staining sequence (PAF) it became apparent that in some individuals, A_2-cells bear few inclusions but these tend to be obscured by the intense background staining. To obtain a clearer picture of the density of PAF-positive inclusions the counterstains were removed by rehydrating and thoroughly washing

in distilled water; slides were then dehydrated and remounted. With only PAF-positive structures remaining, A_1- and A_2-cells were readily estimated. The concentration of inclusions was scored on a scale from 1-4, in which 1 represents few inclusions, increasing to 2, and 3, and with a maximum of 4. The results are given in table 6. From this series it can be seen that in all moths dissected at 3.30 p.m. the neurosecretory cells (A_1 and A_2) are quite uniform and densely staining (fig. 8), which would explain the rather constant picture obtained in the study on ageing. However, the 3 other groups show quite considerable variation; although no clearly defined cycle of secretory activity is evident, in several moths both types of cells reveal secretion of material, and almost all A_2-cell material in a few moths (fig. 8). Nevertheless the fact that secretion does occur at these times, together with the constant appearance of cells with dense granular accumulations fixed in the afternoon demonstrates a circadian cycle of secretion. It is noticeable that when A_1-cells have discharged a considerable quantity of material and are at stage 2, A_2-cells are at stage 3 or 4. Conversely, when A_2-cells are at stage 1 or 2, A_1-cells are at stage 3 or 4. There is an appearance of an inverse relationship between the secretory activity of these 2 types of cells. However, the factors which govern secretion by these cells must be complex.

In preparing moths for this study no attention was paid to feeding; the captured moths used had been exposed to a feeding pad soaked in 1% honey solution. It is likely that the 24 moths that were examined had last fed on different occasions, as later observations showed that when allowed to feed at any time, over a long

Table 6. Concentration of inclusions in A_1- and A_2-cells at timed intervals during a 24 hr. period.

Time	Meth	A_1-cells	A_2-cells	Time	Meth	A_1-cells	A_2-cells
[illegible]	1	4	1		13	4	0
	2	2	4		14	4	1
	3	3	2		15	4	1
9.00 p.m.	4	3	2	8.30 a.m.	16	4	2
	5	2	4		17	4	3
	6	4	2		18	4	4
	7	3	1		19	4	4
	8	2	4		20	4	4
	9	2	4		21	4	4
2.30 a.m.	10	2	3	3.30 p.m.	22	4	4
	11	2	3		23	4	4
	12	2	3		24	4	4

period individuals establish different feeding patterns.

Effect of feeding and starvation upon Secretory Cycle.

In an attempt to elucidate the effect of both feeding and starvation upon the release of chrusosecretory material from A_1- and A_2-cells, two groups of moths each of 28 individuals, were isolated and starved for 5 days. The following day one batch was fed and the other starved for a further 24 hr. Each batch was divided into groups of 4 and one group from each was dissected at 2 hr. intervals commencing at 8.00p.m. In the fed batch, the feeding pad was removed at 4.00 p.m. As in the previous series, the counterstains were omitted, and the density of the inclusions was similiarly scored (table 7 and 8.).

It can be seen that feeding has a marked effect upon the release of neurosecretory material from both A_1- and A_2-cells. The period of illumination extended from 7.00 a.m. to 12.00 p.m. and whether fed or starved it appears that release of material occurs in most individuals after the lights are switched off. Starved moths continue to secrete from A_1- and A_2-cells throughout the night, whereas fed moths secrete from A_2-cells only, and for a much shorter period.

At first it seems somewhat difficult to reconcile these results with the apparent role of A_1- and A_2-cells in P. cynthia ricini; i.e. in control of diuresis and of activity respectively. While the reduced secretory activity of A_2-cells is in accordance with the function attributed to them, the parallel in secretory activity of A_1- to A_2-cells in starved moths and their apparent failure to secrete in fed moths is harder to explain. Possibly,

Table 7. Concentration of inclusions in A_1- and A_2-cells at timed intervals in starved moths.

Time	Moth	A_1-cells	A_2-cells	Time	Moth	A_1-cells	A_2-cells
	1	4	4	2.00 a.m.	15	2	1
	2	4	4		16	1	1
8.00 p.m.	3	4	4		17	2	1
	4	3	4		18	2	1
	5	3	4	4.00 a.m.	19	2	1
	6	3	3		20	1	1
10.00 p.m.	7	3	3		21	2	2
	8	3	44		22	2	2
	9	4	4	6.00 a.m.	23	2	2
	10	2	3		24	2	1
12.00 p.m.	11	2	2		25	2	2
	12	2	2		26	2	2
2.00 a.m.	13	3	4	8.00 a.m.	27	1	2
	14	2	1		28	1	2

as in A_2-cells release of neurosecretory material is inhibited. However in keeping with the postulate; that they have a duiretic function, the reduced rate of secretion after feeding can be explained otherwise. As lipids form the 'flight fuel', activity will result in the production of 'metabolic water', and this may raise the volume of haemolymph, and dilute it, thus stimulating release of a diuretic factor. If this is correct, if would explain the observed activity of A_1-cells. It has been shown that even under dry conditions insects are able to obtain sufficient 'metabolic

Table 8. Concentration of inclusions in A_1- and A_2-cells at timed intervals in fed moths.

Time	Moth	A_1-cells	A_2-cells	Time	Moth	A_1-cells	A_2-cells
	1	4	4	2.00 a.m.	15	4	2
	2	4	4		16	4	1
8.00 p.m.	3	4	3		17	4	3
	4	4	3		18	4	2
	5	4	4	4.00 p.m.	19	4	2
	6	3	4		20	3	1
10.00 p.m.	7	3	2		21	4	4
	8	3	1		22	4	3
	9	4	4	6.00 p.m.	23	4	3
	10	4	2		24	4	2
12.00 p.m.	11	4	1		25	4	4
	12	3	1		26	4	4
2.00 a.m.	13	4	3	8.00 a.m.	27	4	3
	14	4	2		28	4	3

water' to prevent any proportional loss as a body constituent (Buxton and Lewis, 1934; Wigglesworth, 1950).

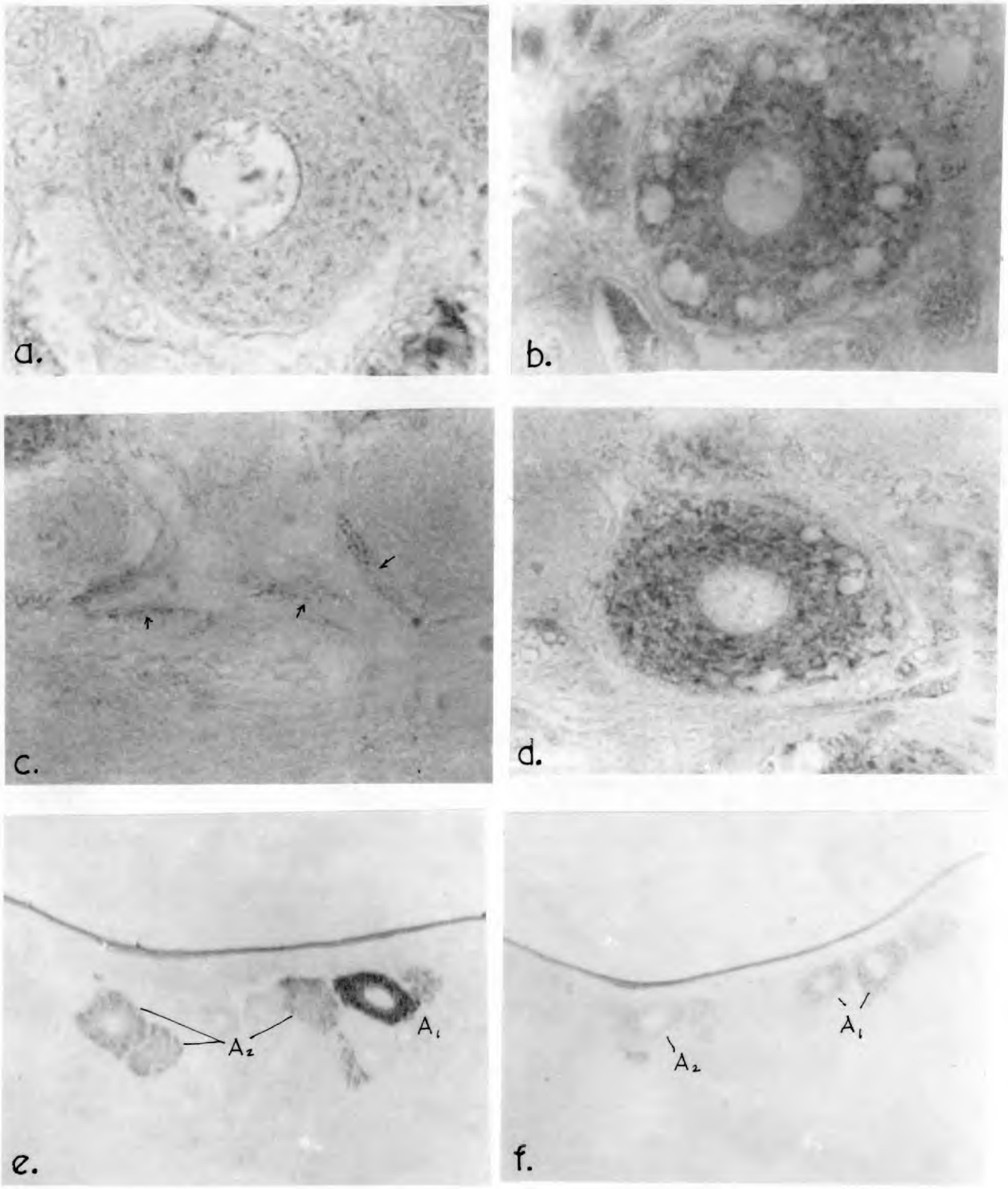

Fig. 8. a.Lateral D-cell in 20-day pupa. b.A_1-cell in 5-day old moth, 24 hr. after water injection. c.Neurosecretory material in axons, 7 hr. after water injection. d.A_1-cell in 5-day old moth, untreated. e.Medial A-cells in starved moth at 3.30 p.m. f. A-cells in starved moth at 3.30 a.m. a.-d. P.cynthia ricini, e. and f. T.pronuba.

Discussion.

The present study confirms that there is a multiplicity of neurosecretory cells and types of cells in the brain of Lepidoptera, as previously reported by Panov and Kind (1963) and Herman and Gilbert (1964, 1965). That, furthermore there is a remarkable uniformity in numbers of A_1-, A_2-, A_3-, C- and D-cells medially and A_4-and B_2-cells laterally, in different species and in most stages of a single species. Studies of the tinctorial affinity and cytomorphology of the various types of cells leaves no doubt that they are quite distinct; the cyclical changes that occur during development confirm the distinction. While some authors believe that cells with different histological appearance merely represent phases in the secretory cycle of a single type of cell (e.g. Brandenburg, 1956, Herlant-Meewis and Pacquet, 1956, Highnam 1959, Kopf, 1957a, Nayar, 1955 and Thompson, 1952), this supposition is quite untenable with regard to Lepidoptera. It is notable that these workers used Azan or CHP but more recently PAF has replaced the 'classical' CHP method. It has become apparent that much of the previous difficulty and confusion in categorising neurosecretory cells can be avoided by the application of the newer technique, together with precise analysis of the results.

The various schemes of classification that have been proposed in the past have been outlined and discussed (pp.), and so far as it has been possible, cells described in previous accounts of the lepidopteran brain have been related to the system adopted here. Acknowledgeing the present wide acceptance of the PAF sequence, the utilisation of an alphabetical system necessitates

drawing a sharp distinction between A- and B-cells. At present there is an outstanding need for this, and based upon PAF staining the only logical distinction is that which Johansson (1958) applied: B-cells never bear fuchsinophil inclusions, whereas A-cell inclusions are always fuchsinophil. This does mean though, that there is an 'A-cell complex' comprising at least 4 distinct types (A_1, A_2, A_3 medially, and A_4 laterally in Lepidoptera), but these cells probably have quite different physiological roles. Raabe's (1965) system includes B_1- and B_2-cells which are fuchsinophil, and would therefore fall into 2 categories of A-cells. The fact that this author differentiated A- and B-cells upon the absence (in the former) and presence (in the latter) of sulphydryl groups does not, per se invalidate the system that places them in the same 'class' of cells. (A-cells), rather it confirms their separate identity. As far as histochemical differentiation is concerned it is worth pointing out that aldehyde fuchsin is used as a histochemical reagent (e.g. Scott and Clayton, 1953, Spicer, 1960, Sulkin, 1960) for mucopolysaccharides. In this respect this primary division of cells would resemble that which is currently accepted for cells of the vertebrate adenohypophysis; into serous and mucoid cells (Herlant, 1964).

To provide a scheme of classification that would embrace all known types of neurosecretory cells from all of the orders of insects, and from brain and ventral cord ganglia poses certain problems. Ultimately, only further confusion will arise, if for example, it is found that A_1-cells of the ventral cord have a different function from A_1-cells of the brain; this must, therefore,

be an area of further investigation. In spite of the difficulties in relating categories of cells designated by one author to those by another, it does become apparent that considerable variation exists in the occurrence of different cells in the Insecta. Among the pterygote orders the presence of lateral as well as medial cells has been widely reported; notable exceptions are found in some Hymenoptera (Thomsen, 1954) and in Blatta orientalis and P. americana (Fuller, 1960) but the presence of neurosecretory cells in the ventral cord is by no means so well known. It is perhaps significant that they have been most frequently reported from orthopteroid insects (Scharrer, 1941, Harker, 1955, 1960, Fuller 1960, Panov, 1962, Freon, 1964a, 1964b, Huignard, 1964, Besse, 1965, Delphin, 1965, Raabe 1965a, 1965b, Chalaye, 1965, 1966). In most of these studies at least 1 type of A-cell is described, and as many as 3 in S. gregaria (Delphin, 1965), whereas only 2 types of A-cell are known to occur in the brain of this species (Highnam, 1961); a single type laterally and another medially. However, neurosecretory cells are known to occur in the ventral ganglia in other orders, most commonly in the sub-oesophageal ganglion, as this is often sectioned with the brain, (e.g. in Diptera; Kopf 1957a, Fraser 1959, Fuller 1960, in Coleoptera; Kirchner, 1960; in Hemiptera: Nayar, 1965, Johansson 1958; in Hymenoptera: Brandenburg, 1956). Ladduwahetty (1962) and Fraser (1959) on the other hand found no neurosecretory cells in the ventral ganglia (including the sub-oesophageal) in D. maculatus and larvae of L. caesar respectively.

Despite the application of CHP, Azan and PAF and adoption of a very comprehensive system of classification, no neurosecretory

cells were observed in either sub-oesophageal - or prothoracic ganglion of larvae, pupae or adults of any of the Lepidoptera examined in this study. It is, therefore surprising that Kobayashi (1957) found no less than 1,172 neurosecretory cells in the ventral cord of B. mori larvae; including 80-100 such cells in the sub-oesophageal, and 79 cells in the prothoracic ganglion. These cells are PAF-negative, but phloxinophil and histochemically are similiar to medial B-cells (= my C_1-cells), they also showed cyclical activity. It is of course possible that B. mori differs from other Lepidoptera that have been studied, and this may well be the case, as Kobayashi further found that lateral cells only became apparent at the fifth instar. This species quite obviously merits further detailed study. Rehm (1951, 1955) has also described neurosecretory cells from the sub-oesophageal ganglion in E. kuhniella and Galleria mellonella, but absent from Pieris brassicae. However, Panov and Kind's (1963) description of P. brassicae does not agree with that of Rehm (1955), the accuracy of Panov and Kind's observations is supported by Herman and Gilbert (1964, 1965) and this study. Rehm's studies, although detailed must be treated with some reservation.

It seems fairly certain that, if there are neurosecretory cells present in the ventral cord of Lepidoptera they comprise a single type. This apparent paucity of cell types in the ventral cord is not surprising when the neuroendocrine system as a whole is compared, with, for example that of S. gregaria. In this species there are 4 types medially + 1 laterally (Highman 1961), and 6 types in the ventral cord (Dolphin 1965). No doubt some

overlap occurs; Dolphin considered 1 type of ventral cord A-cell to be similiar to, if not the same as the medial A-cell. Furthermore, C- and D-cells are likely to be the same. This implies that in S. gregaria there are at least 8 types of neurosecretory cell. Apart from the debatable cells of the ventral cord in Lepidoptera, there are 8 types occurring in the central nervous system, all in the brain. In this context it is of interest to examine Frasers (1959a, 1959b) finding in the brain and ventral cord of L. caesar. Without attempting to fit the cells into any alphabetical system, he makes a good case for considering the cells of each of the 6 groups as distinct. He subsequently described the neurosecretory cells of the ventral cord in the same species, this time applying an alphabetical system and dividing cells into 2 categories of A-cell and 1 B-cell. Although Fraser concluded that the 2 types of A-cell represented different phases in a secretory cycle of a single type of cell, and the B-cell to be an inactive A-cell, his histochemical findings do not support this notion. The possibility the L. caesar has 8 types of neurosecretory can be considered. It would of course be premature at this stage to suggest that 8 types of neurosecretory cell are fundamental to insects in general, before any such conclusions can be drawn, comparative and detailed histological studies will be required for other orders of insects. However it may be stated that in the Lepidoptera the absence of distinct neurosecretory cells in the ventral cord but the presence of at least 8 types in the brain indicates a pronounced cephalisation of central endocrine elements.

Notwithstanding the histological evidence of the multiplicity of neurosecretory cells, there is a justified caution against regarding just any neurone with a typical appearance as having an endocrine function. There is wide agreement that the lateral fuchsinophil and phloxinophil cells as well as the medial fuchsinophil cells are neurosecretory, this immediately includes 5 distinct types of cell in the Lepidoptera. For the remainder of the cells that have been regarded as neurosecretory in this study (B1-, C- and D-cells), their inclusion as such seems justified for the following reasons:- (i) they occur in either lateral or medial groups, (ii) reveal marked cycles of accumulation and secretion, (iii) have fuchsinophil inclusions (D-cells), phloxinophil inclusions (B-cells), or both (C-cells). Even when C-cells are recognised, there is often a reluctance to regard them as neurosecretory (Johansson, 1958, Highnam, 1961, Thomsen, 1965). However, distinct cycles of secretion have only been observed more recently (Delphin, 1965, Siew, 1965b). In this study the cyclical activity of C-cells was clearly demonstrated, occurring at each larval moult, and during metamorphosis. While these cells characteristically bear very small fuchsinophil inclusions, they occur in intimate association with the orange-staining aggregates in P. cynthia ricini (green in other species). Release and synthesis of both materials coincide, thus, it appears that the so called inclusions are part of the secretion which is mainly PAF-negative. Examination of the cytoplasmic volume at different stages of P. cynthia ricini (fig. 7) clearly indicates that medial C-cells in this species are most active during development especially in the

last 2 larval instars and pupae.

Williams (1947) proved that in terminating diapause of pupae of H. cecropia, a brain hormone activates the prothoracic glands, stimulating the production of moulting hormone. This function of a brain hormone has been confirmed by Wigglesworth (1952) in R. prolixus, by Rehn (1952) in Sialis lutaria and by Church (1955) in Cephus cinctus. As Wigglesworth (1934) demonstrated, the effect is not a brief triggering, on the contrary exposure of the prothoracic glands is necessary for several days. In relating the histological evidence of secretory activity of the neurosecretory cells of P. cynthia ricini; the phase of secretion commencing at each moult, stimulates the prothoracic glands for the subsequent moult. The observation of Herman and Gilbert (1964), that all types of neurosecretory cell show cyclical activity during development, is substantiated in this study, which has revealed that 4 types of cell are active at the time of moulting (A_1- and C- medially and A_4- and B_2-laterally). Whether all of these are necessary to activate the prothoracic glands is not known. It must, however, be borne in mind that throughout much of development the corpora allata are also actively secreting. The sequence of events in the endocrinology during development has occupied the attention of numerous researchers over the last 3 decades. The emergent picture has been lucidly presented by Gilbert (1964); stated briefly, moulting hormone induces (i) larval - larval moults when juvenile hormone concentration is high, (ii) larval-pupal transformation when juvenile hormone concentration is low (iii) pupal-adult transformation in the absence of juvenile hormone. In adult insects the gonadotropic

effect of juvenile hormone is well known (reviews by Highnam, 1964, De Wilde, 1964) and the allatotropic effect of a brain hormone has been established in some insects (e.g. Klug, 1958; de Wilde and de Boer, 1961, Ladduwahetty, 1962; Siew, 1965). It is likely that some neurosecretory product has the same effect in larvae, but experimental evidence is conflicting (Wigglesworth, 1964), for in some insects the corpora allata appear to be under nervous rather that hormonal control. Williams (1948) and van der Kloot (1960) found that both lateral and medial neurosecretory products were necessary for continued development in H. cecropia pupae, and that the products must mix to be effective. It is interesting to note that it has recently been suggested that in Schistocerca parranensis the lateral neurosecretory cells control the corpora allata (Strong 1965). During development in P. cynthia ricini, up until the pre-pupal stage the cyclical activity of medial A_1- and C-cells coincides with that of the lateral A_4- and B_2-cells, but at this stage become dissociated. During the 5 day pre-pupal period the lateral cells bear no inclusions until the 5th day, when the C-cells discharge, while A_1-cells show a progressive accumulation throughout the period. Further histological and experimental work will be required before the meaning of these findings becomes clear. As Highnam (1965) has pointed out histological observations alone are of strictly limited value, and in all instances need to be substantiated by suitably designed experiments.

From the species of Lepidoptera that she studied, Rohm (1951, 1955) concluded that the neurosecretory cells are inactive

in the adults. A view supported by Arvy et al (1953) applying to B. mori, although these authors did note release of material associated with oviposition. From their account it is impossible to deduce what type or types of cells are involved. Contrary to their findings, the present investigation has revealed that both lateral and medial neurosecretory cells are active in P. cynthia ricini and T. pronuba. The activity of the different types of A-cell further demonstrates their separate identify.

In an overall consideration it is of interest to compare the 2 species; on one hand the short-lived saturniid reveals a progressive loss of neurosecretory material, in all but the B_2-cells, whereas the long living noctuid shows an accumulation in most cell types. It seems that little synthetic capacity is retained by the neurosecretory cells in P. cynthia ricini, furthermore, apart from A_2-and D-cells there is a reduction in volume of the cytoplasm in the cells of the adult, suggestive of a diminished functional requirement. Examination of pupal brain and comparison with that of the adult shows that following the release of accumulated material before emergence, the A_3-, B_1- and D_2-cells have little if any function in the adult. It is assumed that the pre-emergence phase of secretion from these cells is in some way associated with the pupal-imaginal moult.

The induced release of neurosecretory material from A_1-cells of P. cynthia ricini after injecting distilled water or dextrose solution, and subsequent excretion, is interpreted as hormonal control of diuresis. Highnam, Hill and Gingell (1965) have demonstrated that the medial cells of S. gregaria discharge

their contents when the haemolymph is diluted. Meyer (1960, 1962) has implicated medial A-cells in antidiuretic control, by demonstrating an accumulation of inclusions in I. limbata and P. americana when the insects are hydrated. More direct evidence of secretion of a diuretic factor from medial neurosecretory cells has been provided by Berridge (1966) in Dysdercus fasciatus. Whereas mesothoracic ganglion extracts show only a small amount of diuretic activity in this insect, Maddrell (1964) clearly demonstrated that neurosecretory cells in the fused thoracic ganglion of R. prolixus are the source of diuretic hormone. Unfortunately definitive histological studies are lacking for both species. However Delphia (1965) has shown that in S. gregaria water loss results in release of material from A-cells (A_2- in his classification) in the ventral cord, thus indicating as antidiuretic factor.

With regard to P. cynthia ricini it is of interest to examine the possible diuretic function of A_1-cells at each stadium. The discharge of secretory material from A_1-cells is maximal in larvae at each moult, and also in the mature larva/errly prepupa. Superficially it seems unlikely that diuresis would occur at ecdysis as the hydrostatic pressure of the haemolymph is probably an important aid in splitting the old cuticle, furthermore water losses by transpiration through the cuticle are greatest immediately after moulting (Wigglesworth, 1948). However no excretion occurs during ecdysis and even if diuresis is stimulated, its storage in the malpighian tubules would mean no volumetric loss to the larva. Such a process would, however, increase the concentration of the haemolymph, and in so doing raise the titre of circulating hormones.

The most pronounced release of A_1-cell hormone occurs in the mature 5th instar larva, when distension of the malpighian tubules, accompanied by a marked reduction and concentration of the haemolymph. During metamorphosis, there is, up to the 17th day continued A_1-cell secretion, which again may be correlated with diuresis and production of the liquid meconium, which is stored in the hindgut and largely discharged shortly after emergence.

The circadian cycle of secretion exhibited by A_2-cells in adults is marked by a large efflux of material in T. pronuba, but only slight in P. cynthia ricini. In the latter no material is apparently released during copulation when the moths are inactive. In larvae these cells show little fluctuation in content, but appear to release no material during ecdysis, when the insects are inactive. The A_2-cells do discharge during the pre-pupal stage, it is relevant to note that that the pre-pupae are very active, albeit spinning the cocoon; if they are removed they display a much greater locomotor activity than do disturbed larvae at earlier stages.

In adults of T. pronuba both A_1- and A_2-cells exhibit a circadian cycle of secretion, but the activity of both cells is influenced by nutrition, with respect to the time at which moths last fed and the presence of reserves. Selective ablation of A-cells (both A_1- and A_2) abolishes the endogenous circadian flight rhythm, which provides additional evidence of neurosecretory control of activity. While it is not possible to ablate A_1- or A_2-cells alone, it is most likely that A_2-cells produce the flight activating factor. A circadian cycle of secretion by medial neurosecretory

cells was first demonstrated by Klug(1958) in Carabus nemoralis, in which complete discharge of stainable material was noted in fully active beetles followed by rapid resynthesis when inactive. A similiar but bimodal cycle occurring in medial cells of Drosophila melanogaster has been reported by Rensing (1964, 1965), which can be correlated with locomotor activity and oxygen consumption. Neither author, though, gives sufficient histological details to permit classification of the neurosecretory cells, other than that they are PAF-positive, and therefore A-cells.

Evidence pointing to control of locomotor of flight activity by A-cells is to some extent difficult to reconcile with the well established dynamics of secretion by these cells in females of some species, in relation to reproductive cycles. Highnam (1962) has shown that in the female S. gregaria there is accumulation without release of material until copulation. However, inactivity does not accompany failure to secrete as it does in the case of T. pronuba, but Highnam et al (1965) have shown that these cells are also involved in diuretic control. As Highnam (1965) suggests, these cells probably have a dual role, from a functional point of view the medial A-cells in S. gregaria would correspond to A_1-cells in Lepidoptera. Whether the A-cells do have numerous functions; control of protein synthesis (Thomsen, 1952, Highnam 1962, Thomsen & Moller 1963), triglyceride and glycogen synthesis (Van Handel and Lea, 1965), diuresis (Highnam et al 1965, Berridge, 1966), antidiuresis (Mayer, 1960, 1962), locomotor activity (Klug, 1958, Rensing, 1964, 1965), activation of corpora allata (Highnam, 1964), activation of prothoraci glands (Wigglesworth, 1952, Williams, 1947),

or, some of those functions are controlled by other cells is not clear. Certainly there are sufficient number of different neurosecretory cells in the Lepidoptera and S. gregaria that have been described, to permit such 'division of labour'. These are problems which await future investigation; from this study evidence is presented to suggest that A_1- and A_2-cells are involved in diuresis and flight activity respectively.

V HISTOCHEMISTRY

1. Introduction.

Histochemical studies have been conducted upon neurosecretory cells of various insects: Ephestia kuhniella, Galleria mellonella and Pieris brassicae (Rohm, 1955); Iphita limbata (Nayar, 1955); Lucilia caesar (Fraser, 1959a, and b); Blabera fusca (Brousso, Idelman and Zagury, 1958); Bombyx mori (Kobayashi, 1957, Ganguly and Basu 1962) Locusta migratoria (Chalaye, 1965); Periplaneta americana (Pipa, 1962); and the comparative study of Arvy and Gabe (1962) comprising 15 species from 9 orders, including G. mellonella and B. mori. Only Fraser (1959a, and b), Nayar (1955), Kobayashi (1957) and Chalaye (1965) specify the types of cells involved. Unfortunately all of these are limited by the tests applied; Fraser consentrated upon the carbohydrate and lipid content, while Chalaye utilised three procedures for demonstrating sulphydryl groups, and 2 for carbohydrates. Nayar gave no consideration to any carbohydrate component other than glycogen, and Kobayashi confined his attention to carbohydrates and lipids.

The conclusions reached regarding the chemical composition of neurosecretory materials are understandably varied and are listed below:-

(i) phospho-lipoprotein complex (Rohm, 1955); (ii) phospholipid and protein (Nayar, 1955); (iii) Lipid and phospholipid, but no protein (Kobayashi, 1957); (iv) mucopolysaccharide or mucoprotein (Ganguly and Basu, 1962); (v) sulphydryl-rich lipoprotein (Brousso et al 1958) (vi) mucopolysaccharide in some cells, glyco- or phospholipid, or lipoprotein in others (Fraser, 1959a and b); (vii) lipo-

fuscin in A-cells (Pipa, 1962); sulphydryl-rich glycoprotein (Arvy and Gabe, 1962). Chalaye (1965) clearly demonstrated that three types of cell bearing PAF-positive inclusions, only one proved to be rich in sulphydryl groups; all were positive to Alcian blue, but only these rich in sulphydryls gave a strong reaction. As all types of cells were PAS-negative they obviously differ from 'A' cells in the brain of L. migratoria described by Arvy and Gabe (1962).

The most comprehensive study undertaken, with the most accurate tests is that of Arvy and Gabe (1962). However, these authors failed to take the multiplicity of cell types into account. Their histological criteria i.e. PAF-positive, and staining red with Azan include at least two categories of cells in the Lepidoptera, the A_1- and A_2-cells demonstrated in this study.

The histochemical- and staining reactions of the brain neurosecretory cells of T. pronuba were investigated in the hope of explaining the chemical basis of the histological differentiation. Selected tests were also applied to several other species.

2. Preparation of Tissues and Staining Procedure.

Initially brains were treated with fixatives appropriate to the tests and reactions that were to be conducted. For example cadmiumcalcium-formalin for lipids, acridine-ethanol for mucopolysaccharides and Carnoy for nuclei acids (Pearse, 1960) were used. Unfortunately most of these special fixatives result in extremely poor preservation and all tests had to be repeated after fixation in Bouin. Provided excess picrate from Bouin is removed, results are essentially similiar but preservation is far superior. Embedding and sectioning procedures were the same as those used for the histological work, serial sections were mounted on acid-cleaned slides, and all sections from each brain were subjected to each test.

The staining procedures and histochemical reactions were conducted according to the methods given by Pearse (1960). Nucleic Acids: Pyronin/Methyl green of Kurnick (1955), Gallocyanin-chromalum (Einarson, 1951), and controlled by hydrochloric acid hydrolysis.

Proteins: Chloramine-T Schiff (Burstone, 1955), Mercury Bromphenol Blue (Bonhag, 1955); Arginine: Sakaguchi reaction (Baker, 1947); Tyrosine: Millon reaction (Baker, 1956); Sulphydryl and Disulphide: Ferric-ferricyanide (Chevremont and Frederic, 1943), RSR reagent (Bennett, 1951), DDD reagent (Barrnett and Seligman, 1952); Neotetrazolium-BT (Pearse, 1954); reduction of disulphide linkages accomplished with sodium thioglycollate buffered to pH 8.0 and sulphydryls blocked with N-ethyl-maleimide at pH 7.4.

Carbohydrates: PAS reaction of McManus (1948), controlled by acetylation and saponification, for unsubstituted glycosides; Toluidine Blue Method (Hess and Hollander, 1947) and Alcian Blue (Steedman, 1950) for acid mucopolysaccharides; sulphation technique of Moore and Schoenberg (1957) followed by paraldehyde fuchsin for neutral mucopolysaccharides and glycoproteins.

Lipids: Sudan Black B method (Cluffellt and Putt, 1951) and Oil Red O (Lillie, 1954).

In addition, three techniques quoted by Barka and Anderson (1963) were used: Diazotised Saffranin for 5-hydroxytryptamine (Lillie, Burtner and Greco-Henson, 1953); the Astra Blue method for acid mucopolysaccharides (Pisch, 1958); and Potassium iodate method for adrenalin (Hillarp and Hokfelt, 1955).

After glutaraldehyde fixation the Tetrazolium-, and Ferric-ferricyanide reactions for nor-adrenalin (Hopsu and Makinen), and differentiation procedures for indoles of Solcia, Sampetro and Vassalle (1966), were applied.

3. Results.

After application of some stains and tests no 'background' staining of either non-neurosecretory neurones or neuropile occurs. However, the staining reactions of neurones other than neurosecretory cells, together with those of the tracheae, neurilemma and the neurosecretory cells, are included in Table 9.

Nucleic Acids: While RNA is present in all neurosecretory cells, there it occurs in a variable concentration; no importance is attached to its occurrence in this study. On one hand Bouin is not a very suitable fixative, and on the other, there is no evidence

from this study, or any previous ones that RNA is a component of any neurosecretory material. It has become apparent, though, that B_1-cells in the 'pre-secretion phase' contain considerable amounts of RNA as compared with the phase B_1-cells in which they are packed with inclusions. In table 10 the constituents of the two phases of B_1-cells are compared. Quite clearly the smaller cells with their ellipsoid nuclei are at a stage in which RNA and proteins, probably structural proteins and enzymes associated with ribosome/Golgi synthetic mechanism, are abundant. These diminish markedly when the neurosecretory material accumulates. Despite the rapid elaboration of inclusions in A_1- and A_2-cells during a 24 hour period, these cells have a comparatively low concentration of RNA.

Proteins: The distribution of protein revealed by Chloramine-T Schiff closely resembles that of Bromphenol blue (fig. 9). The former reveals terminal amino groups and unbound amino groups in side chains of arginine, lysine, glutamine and asparagine. The mechanism of reaction of Bromphenol blue is not understood (Pearse, 1960). It is assumed here, that it reacts with free amino groups, as ionisation of carboxyl groups would be suppressed, but protonation of amino groups enhanced in the axid dye solution.

Both the DMAB reaction for tryptophan and Millon reaction for tyrosine were carried out on sections of *A. ipsilon* *D. porcellus* and *P. cynthia ricini* in additional to *T. pronuba* (fig. 9). Only in *P. cynthia ricini* was there any appreciable concentration of tyrosine in A_1-cells as well as in A_2-cells, and in all

species tryptophan is confined to A_2-cells. The distribution of sulphydryl groups (cysteine) is uniform throughout the brain, but in very low concentration. After thioglycollate treatment cystine is reduced, and staining with RSR reagent shows a slight increase in sulphydryls in all cells with a comparatively greater increase in A_2-cells. However, this is not revealed by the DDD method after reduction (fig. 9). Similiarly, arginine has a very uniform distribution, with no high concentration in any neurosecretory materials. In addition to reacting with sulphydryl groups, Neotetrazolium BT, also reacts with other reducing groups, including sugar, lipid- and lipofuscin-aldehydes (Pearse, 1960). The intensity of the reaction (fig. 9), certainly implicates more than cysteine and cystine. Likewise ferric-ferricyanide reacts with other reducing groups besides sulphydryls. It is doubtful if lowering the pH as Barrnett and Seligman (1954) suggested is in itself sufficient to confer the specificity to the Tetrazolium reaction than has been claimed.

Carbohydrates: The A_2-cells alone give a positive reaction with PAS (fig. 9), which is abolished by acetylation and restored by saponification. The reaction is strong in A_2-cells of *A. ipsilon* but weak in A_2-cells of *P. cynthia ricini*. As α-amylase does not affect the reaction the polysaccharide cannot be glycogen. Staining with Toluidine blue at pH 2.5 showed a complete absence of metachromasia, with or without oxidation. Similiarly there is no affinity for Alcian blue at low pH. Barka and Anderson (1963) prefer Astra blue for the demonstration of strongly ionising cationic groups and using this technique A_2-cells have slight affinity for

the dye after oxidation, but it becomes leached out during dehydration. As the affinity is abolished by reduction in bisulphite solution the reacting groups cannot be sulphinic- or sulphonic acids, but are most likely to be carboxyls of aldehydes; the latter reacting to form bisulphite complexes. Ionisation of the latter would produce cationic groups for which the dye would have affinity.

Sulphation introduces sulphuric acid groups into both substituted and unsubstituted polysaccharides. In practice this technique must be carefully carried out; exposure of the sections to the sulphuric-/acetic acid mixture results in extensive hydrolysis after 3 minutes. The use of paraldehyde fuchsin to reveal introduced sulphuric acid groups showed that nearly all cells and other structures that are fuchsinophil after permanganate oxidation are also fuchsinophil after sulphation (fig. 10).

Lipids: All cells in the brain, the neurilemma, and neuropile show some sudanophilia. Affinity for either Sudan Black B or Oil Red O is no greater in neurosecretory cells than it is in many other neurones. In the former the stainable material is not granular, on the contrary, in A_2-cells it is clear that the dye is taken up between the flakes of neurosecretory inclusions (fig. 10).

Biogenic Amines: A strongly positive reaction with diazotised saffranin indicates the presence of 5-hydroxytryptamine (5-HT), which is confined to A_2-cells in all species studied (fig. 10). This was not confirmed by the tests for indoles (DMAB and xanthydrol) when applied to glutaraldehyde fixed sections. Although this fixative produces little shrinkage, and good preservation appears to be obtained after dehydration and wax embedding, application of the

PAF-sequence showed a marked loss in staining. The possibility of inadequate fixation cannot, therefore, be overlooked. Similiarly the negative result with ferric ferri-cyanide may not necessarily indicate the absence of nor-adrenalin. It is probably significant that the Tetrazolium reaction (Na tetrazolium-B1) was much weaker than after Bouin or buffered formalin fixation.

4. Stains Specific to Neurosecretory Material.

Reactions of the stains specific to neurosecretory material; PAF, paraldehyde thionin (PAT) of Paget (1959), and Victoria Blue (VB) of Dogra and Tandan(1961), are included in Table 9. Some analysis has been made of the effect of oxidising and reducing agents. Their specificity indicates their potential use as precise histochemical reagents. Indeed they permit a degree of differentiation that is unparalleled by and accepted histochemical techniques. In comparing the results of the 3 methods, it can be seen that specificity is in the order: VB PAT PAF. Whereas VB stains only A_2-cells, neurilemma and tracheae, PAT stains A_4-cells in addition, and PAF staining all categories of A-cells, inclusions of C- and D-cells as well as neurilemma and tracheae. However, distinction must be made between specificity and sensitivity. Quite clearly, PAF has affinity for sulphuric acid groups (from sulphation: fig. 10); sulphonic and sulphinic acids (oxidation of beta-cells of pancreas); aldehydes (engendered by oxidative deamination: fig. 10). Paraldehyde-thionin has affinity for aldehydes where they occur in sufficient concentration (permanganate, or performic acid oxidation + reduction) but very little affinity for sulphuric acid groups (sulphation). While VB has selective affinity for the bisulphite-aldehyde complexes arising from oxidation of 1, 2-glycols (by mild permanganate oxidation, or performic acid + reduction: fig. 10), it has no affinity for sulphuric acid groups. Staining of aldehydes by PAF is much more intense that by VB, and both are more intense than PAT. The importance of aldehydes groups in staining of neurosecretory material by any of these three methods, is

Table 9. Staining and Histochemical Reactions of the Neurosecretory cells of T. pronuba.

Stain or reaction	A_1	A_2	A_3	A_4	B_1	B_2	C_2	Other Neurones	Neurilemma.	Tracheae
Bromphenol blue	2	3	2	1	2	2	1	1	1	1
Chloramine-T Schiff	2	3	2	2	2	3	2	1	2	1
Neotetrazolium-BT	2	3	1	2	2	2	2	1	2	1
Millon-Baker	±	2	±	±	±	±	±	±	±	±
DMAB	0	2	0	0	0	0	0	0	1	0
Xanthydrol	0	2	0	±	0	0	±	0	1	0
Sakaguchi	1	1	1	1	1	1	1	1	1	1
Ferric-ferricyanide	1	2	±	1	1	1	1	±	±	±
Reduction/Ferric-ferricyanide	2	3	1	2	2	2	2	1	1	1
Oxidation/Ferric-ferricyanide	2	3	±	3	±	±	2	1	2	2
RSR	1	1	1	1	1	1	1	1	1	±
Reduction/RSR	2	3	2	2	2	2	2	2	2	±
DDD	1	1	1	1	1	1	1	1	1	±
Reduction/DDD	2	2	2	2	2	2	2	2	2	±
Periodic acid-Schiff	0	2	0	0	0	0	0	0	±	±
Permangamate-Schiff	0	2	0	0	0	0	0	0	±	±
Alcian blue	0	0	0	0	0	0	0	0	0	0
Oxidation/Alcian blue	0	0	0	0	0	0	0	0	0	0
Astra blue	0	0	0	0	0	0	0	0	0	0
Oxidation/Astra blue	0	0	0	0	0	0	0	0	0	0
Metachromasia	0	0	0	0	0	0	0	0	0	0

Stain or reaction	A_1	A_2	A_3	A_4	B_1	B_2	C_2	Other neurones	Neurilemma.	Tracheae
Diazotised saffranin	0	3	0	0	0	0	0	0	0	0
Permanganate/PAF*	3	3	3	3	0	0	1	0	3	3
Sulphation/PAF	3	3	0	1	0	0	0	0	3	3
Chloramine-T/PAF	3	3	3	3	2	2	3	2	3	3
Performic acid/PAF	3	3	2	2	0	0	0	0	3	3
Permanganate/ Acetylation/PAF	1	1	0	1	0	0	0	0	1	1
Sulphation/VB	±	1	0	0	0	0	0	0	1	1
Permanganate/VB	±	3	0	0	0	0	0	0	3	3
Performic acid/VB	0	1	0	0	0	0	0	0	1	1
Performic acid/ Reduction/VB	0	3	0	0	0	0	0	0	3	3
Sulphation/PAT	0	±	0	±	0	0	0	0	1	1
Permanganate/PAT	±	3	0	2	0	0	0	0	3	3
Performic acid/PAT	0	2	0	0	0	0	0	0	2	2
Performic acid/ Reduction/PAT	±	3	0	2	0	0	0	0	3	3

0 = negative reaction; ± = doubtful result; 1, 2, 3, = positive reactions (weak, intermediate and strong). * = counterstains ommitted.

Table 10. Comparison of Appearance and Staining Reactions of the 2 Phases of B_1-cells in *A. ipsilon* and *T. pronuba*.

	Inclusions present.	Inclusions absent.
Dimension (means in μ).	Cell 14.0; nucleus 7.5	Cell 10.0; nucleus 5.0
Appearance of chromatin	sparse and aggregated	Well dispersed
Appearance of cytoplasm	some peripheral vacuoles.	never vacuolated
PAF	numerous green inclusions.	Inclusions absent
Azan	reddish-blue inclusions	Colourless or grey
CHP	phloxinophil	pale grey-blue
Pyronin/Methyl Green	little RNA present	RNA abundant
Bromphenol blue	strong reaction	mild reaction
Basiphilia	nil	marked.

confir' d by acetylation; which abolishes staining by PAT and VB and markedly diminishes affinity for PAF (fig. 10). Thus PAF has the greatest sensitivity but least specificity, however, the latter is increased by using the stain after a precise period of ripening and discarding it when selectivity diminishes. On the other hand the other two dyes are much more stable, and are self-limiting.

(5) Enzyme Digestions.

The availability of relatively pure enzymes from commercial sources, permit their use in augmenting histochemical studies. Classically, ribonuclease, x-amylase and hyaluronidase are used to confirm the presence of RNA, x-1, 4-glucan residues, and hyaluronic acid respectively, that are indicated by histochemical staining methods. As enzyme specificity is extremely high, they provide very sensitive reagents.

In this study selected 'glycolytic' and 'proteolytic' enzymes were used, to substan' ate, and extend the histochemical findings.

Enzymes were purchased from Sigma Chemical Company in the purest grade available. These include, trypsin, pepsin chymotrypsin, clostridiopeptidase A (= collagenase), x-amylase (= diastase), B-glucuronidase, hyaluronidase and neuraminidase (= sialidase).

Sections of Bouin fixed brains were used in each instances; they were thoroughly washed in lithium carbonate solution to remove any residual picrate, followed by rinsing in several changes of distilled water, and finally immersed in a buffer solution of the same composition as that in which each enzyme was dissolved.

Rapid hydrolysis of sections by proteolytic enzymes, together with the tendency for sections to become detached during extended incubation (3 hours or more), required repeating the treatments at various concentrations for shorter periods of time. In order to eliminate the chance of hydrolysis by contaminating enzymes, ideally very low concentrations should be used. In practice it was found that sections were less easily detached when treated for short intervals, consequently higher concentrations of enzymes had to be used. After a series of trials, the following concentrations and incubation times proved most suitable. (The numbers in parenthesis and nomenclature are taken from the Enzyme Commission Report (Dixon and Webb, 1964)).

Trypsin (3.4.4.4.): Hydrolysing peptides at bonds adjacent to aromatic or dicarboxylic amino acids. 1 mg/ml in 0.05 M glycine buffer pH 2.2. Incubated at 37°C for 1.5 hours.

Chymotrypsin (3.4.4.5.): Hydrolysing peptides at bonds involving carboxyl groups of aromatic amino acids. 1 mg/ml in 0.05 M phosphate buffer pH 7.0. Incubated at 37°C for 1.5 hours.

Clostridiopeptidase A (3.4.4.19.): Hydrolysing peptides containing proline. 2 mg/ml in 0.05 M phosphate buffer pH 7.4. Incubated at 37°C for 4 hours.

α-Amylase (3.2.1.1.): Hydrolysing α-1, 4-glucan links in polysaccharides containing 3 or more α-1, 4-linked D-glucose units. 1 mg/ml in 0.02 M phosphate buffer pH 6.0 (+ 0.05 M NaCl). Incubated at 25°C for 2 hours.

β-Glucuronidase (3.2.1.31.): Hydrolysing β-D-glucuronides. 1 mg/ml in 0.05 M phosphate-citric acid buffer pH 5.0. Incubated at

37°C for 3 hours.

Hyaluronidase (3.2.1d): Hydrolysing links between 2-acetylamino-2-deoxy-D-glucose and D-glucuronate. 0.5 mg/ml in 0.1 M phosphate buffer pH 6.9. Incubated at 37°C for 4 hours

Neuraminidase (3.2.1.18.): Hydrolysing terminal α-2, 6-links between N-acetylneuraminic acid 2-acetylamino-2-deoxy-D-galactose. 0.04 mg/ml in 0.05 M acetate buffer pH 5.5 (+0.005 M $CaCl_2$). Incubated at 37°C for 4.5 hours.

RESULTS.

The effects of the enzymes are given in Table II. Assessment was made after staining with the complete PAF sequence, after comparing untreated sections and control sections incubated in buffer solution without enzyme. Slight reduction in staining was noticed in some controls which were allowed for in analysing the hydrolytic effects of the enzymes.

Trypsin rapidly removes practically all structure other than trachoae, neurilemma and A_2-cell inclusions (fig. 11). Some proteinaceous material is also removed from the neurilemma and A_2-cells, as they lose their affinity for light green. The fraction lost from A_2-cells contains tryptophan, but tyrosine residues still remain (fig. 11). The A_2-cell material is largely retained within the axons, this enzyme, could therefore, provide a means of differentiating neurosecretory materials under transport.

The effect of pepsin (fig. 11) and chymotrypsin are similiar in that both hydrolyse the tissue proteins much more slowly, but both remove A_1-, A_3- and A_4-cell inclusions. Pepsin partially removes the light green-staining fraction from A_2-inclusions, leaving the material negative to Millon's reagent but positive to DMAB, whereas chymotrypsin has little effect, leaving both tyrosine and tryptophan residues intact. All other types of neurosecretory cells are fairly rapidly hydrolysed by pepsin and trypsin, but B_1- and C_1-cells are only partially affected by chymotrypsin.

Table 11. Effect of Enzyme Digestions as Revealed by the PAF-Sequence, and Millon-1 and DMAB Reagents.

Enzyme and reagent	A_1	A_2	A_3	A_4	B_1	B_2	C_2	Other neurones	Neurilemma	Tracheae
Trypsin/PAF	3	1	3	3	3	3	3	3	1	1
Trypsin/Millon	-		-	-	-	-	-	-	-	-
Trypsin/DMAB	-		-	-	-	-	-	-	-	-
Pepsin/PAF	2	1	1	1	2	2	2	1	2	0
Pepsin/Millon	-		-	-	-	-	-	-	-	-
Pepsin/DMAB	-		-	-	-	-	-	-	-	-
Chymotrypsin/PAF	3	1	1	1	2	2	2	1	2	0
Chymotrypsin/Millon	-	0	-	-	-	-	-	-	-	-
Chymotrypsin/DMAB	-	0	-	-	-	-	-	-	-	-
Clostridiopeptidase/PAF	2	0	0	0	0	2	0	0	2	0
α-Amylase/PAF	0	0	0	0	0	0	0	0	0	0
β-Glucuronidase/PAF	2	0	0	0	0	0	0	0	0	0
Hyalurouidase/PAF	2	0	0	0	0	0	0	0	0	0
Neuraminidase/PAF	1	0	1	1	0	0	0	0	0	0

0 = no effect; 1 or 2 partial hydrolysis; 3 complete hydrolysis; - = unassessed because of limitations of reagent.

Clostridiopeptidase quite selectively hydrolyses A_1- and B_2-cell inclusions (fig. 11), and the light green-positive material in C_1-cells. All other cells and structures of the brain

are unaffected. It is of interest that there is selective hydrolysis of some light-green-staining proteins, indicating chemical differences that are not evident after any staining procedure.

X-Amylase has no effect, that could be detected with PAF, PAS, or Azan. Some reduction of A_1-cell inclusions occurs from treatment with glucuronidase (fig. 11) and hyaluronidase, and the pattern of hydrolysis is similiar in both. It is of course possible that the samples of both enzymes were contaminated with some proteolytic enzymes. However the slides subjected to oxidation before treatment showed very little loss of material and as 1, 2 glycols are most susceptible to Malapradian oxidation, proteolysis seems less likely. Moreover, only A_1-cells showed any significant change, it therefore seems most likely that the reduced staining was due to hydrolysis of the polysaccharide moiety.

The effect of neuraminidase, in selectively and substantially reducing fuchsinophilia of A_1-cells is interesting. As this enzyme was employed at a very low concentration (0.04 mg/ml) there is little likelihood of the effect due to protease. Consideration must then be given to the possible presence of N-acetylneuraminic acid as a component of the neurosecretory material (fig. 11).

These results clearly underline the differences in composition of the various neurosecretory materials, and provide a means of characterising and investigating them that has remained unexploited.

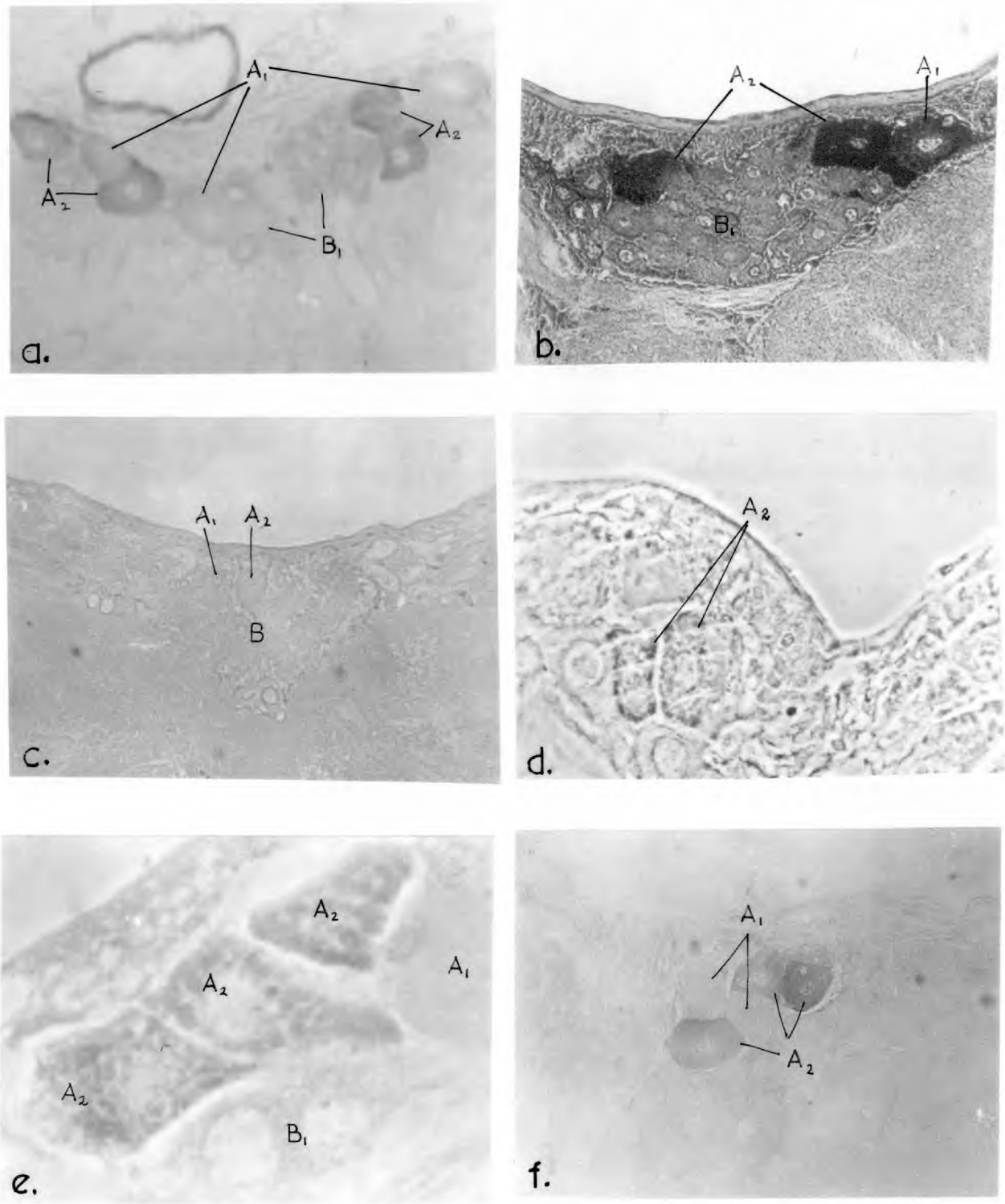

Fig. 9. Histochemical- and staining reactions of the medial neurosecretory cells of T.pronuba. a.Bromophenol blue. b.Neotetrazolium. c.DDD. d.Millon-Baker (phase contrast). e.DMAB (phase contrast). f. PAS.

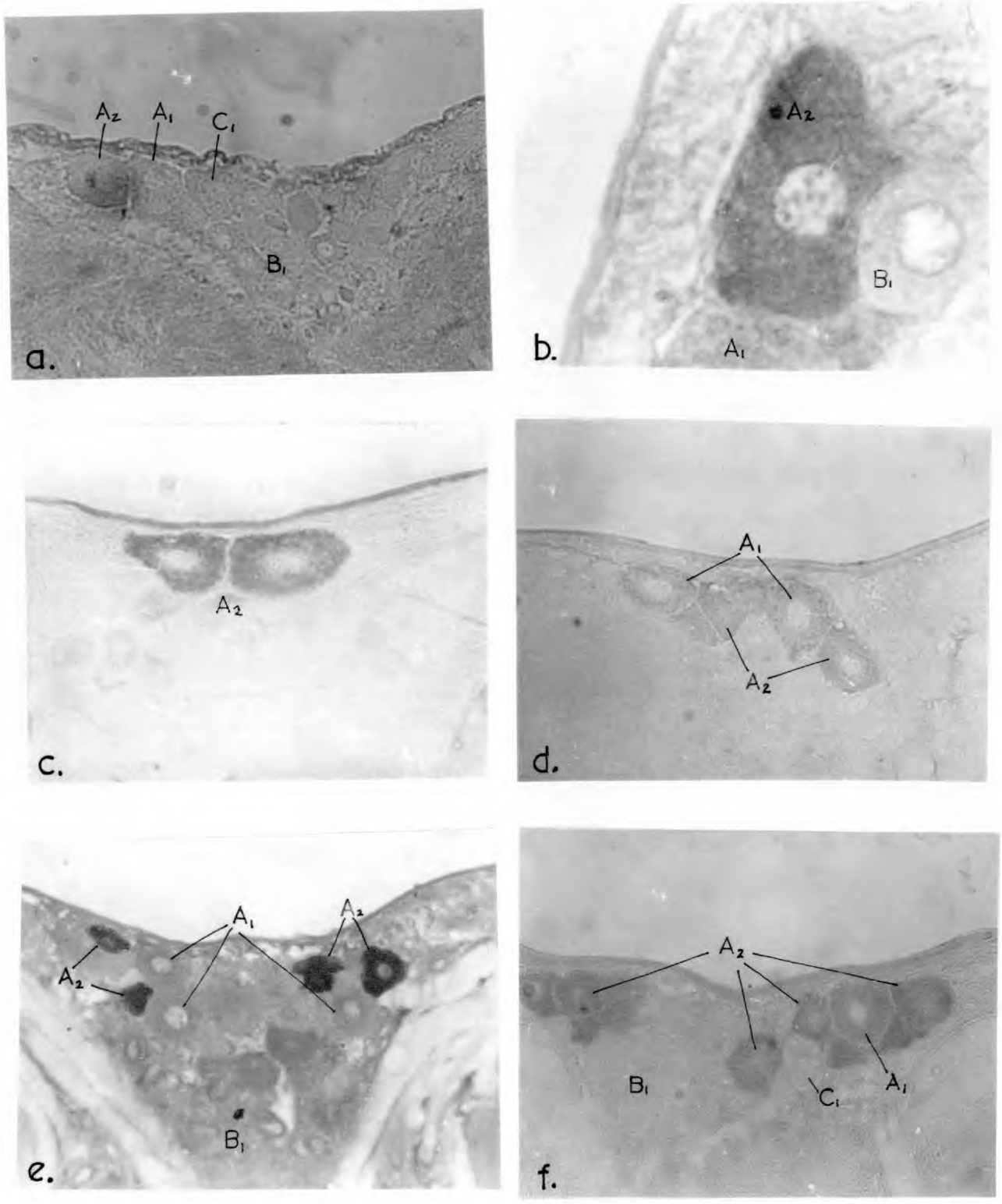

Fig. 10. Histochemical- and staining reactions of the medial neurosecretory cells of T.pronuba. a.Sudan black. b.Diazotised saffranin. c.Performic acid, bisulphite rinse, Victoria blue. d.Sulphation. PAF. e.Chloramine-T. PAF. f.Acetylation. PAF

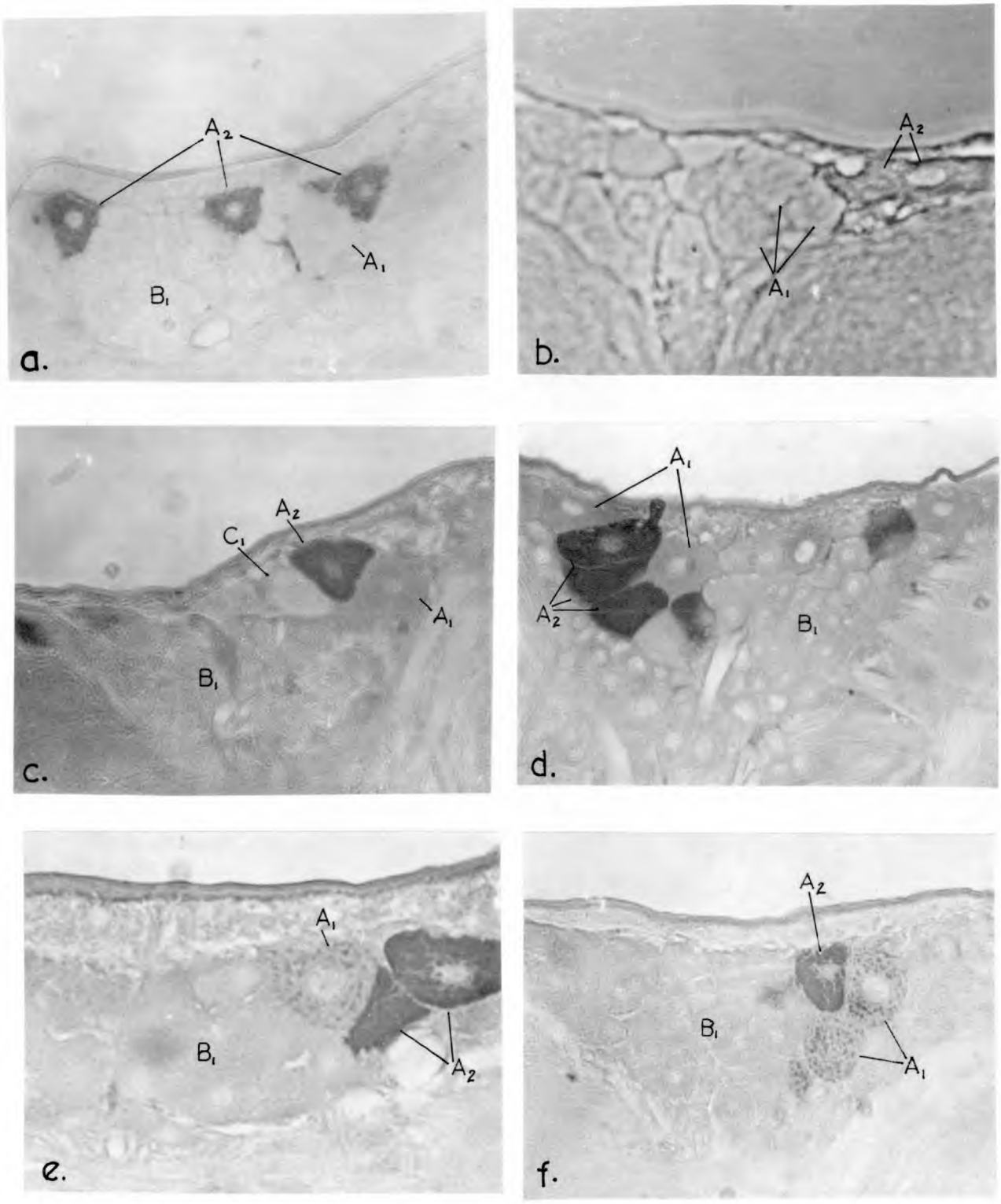

Fig. 11. Effect of enzyme digestions upon the medial neurosecretory cells of T.pronuba. a.Trypsin, PAF. b.Trypsin, Millon-Baker (phase contrast). c.Pepsin, PAF. d.Clostridiopeptidase, PAF. e.Glucuronidase, PAF. f.Neuraminidase, PAF.

Discussion

The low lipid content of all neurosecretory cells is consistent with the findings of Arvy and Gabe (1962). However, many lipoid materials would not remain after Bouin fixation and wax embedding. The faintly sudanophil material that is present, lies between aggregations of neurosecretory material in A_1- and A_2-cells. As no neurosecretory material were sudanophil even after cadmium-calcium-formalin fixation it is concluded that in T. pronuba, at least, there is no lipid component.

When sections are stained with PAF, ribonucleic acid contributes to the dye affinity of only B_1-cells, and then only the phase where inclusions are absent.

Total protein concentration cannot be demonstrated by any single method and can only be deduced after staining for component amino acids and unbound amino-groups. Both Chloramine-T Schiff and Bromphenol blue visualise the latter, and they are present in all neurosecretory materials. However the greatest concentration occurs in A_2- and A_4-cells. By blocking amino groups by acetylation it was demonstrated that they are to some extent responsible for the affinity shown towards light green in the PAF sequence. The relatively low but uniform distribution of arginine, indicates that basic groups of lysine, hydroxylysine or histidine contribute mainly to the acidophil properties of many neurosecretory cells. As B_1- and B_2-cell inclusions are readily susceptible to tryptic digest, a fairly high concentration of lysine can be expected. Unfortunately carboxylic acid groups cannot be satisfactorily demonstrated by present histochemical techniques, and the possibility

remains that some of neurosecretory materials, especially the protein fraction of A_1-cells may be rich in aspartic- and glutamic acids.

The presence of sufficiently high concentrations of tyrosine and tryptophan to give a positive reaction by the methods used, characterises A_2-cells in noctuids and Sphingids. However, the A_1-cells in P. cynthia ricini also give a faint, but nevertheless, a positive reaction for tyrosine. When this is considered together with the higher concentration of arginine, and apparent absence of tryptophan in this species, it seems likely that there are specific differences in the amino acid composition of some neurosecretory materials.

Results of the enzyme digestion studies provide evidence that both tyrosine and tryptophan are protein-bound in T. pronuba and D. porcellus, as tyrosine is in P. cynthia ricini. It was earlier postulated that the strongly positive enterochromaffin reaction (diazotised saffranin) revealed stored 5-HT and the comparatively high tryptophan content, of A_2-cells, indicated reserves of this amino-acid, as the precursor of 5-HT, Hinks (1967). An intense enterochromaffin reaction would indicate the presence of 5-HT in these cells, but in the light of the enzyme digestions, the above hypothesis has to be modified. As chymotrypsin rapidly abolishes the affinity for diazotised saffranin, but leaves tryptophan intact, it is unlikely that the latter exists in the cytoplasmic pool of reserves, but is an integral part of the A_2-cell neurosecretory material.

The specificity of diazotised saffranin ofr 5-HT is, of

course questionable; all of the five methods used to demonstrate this amine quoted in Pearse (1960) depend upon reduction of the chromophore by the -5-hydroxyl group. As Pearse points out, ferric-ferricyanide is not only reduced by 5-HT, but also by melanin, lipofuscin sulphydryls and phosphoglyceracetals. However, melanin is visible as a pigment, and concentrations of both lipids and sulphydryls have been shown to be low, and can therefore be discounted. In my opinion reducing sugars, are also effective, as they are in reducing tetrazolium salts; further research is required to decide whether the positive enterochromaffin reaction, is, in this instance due to reducing sugars or to 5-HT as suggested.

The demonstration of cysteine and cystine in neurosecretory cell inclusions has had a considerable influence upon interpretation of the chemical nature of the material, and has stimulated the use of ^{35}S-cystine in conjunction with autoradiography as a means of studying the dynamics of synthesis and secretion. (Highman, 1962; Dolphin, 1963; Siew, 1963, 1965). Steedman (1950) introduced Alcian blue 8GS as a histochemical stain for acid mucopolysaccharides; it interacts with carboxyl and sulphate groups (Scott, Quintarelli and Dellovo, 1964; Quintarelli, 1965). Adams and Sloper (1955) adopted the technique for the demonstration of cysteine-rich neurosecretory material in the vertebrate hypothalamus. The chemical basis of the reaction rests upon the oxidation of cysteine/cystine to produce sulphonic- or sulphinic-acids, which behave as sulphated mucopolysaccharides do towards Alcian blue in acid solution. Similiarly the use of aldehyde-fuchsin for demonstrating the insulin-containing beta-cells of the pancreas rests upon the

production of sulphinic- and sulphonic acids by permanganate/ sulphuric acid oxidation. (Scott and Clayton, 1953; Kristberg, Lester and Lazarow, 1966). Sloper (1958) inferred that oxidised cysteine and cystine was responsible for the affinity shown by neurosecretory material for paraldehyde-fuchsin and chrome-alum-haematoxylin. However, in comparing PAF and PAS reaction in various tissues Scott and Clayton (1953) concluded that PAF stained both acidic groups and aldehydes.

Sloper (1958) mentions that Crystal violet-Dextrin-Resorcin substituted for Alcian blue gives improved results; and this formed the basis of Dogra & Tandan's (1964) Victoria blue-Dextrin-Resorcin-iron lake (VB). They showed that after performic acid oxidation this dye is very specific towards both vertbrate and invertebrate neurosecretory materials. Applied to the brain of Sarcophaga ruficornis and compared with PAF, Tandon and Dogra (1966) showed that not all PAF-positive cells reacted with VB, and suggest that the increased specificity of the latter renders it superior. Recently, Dogra (1967), has stated that the B-cells of Kobayashi (1957), Johansson (1958), Mitsuhashi and Fukaya (1960), Highnam (1961), Ewen (1962), McLeod and Beck (1963) and Mitsuhashi (1963), are not only contradictory but conspicuous by being negative towards VB. He puts forward the view that the absence of cystine as indicated by this technique may exclude the possibility that these cells are neurosecretory.

Of the methods currently used to demonstrate sulphydryls, DDD and RSR reagents are generally accepted as more reliable (Pearse, 1960; Barka & Anderson, 1963). Ferric-ferricyanide and

Tetrazolium salts are also used, but readily react with other reducing substances. From the histochemical results of this study it is apparent that these two groups of reagents act differently; DDD and RSR giving a weak reaction in practically all cells of the brain, whereas ferric-ferricyanide and Neotetrazolium stain A_2-cells particularly intensely, as do PAS, and VB after oxidation and bisulphiterinse. The fact that VB fails to stain any neurosecretory cells after performic acid oxidation alone rules out any significant concentration of sulphydryls or disulphide. Nevertheless in some species there is a sufficiently high concentration to produce an intense reaction with Alcian blue, after oxidation (Brousse et al, 1958; Sloper, 1957; Chalaye, 1965; Delphin, 1965; Siew, 1965c; Naisse, 1966). As Delphin (1965) found that two out of three types of PAF-positive cells stained with Alcian blue, and Chalaye (1965) found that one out of three stained strongly and the other 2 weakly, and all cells in the brain of the Lepidoptera used in this study were negative, it is evident that there is a considerable variation in sulphur content of neurosecretory materials. Therefore, methods relying upon the visualisation of oxidised sulphonic- or sulphinic acids will reveal only a fraction of the neurosecretory cells in some species and none in others. It would be quite invalid to use this as the sole criterion for neurosecretion.

The technique of staining with PAF after sulphation is used to demonstrate neutral mucopolysaccharides, or glycoproteins (Sulkin, 1960). Theoretically, hydroxyl groups of serine, threonine, hydroxylysine and hydroxyproline could be expected to react, but in

tissue sections they are unreactive (Pearse, 1960). From the results obtained in T. pronuba, inclusions of A_1-, A_2-, A_4- and C_2-cells gave a positive reaction. As A_1-, A_4- and C_2-cells are PAS negative, it is apparent that the carbohydrate moiety comprises substituted hexoses, i.e. hexosamines and N-acetylhexosamines. These sugars are less easily oxidised; periodate oxidation is hindered by protonation of a free amino-group (Brimacombe and Webber, 1964). Nevertheless, cleavage of the bond between carbons 3 and 4 will occur in N-acetyl-hexosamines (Neuberger and Marshall, 1966). This may possible explain why the oxidation products of these substituted sugars are PAS-negative, but PAF-positive, and also why the stronger oxidising agent, permanganate is preferred for use with PAF.

The intensity of PAS-staining correlates well with hexose content, but only when 1, 2-glycols are available for oxidation (Curran, 1965). Seemingly, the distance between 1, 2-dialdehydes allows condensation with the 2 open arylamine groups in a molecule of fuchsin leucosulphonic acid, but in a linear polymer of substituted hexoses the distance between adjacent aldehydes (1/ hexose residue), will be too great. Alternatively, if a single bond is formed a quinone structure will not develop, and no colour will be evident (Kasten, 1960). Paraldehyde-fuchsin is prepared from basic fuchsin, which consists of a mixture of tri-amino-tri phenyl-methane derivatives (Conn, 1961; Gurr, 1960). During 'ripening' of the prepared stain, hydrochloric acid catalyses depolymerisation of paraldehyde to free acetaldehyde, which condenses with the aryl-amino groups forming azamethines (Bangle, 1954). When

all amino groups and open condensation with tissue aldehydes does not occur in the staining time normally employed. Neither does it when the dye has 'aged', i.e. when all amino groups have reacted with acetaldehyde. It would therefore seem probable that the reactive dye is mono-acetaldehyde pararosaniline chloride. With one remaining aryl-amino group, this dye would react with single aldehydes resulting from oxidation of amino sugars.

Paraldehyde-fuchsin quite clearly reacts with tissue aldehydes, as evident after oxidative deamination, however in the demonstration of structures after permanganate oxidation, the reducing rinse will result in formation of a bisulphite addition product. This may dissociate in the acid dye solution leaving free aldehyde, or, may ionise as would sulphinic- or sulphonic-acid. The latter mechanising would explain the importance of the bisulphite rinse in obtaining a positive reaction with VB, regardless of the oxidant employed.

From the enzyme digestions if appears that all types of neurosecretory materials have a protein or peptide backbone. A_1- and C-cell inclusions have in addition a carbohydrate fraction. The rapid hydrolysis of A_1-cell inclusions by the proteolytic enzymes indicates that the protein component is rich in phenylalanine, (chymotrypsin), or dicarboxylic acids (pepsin); lysine (trypsin) and proline (clostridiopeptidase). Only the last enzyme had any appreciable affect upon A_2-cells, and it may be deduced that these have a significant proline content. This enzyme also differentiates B_1- and B_2-cell inclusions, the latter being rapidly hydrolysed but the former unaffected. It has already been suggested

that reduced staining of A_1-cells after treatment with B-glucuronidase or hyaluronidase may be due to the presence of traces of proteolytic enzymes. Interpretation of the results as indicating the presence of glucuronate, glucuronide, or 2-acetylamino glucose, on this basis must be tentative. On the other hand, the effect of neuraminidase is less doubtful, as the enzyme was prepared at a low concentration. While neuraminic acid has never been identified in any insect tissue, it is a common component of vertebrate adenohypophysial glycoproteins (Papkoff, 1966). Insect glycoptroteins, other than chitin, have received scant attention in the past (Wyatt, 1967).

It is concluded that the PAF-positive neurosecretory materials are glycoproteins, differing from one cell-type to another in both composition of the protein backbone, and the sugars of the carbohydrate side chains. The latter show both qualitative and quantitative differences. Carbohydrate to protein ratios are in the following order: $A_1 > A_2 > A_4 > C_2 > C_1$. B-cell neurosecretory materials consist solely of protein or peptide. The results therefore support the differentiation and classification of the cells, based upon PAF staining. The basic division into A- and B-cells is justified, but although it would be logical to include C- and D-cells as further types of A-cells, at present it is more convenient to adhere to the alphabetical system, that is consistent with cyto-morphological distinctions evident after PAF staining.

VI. EXPERIMENTATION.

1. Apparatus for recording flight activity.

In the design and construction apparatus recording activity (actograph) difficulties are encountered in producing a readily assessed record of activity without imposing any significant disturbance. The types of actograph described and reviewed by Cloudsley Thompson (1955 and 1961), can be used to record ambulatory movement. Recording flight activity, particularly of nocturnal insects presents special problems, as even brief exposure to any part of the range of electro-magnetic radiation, from ultra-violet to far infra-red can induce a response. The effect of intermediate and far infra-red radiation upon nocturnal moths has been described by Callahan (1965).

Substrate vibration can have a similiar effect, but may only become apparent in unnatural situations, for example in continuous light or darkness. However a constant environment is a pre-requisite in any test for innate, endogenous or persistent rhythms.

Edwards (1960) has described an actograph for continuous recording of flight activity, in which the insects are enclosed in a Faraday cage and the charge generated on the wings during flight is detected by an electrostatic probe. The signal is amplified and graphically recorded. While this instrument is very suitable for flying insects, disadvantages lie in its high cost and the need to adjust the size of the cage and the input resistance to the size of the insects. As an alternative, the apparatus described below was constructed.

The cage containing the moths is fitted with microphones and the sound produced by moths flying near or against the walls is recorded; the apparatus is therefore, an audiometric actograph. The principle of audio-frequency recording of insect activity was first applied by Park (1937) but his description is rather obscure and contains no details of the flight chamber. Since then, audio-frequency recording has been confined to analysis of insect 'songs' (Busnel, 1964).

The flight chamber consists of a wooden frame 30 x 30 x 30 cm with taut tracing paper walls on three sides, top and bottom, and cellulose acetate on the remaining side. The latter provides a window facing an Osram strip light which is fastened to the outer box. The side opposite the cellulose acetate windows is hinged and served as a door. The outer box consists of a stout frame covered with "beaver board"; this effectively insulates the microphones from any extraneous sound.

In order to minimize vibration the flight chamber is suspended by elastic bands. Five crystal microphones are affixed centrally on the top and 4 walls. Leads from the microphones and light transformer are taken through a rubber grommet in one side of the outer box. The common lead from the microphones feeds the signal to a simple 2 valve amplifier, through a diode rectifier to a Fielden Servograph. Maximum deflection of the recorder pen is adjusted by the potentiometer in the amplifying circuit, according to the number of insects in the flight chamber and the strength of the signal produced at maximum activity. The circuit to the strip light incorporates a Venner time switch with fittings for

6 switch leaves, thus permitting 1, 2 or 3 phases (light + dark) in a 24 hour cycle. The apparatus is illustrated in figure 12.

2. Preliminary results and test for endogenous rhythms.

Initially diurnal and nocturnal species were tested in the actograph. Diurnal species indlude, Pieris brassicae Linn, Vanessa urticae Linn, and Zygaena filipendulae Linn. The time switch to the light was adjusted to give a 16 hour photophase (7.00 a.m.-11.00 p.m.). The amount of activity and flight pattern showed little variation when a feeding pad was present or absent. When fed, a pad of cotton wool soaked in 1% solution of honey or sucrose was placed in a polystyrene container inside the flight chamber.

To test for endogenous flight rhythm the actograph was put in a constant temperature room and the insects exposed to continuous illumination for period in P. brassicae and V. urticae extends for the whole time of illumination with no peak of activity corresponding to the time of illumination in natural conditions. Both P. brassicae and V. urticae fly only when illuminated and appear to require untra-violet light. When a Phillips warm white (80 W/29) strip light was fitted no flight occurred; this light does not emit the ultra-violet range.

Under continuous illumination Z. filipendulae exhibits a peak of maximum activity coincident with the activity when exposed to the normal scoto- and photo-phase, nevertheless, some activity occurs throughout the 24-72 hour period of illumination. These 3 species are strictly diurnal and no flight occurs in the absence of illumination. Further experiments in which P. brassicae was subjected to 6 hour photophase alternated with 6 hour scotophase

for 72 hours reveals that this species will fly during any period of illumination regardless of the time (fig. 13). While Z. filipendulae shows some evidence of an endogenous circadian flight rhythm, none is apparent in P. brassicae or V. urticae.

Many nocturnal moths were examined, those were collected from light traps maintained at Silwood Park. After capture, species were sexed and separated and confined in muslin walled cages in a 20°C constant temperature room; exposed to a 16 hour day (7.00 a.m. - 11.00 p.m.). Test conditions were essentially the same as those used for the diurnal species, except that the light source was disconnected and the room in which the actograph was used was blacked out. This stage of recording was carried out as a preliminary to selecting a species for further experimentation; various other factors had, therefore, to be taken into account. As various surgical procedures were envisaged to investigate endocrine effects, smaller noctuids, notodontids and geometrods were rejected as they showed a low survival rate after incisions were made in the head capsule and cervical region. Larger noctuids such as Autographa gamma, Diataraxia oleracea and Noctua pronuba fulfill this requirement, and also have a distinct pattern of nocturnal flight. Figure 14 shows recordings from A. gamma and D. oleracea; it is interesting that both species are also diurnally active. Weak bursts of diurnal flight is characteristic of D. oleracea, and a marked overrun of vigorous flight into the photophase commonly occurs in A. gamma. In neither case is it likely that the bursts of diurnal flight result from disturbance within the actograph; A. gamma can often be observed flying and feeding

during the day. Recordings taken during 24-72 hours continuous darkness show that D. oleracea, A. gamma and T. pronuba have a persistent endogenous circadian flight period (fig. 14), whereas the diurnal flight activity is promoted by light following the scotophase, and no circadian rhythm during 72 hours continuous light is detectable.

In each experiment it was found practical to use more than one moth. When activity was recorded continuously for 3 days, or as long as 14 days, the flight chamber could be left undisturbed even if one moth died. However, when too many moths were put in the flight chamber mutual disturbance became a significant factor; 3-5 moths was the most suitable number and as the availability of moths varied, 3 moths were used in each experiment.

The flight chamber obviously presents an unnatural environment inasmuch as the space for free-flight is comparatively small. For this reason large rapidly-flying moths such as sphingids were not used. Initial trials with Mimas tiliae Linn and Smerinthus ocellatus Linn proved unsatisfactory as they became badly damaged after several nights. In this respect the noctuids used are ideal in that very little damage through contact with the walls of the flight chamber occurs even after several weeks. The damage sustained by sphingids in prolonged experiments depends upon certain flight characteristics which are absent in the Saturniidae; groups of 3 male P. cynthia ricini and H. cloveri were repeatedly used and lived as long as controls confined to large muslin walled cages.

In considering of the activity patterns of the species tested, the more clearly demarcated nocturnal flight period of T. pronuba made this species the most suitable for further experimentation. Difficulties were encountered in establishing a breeding culture, but during the summer months large numbers became available from the light traps. Furthermore, some individuals survive for 2-3 months under laboratory conditions. From the point of view of surgical procedures, the Tindall effect exhibited by the medial neurosecretory cells (A cells) is a distinct advantage, and facilitates the selective ablation of these cells with a high degree of accuracy. This property is shared by several other species of Noctuidae, but the field populations at Silwood Park (as indicated by light trap captures) are very much smaller than that of T. pronuba.

The experiments detailed in subsequent sections were applied mainly to T. pronuba, but when time permitted selected experiments were also made with, A. ipsilon, A. gamma and P. cynthia ricini (where these were considered particularly significant).

It is likely that activity rhythms in females are complicated by endocrine changes associated with reproductive cycles, and oviposition behaviour, therefore, males alone were used in all experiments.

Effect of Feeding.

The time of onset of flight, and its duration and amplitude proved quite uniform, in unfed males (T. pronuba), collected from the light traps and recorded within 72 hours. Whereas moths that had been exposed to a feeding pad varied considerably; frequently showing a marked reduction in activity and a shift

in the peak flight time. In [illegible] there was no flight, or it was of brief duration, in the photophase alone.

This suggests that feeding has a marked effect upon the circadian flight rhythm. To investigate this, freshly captured moths were isolated and starved for 48 hours. Three moths were transferred to the flight chamber of the actograph and flight activity was recorded for further period of 24 hours of starvation. With few exceptions vigorous nocturnal flight occurred. A feeding pad was then placed inside the flight chamber, and removed 24 hours later, when the moths were examined to assess the extent of feeding in each moth. After 3 days starvation they usually feed until satiated. Individuals weighed prior to, and immediately after feeding often show a 30% gain in weight. However, a relatively large volume of liquid imbibed can be gauged by the distended abdomen. Occasionally moths were encountered which would not feed. Upon dissection a pathological condition was recognised, in that the oesophagus was partially or completely occluded by a plug of melanised tissue. Diseased moths do not usually survive the initial period of starvation.

A typical activity pattern extending over the pre-feeding, feeding and starvation periods is shown in figs. 15 and 16 they depict an uninterrupted record from the same three moths. The marked reduction in activity during the feeding period always occurs. Obviously no flight takes place when the moths are feeding and it is possible that feeding occurs at intervals throughout the night. The low levels of flight activity during the 2nd and 3rd nights suggests that some inhibitory process

has taken place as sporadic diurnal flight occurs during the photophases of the first 72 hours it is apparent the only nocturnal flight is being inhibited.

On the third night there is a considerable increase in activity with further increased during the 4th and 5th nights. Very little increase in activity occurs after the fifth night, and the feeding/starvation sequence can then be duplicated with striking regularity.

The commonly occurring burst of activity commencing at 8.00-8.30 a.m. is associated with switching on of the light. It is noteworthy that this activity is also affected by the nutritional status. On the first and second days it is absent but reappears on the third day and reaches a peak on the following day. The fourth day appears as a transition point and in some individuals the amount of activity recorded between 8.00-9.00 a.m. exceeds that of the nocturnal phase. The possibility that this morning burst of activity is an unnatural one, induced by the sudden dark-light transition cannot be overlooked. However, a dawn flight is a common feature of nocturnal moths and is probably represented by this brief burst of activity but enhanced by the experimental abrupt transition.

In these experiments a 1% solution of honey was used as the food sources at lower concentrations the quantity imbibed varies. Higher concentrations produce a longer period of inihition.

It is apparent that nutritional status is an important factor in determining flight activity. In all subsequent experiments this factor was taken into account, and, utilising the

uniform activity pattern and marked nocturnal habits exhibited by T. pronuba after 4-5 days starvation, moths were starved for a standard 4 day period.

(4) Effect of Age.

As only a limited number of moths became available from laboratory reared stocks a large proportion of the experimental work had to be carried out on specimens collected from the light traps. No method was found for assessing the age of these, but age is a factor which must be considered. Edwards (1962) found that changes in the flight activity pattern occurred in 4 out of the 7 species he studied.

A group of 20 pupae from laboratory reared T. pronuba were isolated and later, moths divided into age groups after emergence. The first 5 days activity was recorded from 4 groups of 3 moths which were unfed. Thereafter activity was recorded for 2 consecutive days, representing days 4 and 5 after each exposure to a feeding pad. This was continued for 60 days from the time of emergence.

After 4-5 days from the day of emergence the full flight activity had developed and very little variation was shown by the different groups of moths. There was no shift in the time of onset of nocturnal or diurnal activity. Only the amplitude and duration of flight activity showed any change; there was a gradual increase in the first 4 days, and a fall off as the moths aged.

(5) Effect of Continuous Illumination.

From the experiments on feeding and starvation it became evident that some diurnal flight always occurs in Noctua. From the feeding-starvation experiments the extent of diurnal flight was found to vary, and that variation was related to the nutritional status of the moths. Initial records of activity for periods varying from 48 to 72 hours under continuous illumination gave no indication that diurnal flight has a circadian rhythm.

The effect of continuous illumination was further investigated upon both 5-day starved and freshly fed moths. In each experiment a continuous record was maintained for 7 days. With continuous illumination temperature did not fluctuate and remained at 20°C or 25°C. The 7-day activity records showed no difference at either temperature. Only during the first day was any difference apparent between starved and fed moths; those that had been fed showed a small burst of activity between 7.00 and 10.00 a.m. On the other hand starved moths showed a small burst of activity, in 3 out of 4 groups; this occurred between 11.00 p.m. and 2.00 a.m. In both starved and fed moths very little or no activity occurred during the following 6 days. This reduced activity was sporadic and had no circadian rhythm. It is quite apparent that exposure to continuous illumination has a marked inhibitory effect.

(6) Effect of Continuous Darkness.

It has already been noted that under constant darkness for 48 or 72 hours a circadian cycle of flight activity persists, the onset and duration coinciding approximately with the normal nocturnal rhythm. These experiments were extended in different batches of moths, their activity being continuously recorded for as long as 14 days. After 7 days however, moths began to die of starvation and it became impractical to extend recording beyond 8 days. Figures 17 and 18 shows a continuous record of one group of 3 moths for a period of 8 days. These had been starved for 24 hours after capture from the light traps. During the first 24 hours in the actograph an 11 hour scotophase was given, which corresponded approximately to field conditions at that time of the year. This was followed by a period of continuous illumination lasting until 5.00 p.m. on the second day, at which time the light was switched off. The flight activity occurring in the following 5-days was recorded in constant darkness.

The nocturnal rhythm quite clearly persisted and showed a circadian rhythm. It is noteworthy that the moths become re-entrained to commence flight just after 5.00 p.m. i.e. at the time when the light was switched off. This effect diminished with time 2nd by the 5th 'day' of constant darkness the first strong burst of activity returned to 9.30 p.m. The entraining effect of the light-dark transition is clearly demonstrated in fig. 18., in this instance the same moths were exposed to a 1 hour illumination period from 1.00-2.00 p.m. Further experi-

ments were conducted with separate groups of moths, when the light was switched off at 9.00 a.m., mid-day and 1.00 a.m. following 1 hour illumination, after several days constant darkness. In each instance re-entrainment occurred, but the effect was less marked and the normal nocturnal periodicity commencing about 9.30 p.m., resumed after 1 or 2 days.

(7) Effect of Phase Reversal.

In the above experiments an endogenous circadian flight rhythm was demonstrated, and also the entraining effect of the light-dark transition, introduced at a time which would not occur naturally. The effect of complete phase reversal was investigated; introduced after several days exposure to the usual 8 hour scotophase (11.00 p.m.-7.00 p.m.). To introduce reversed phases, the new scotophase (11.00 a.m.-7.00 p.m.) must be preceded either by a short photophase (7.00-11,00 a.m.) or by a 28 hour photophase. Alternatively, a normal scotophase that ends at 7.00 a.m. can be extended to 7.00 p.m., which means preceding the reversed phases by a 20 hour scotophase.

The effect of these 3 conditions before the reversal of the phases was investigated, by recording flight activity for 4 days. Following a 28 hour photophase a near-normal pattern of activity is exhibited, occurring in the new scotophase (fig. 19). The new scotophase is adopted on the first experimental "night" and similiar patterns of flight occur on the 2nd, 3rd and 4th. 'nights.' When the reversed phases are preceded by a 20 hour scotophase, the new scotophase is likewise adopted from the first 'night' with closely similiar patterns of flight on

the subsequent 'night'. However, a 4 hour photophase preceding the reversed phases elicits very little or no activity during the first reversed scotophase, but during the 2nd, 3rd and 4th 'nights', near-normal levels of activity again occur (fig. 19). Seemingly the moths must be exposed to a certain duration of photophase before re-entrainment to a reversed or shifted scotophase can take place. Experiments in which photophases of 5, 6 or 7 hours duration were given have shown that at least 6 hours darkness are necessary at a temperature of 20°C. Separate groups of moths were used in each experiment which was repeated at least 3 times. In further experiments moths were exposed to reversed phases following a 28 hour photophase, and activity was then recorded in constant darkness for 48 hours (fig. 20). While the reversed scotophase is adopted, the levels of activity are always lower than in those in which the scotophase is set at a natural time. The appearance of bursts of activity occurring 11.00 p.m. and 7.00 a.m. in constant darkness again demonstrates the endogenous rhythm, but there is a distinct tendancy to shift back to the original time commencing at 11.00 p.m.

These results imply that some internal process operates in controlling the circadian flight rhythm in a manner similiar to that which Harker (1956b and 1960a) has demonstrated in P. americana.

Assuming the release of an activating factor at the onset of the endogenous rhythm, it is apparent that it only promotes flight activity when the moths are in darkness, or, that it is

released at the onset of darkness. The transition from light to darkness appears to set the 'internal clock', which is then released at this time on each subsequent day or night in a circadian rhythm when constant darkness is maintained. Furthermore the efficiency of entrainment increases progressively as conditions approach the natural dusk.

(8) Effect of Chilling.

Harker (1960b) showed that when intact cockroaches were chilled to 3°C, a delay equivalent to the duration of chilling occurred before the next period of activity. A similiar effect was obtained when the sub-oesophageal ganglion alone was chilled.

Moths were taken from a normal light-dark cycle (11.00 p.m. - 7.00 a.m. scotophase), at midnight and chilled at 2°C for 12 hours. They were then transferred to the flight chamber of the actograph in which the scotophase had been reversed (11.00 a.m. - 7.00 p.m.). After one complete cycle (24 hours) further activity was recorded in constant darkness for 72 hours (fig. 21). The persistence of a circadian flight rhythm commencing between 11.00 a.m. and midday indicated that the endogenous rhythm was shifted by 12 hours. In contrast to reversing the scotophase without chilling, a permanent reversal in the endogenous rhythm had taken place.

(9) Effect of Ablating Selected Areas in the Brain.

a. Medial Neurosecretory Cells.

The "Tindall effect" exhibited by both A_1- and A_2-cells makes it possible to ablate selectively these cells with a fairly high degree of accuracy and with a minimum amount of damage to the adjacent neurones.

The operation was performed upon moths that had been starved for two days. Ether and chloroform anaesthesia resulted in a high post-operative mortality. Chilling to 2°C and CO_2 anaesthesia proved quite satisfactory, immobilising the moths for longer periods, and allowing full recovery. Anaesthetised moths were rapidly transferred to a cooled plasticine platform which had been moulded to fit the ventrum. The thorax was held in position with a plasticine strap and beads of plasticine were moulded around the compound eyes and frons, thus firmly securing the head. A small area of cuticle above the brain was removed, and the exposed dorsal tracheae moved to one side. Gentle pressure on the abdomen maintained a pool of haemolymph covering the brain. By releasing the abdomen the level was lowered below the neurosecretory cells which were immediately cauterized, and the brain was then again bathed in haemolymph. The excised piece of cuticle was extremely difficult to replace with out damaging the brain; as an alternative small pieces of Kleenex tissue were used. These were soaked in Ringer solution (Hoyle, 1953), and immediately after cauterisation a piece was stretched across the cut edges of the cuticle. Sealing was effected by moulding bees wax over the moistened tissue and over the edges of the

cuticle, using a warm needle.

In the above, and in subsequent operations, ablation was accomplished with a battery operated micro-cautery. Briefly, the instrument comprises a heater filament formed from a loop of 47 SWG platinum wire mounted in a plastic handle, through which are carried wires connected to a 1½ volt battery. Current is controlled by a potentiometer and monitored by an ammeter. The platinum wire loop was bent to form an effective cauterising tip approximately 60μ in diameter.

Operated moths were isolated in humid containers for at least five days, as it was found that individuals living that long usually lived for at least a further five days. Groups of 3 moths were transferred to the actograph, and their activity was recorded for several consec utive days. In each instance there was no activity during the scotophase but some flight usually occurred during the photophase (fig. 22). This indicates that the ability to fly was not abolished, but there is a definite loss of the factor that iniates and maintains nocturnal flight.

b. Other Areas of the Brain.

Identical operations were carried out on three selected areas of the brain:- area immediately lateral to the A-cells, fronto-lateral area, and posterior area. In the first two, the areas were cauterised on both sides of the brain, and in the last a single medial area. Care was taken to avoid damaging the medial cells, and the total areas ablated were approximately equivalent to that of the A-cell ablation.

Results of all three experiments were essentially similiar:- apart from a general reduction in flight amplitude, the nocturnal rhythm was unaffected (fig. 22).

Post-mortem Examination.

At the end of each experiment, moths were dissected to assess the extent of the operations. One or more of the brains were fixed, sectioned and stained with PAF. While A-cell ablation was conducted quite successfully, some underlying B_1-cells were inevitably damaged. Out of the 82 B_1-cells in this species, between 40 and 60 cells survived the operation. The wound becomes partially sealed by the cautery, but adjacent cells do degenerate and become melanised, and this increases in successive days after the operation. Sections of brains in which areas adjacent to the A-cells were ablated, in no instance revealed any necrotic changes in the medial neurosecretory cells. Lateral ablation resulted in loss of the A_4-, B_2- and C_2-cells, but this does not seem to impair flight activity or rhythmicity any more than does ablation to non-neurosecretory neurones.

(10) Effect of Injecting Pharmacologically Active Substances.

a. A-cells Ablated.

5-Hydroxytryptamine. Previous experiments have implicated one or more types of medial A-cell in the control of the circadian flight rhythm. When this is considered together with histological demonstration of a circadian cycle of secretion by A_1- and A_2-cells, and histochemical indication of 5-HT, it is feasible that this substance has a function in the initiation and maintenance of nocturnal flight activity. This hypothesis was

tested by injecting 5-HT into moths in which the A-cells had been ablated. Solutions of this amine were prepared by dissolving it in distilled water or Ringers solution, and serially diluted over a range 1 part in 10^{-5} to/in 10^{-8}. At each concentration the moths received 1 µl. Activity was recorded for 24 hours before injection, and from 2-4 days afterwards. The actograph light was set to switch off at 11.00 p.m. and the injections given one hour earlier.

At all concentrations 5-HT injection consistently produced nocturnal flight activity, in moths that had previously been shown to be inactivie. There was a noticeable relationship between concentration and total activity; moths given higher dosages were more activie. At the two lower concentrations moths were active only during the night after the injection, but at the higher concentrations (1×10^{-6} and 1×10^{-5}), some activity also occurred during the following night (fig. 23).

Other Biogenic Amines. Moths were prepared by the method given above and injected with 1 µl samples of the following substances:- adrenaline, nor-adrenaline, dopamine, tyramine and tryptamine. Four concentrations of each amine were prepared, ranging from 1 part in 10^{-5} to 1 in 10^{-8}. They all failed to restore nocturnal flight activity, and whereas some diurnal flight occurs just after the lights switch on, there was none in moths injected with adrenaline and nor-adrenaline.

b. Unoperated Moths.

5-Hydroxytryptamine. This was administered at two concentrations; 1 part in 10^{-5}, and 1 in 10^{-2}. The moths used in these experi-

ments had been starved for 3 days, and injections were made at 10.00 p.m. i.e. one hour before the light in the actograph switched off. Records of activity of one group of 3 moths are shown in figure 24; During the first 24 hours before the injection, these moths displayed an activity pattern consistent with the fact that they had last fed approximately 72 hours earlier i.e. total activity was approximately equally divided between nocturnal and diurnal flight. Handling disturbed the moths, consequently they were placed under a bright light for 30 minutes to inhibit flight, and only then introduced into the actograph. On the first night after injection activity is considerable. It is slightly reduced during the second night, and more so during the third night. Some increase in nocturnal flight is to be expected even without injection, but consistently became greater in the groups of moths injected with 5-HT at low concentration. The subsidence of activity during successive nights after the initial injection provides further evidence of the stimulatory effect of 5-HT. However, when 5-HT administered at the higher concentration (1 μl, 1 part in 10^{-2}), activity is markedly depressed (fig. 25).

Other Biogenic Amines. Adrenaline, nor-adrenaline, dopamine, tyramine and tryptamine, were injected. Each moth received 1 μl, and two concentrations were tested; 1 part in 10^{-5} and 1 in 10^{-8}. Dopamine, tyramine and tryptamine had no measurable effect, whereas both adrenaline and nor-adrenaline inhibited flight activity. The duration of inhibition depended upon the concentration; at 1 in 10^{-5} activity was abolished during the first

night; but gradually recovered during the following two nights and was completely restored during the fourth night. At a concentration of 1 in 10^{-8}, activity was depressed during the first night only.

Reserpine. As this alkaloid is known to stimulate the release of 5-HT and catecholamines in vertebrates (Shore, 1962; Axelrod, 1964) it was interesting to examine its effect upon flight activity in T. pronuba. Dosages of 1 µl of 1 in 10^{-6}, 1 in 10^{-4} and 1 in 10^{-2} were injected. At the highest concentration activity was completely depressed during the 4-day test period; and considerable depressed at 1 part in 10^{-4}. However at the concentration of 1 part in 10^{-6}, the opposite occurred and activity was considerable enhanced. (fig. 25).

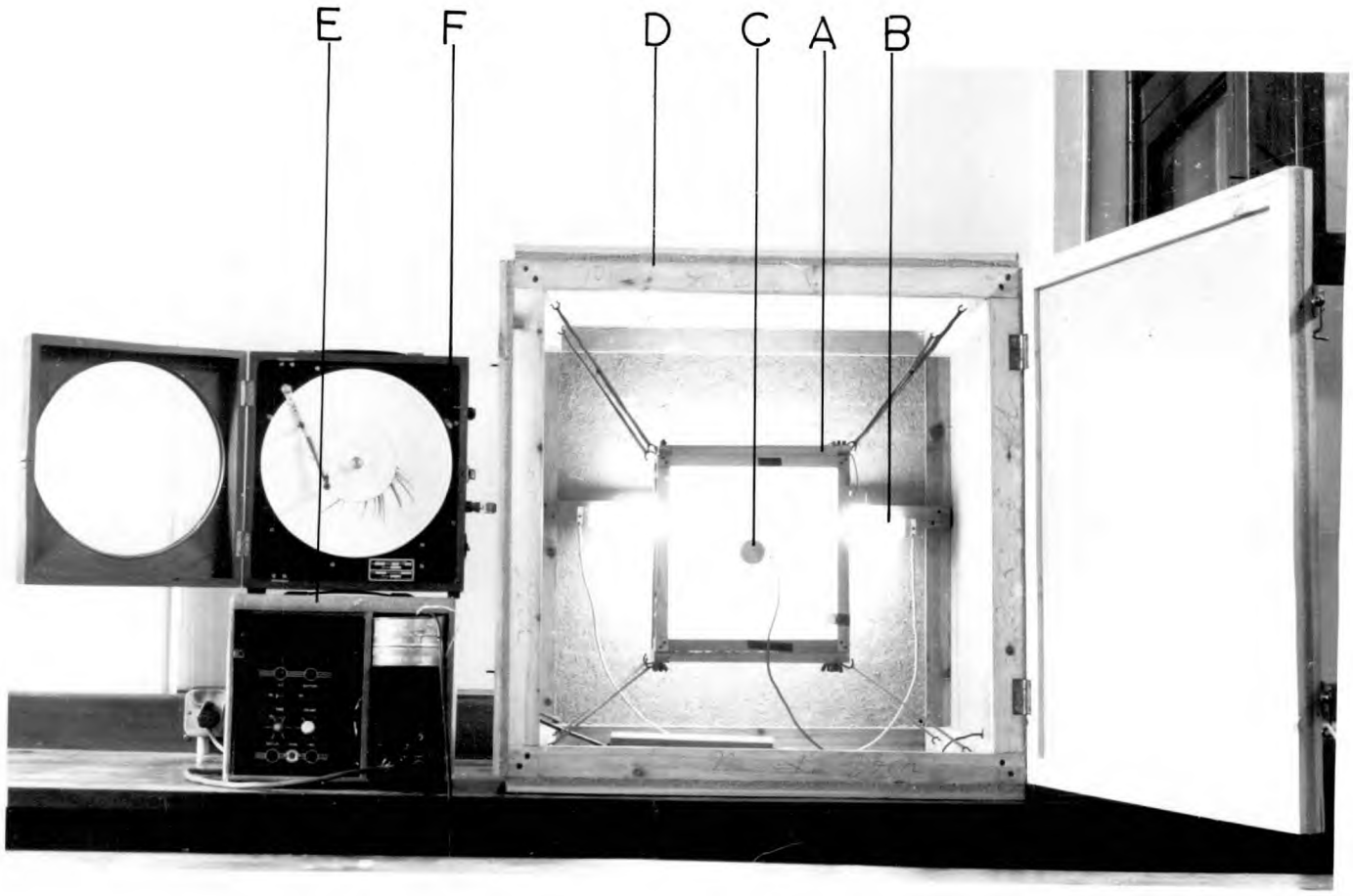

Fig. 12. Actograph. A, flight chamber; B, strip light; C, microphone-insert; D, sound-insulating box; E, amplifier; F, Fielden Servograph.

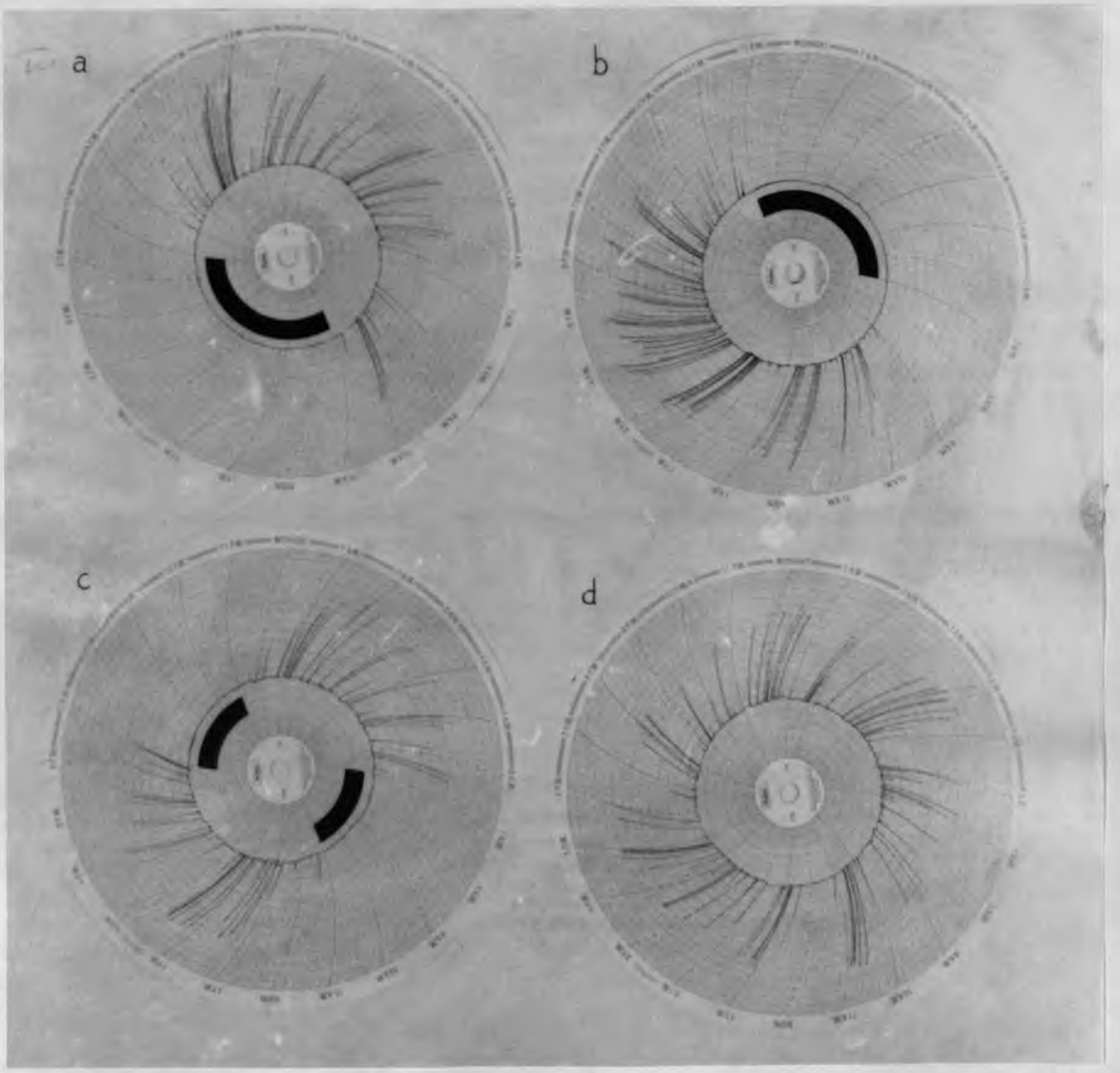

Fig. 13. Flight activity records of Pieris brassicae. a. Reversed scotophase. b. Normal scotophase. c. Two scotophases d. Continuous illumination.

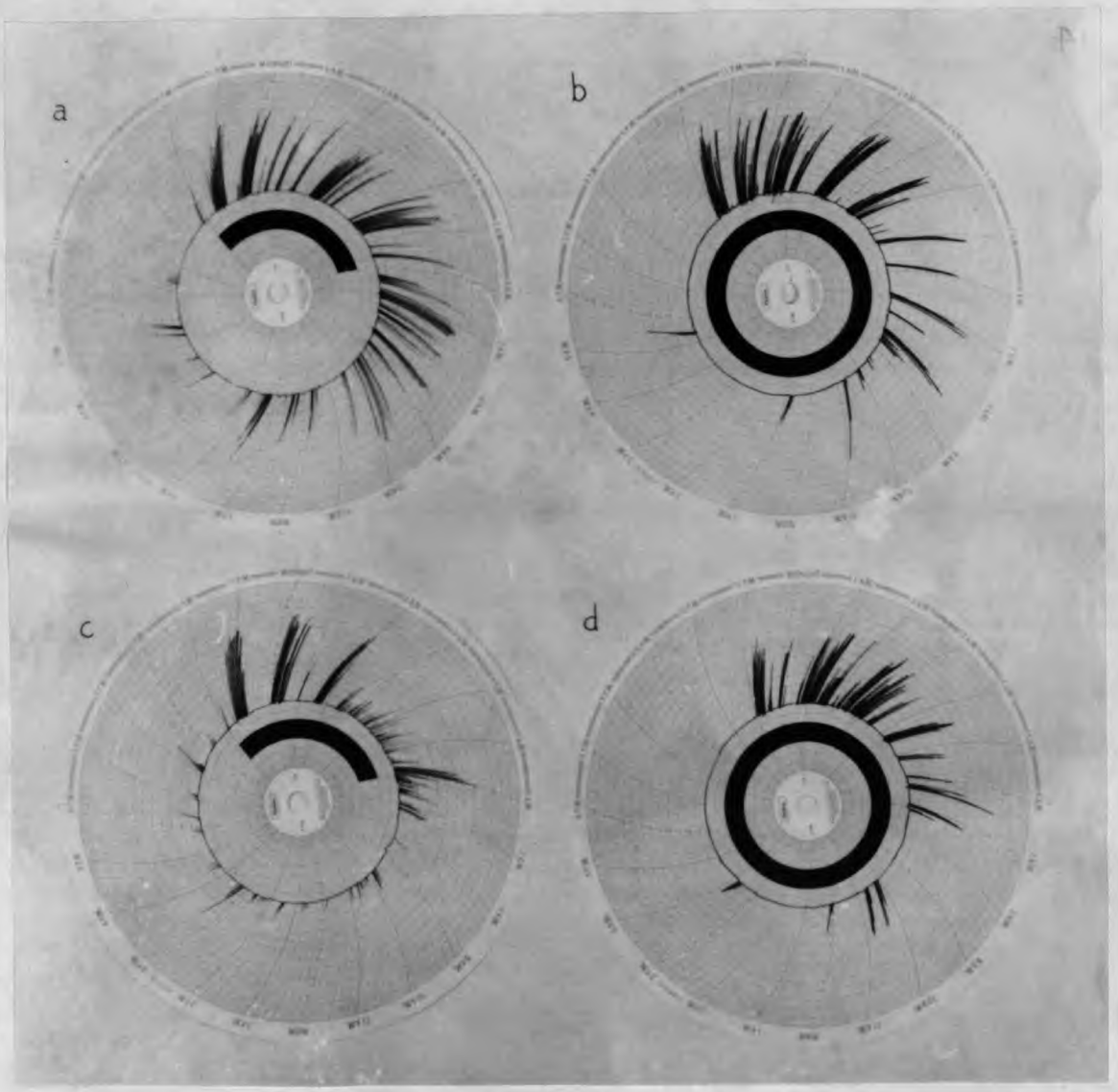

Fig. 14. Flight activity of Autographa gamma (a. & b.) and Diataraxia oleracea (c. & d.). Normal scotophase and constant darkness.

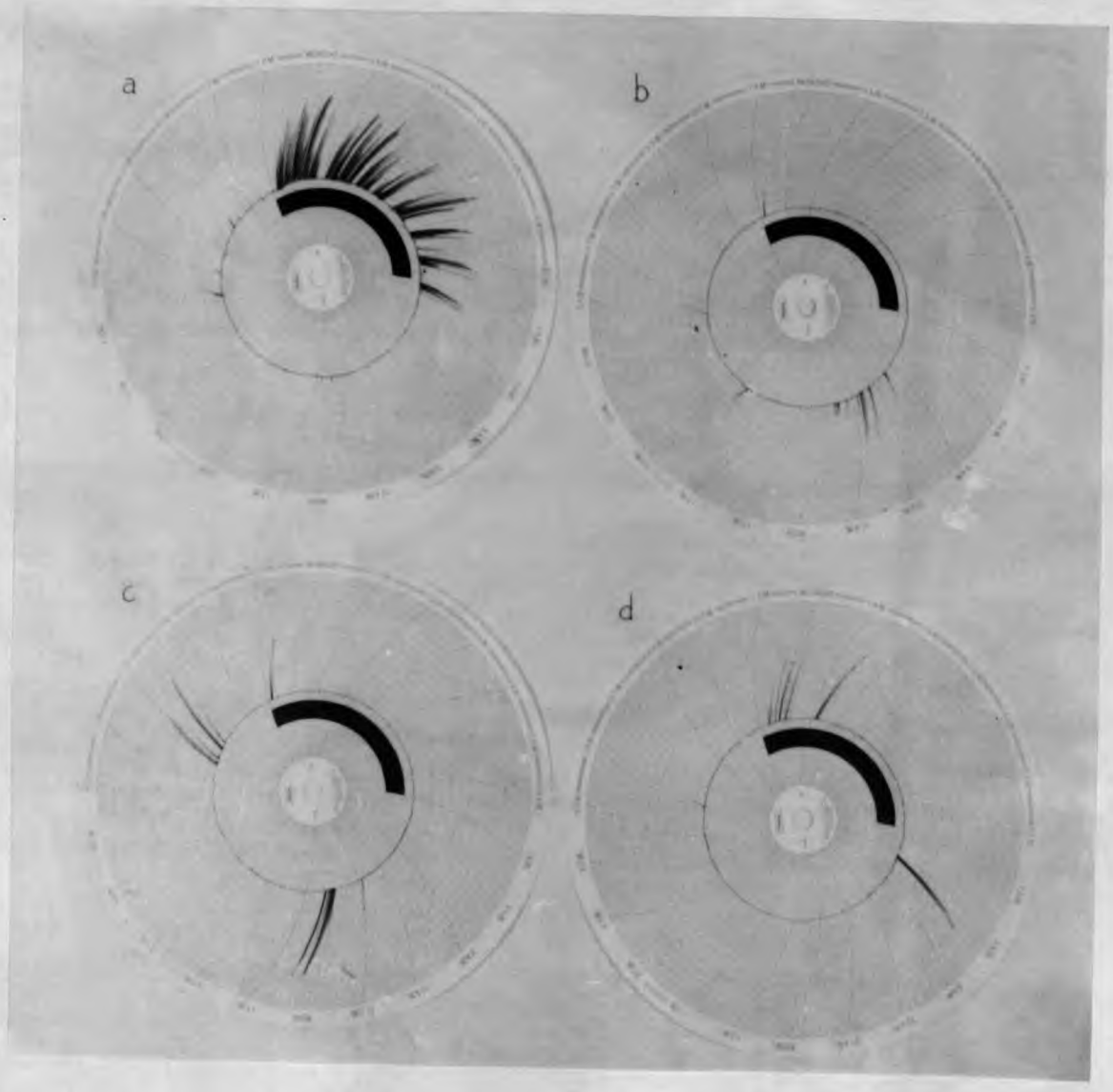

Fig. 15. Effect of feeding upon flight activity of Triphaena pronuba. a. Unfed. b.-d. The same moths fed during the scotophase in b.

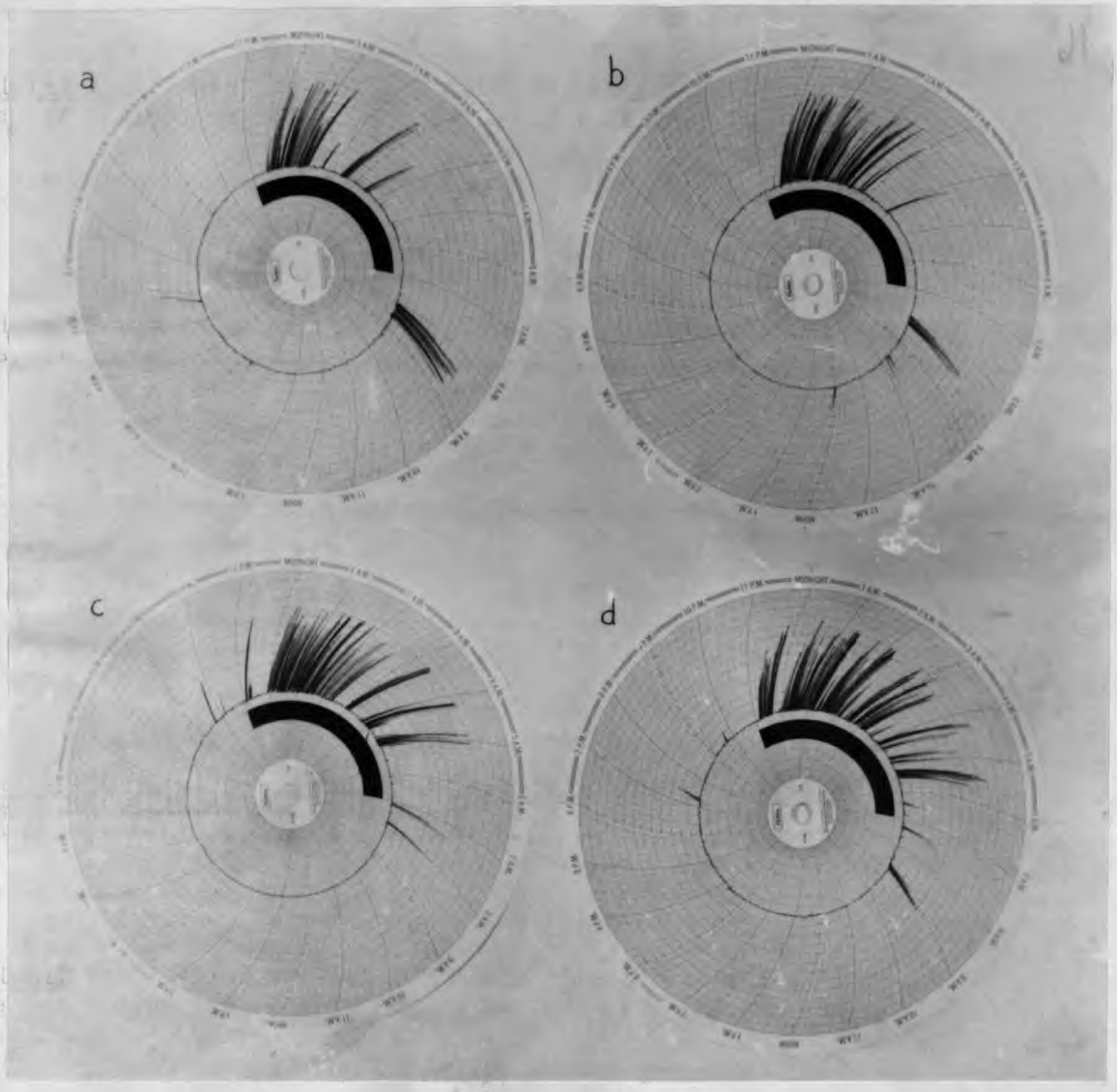

Fig. 16. Activity records of the same 3 moths shown in fig. 15 (unfed; note the restoration of nocturnal flight).

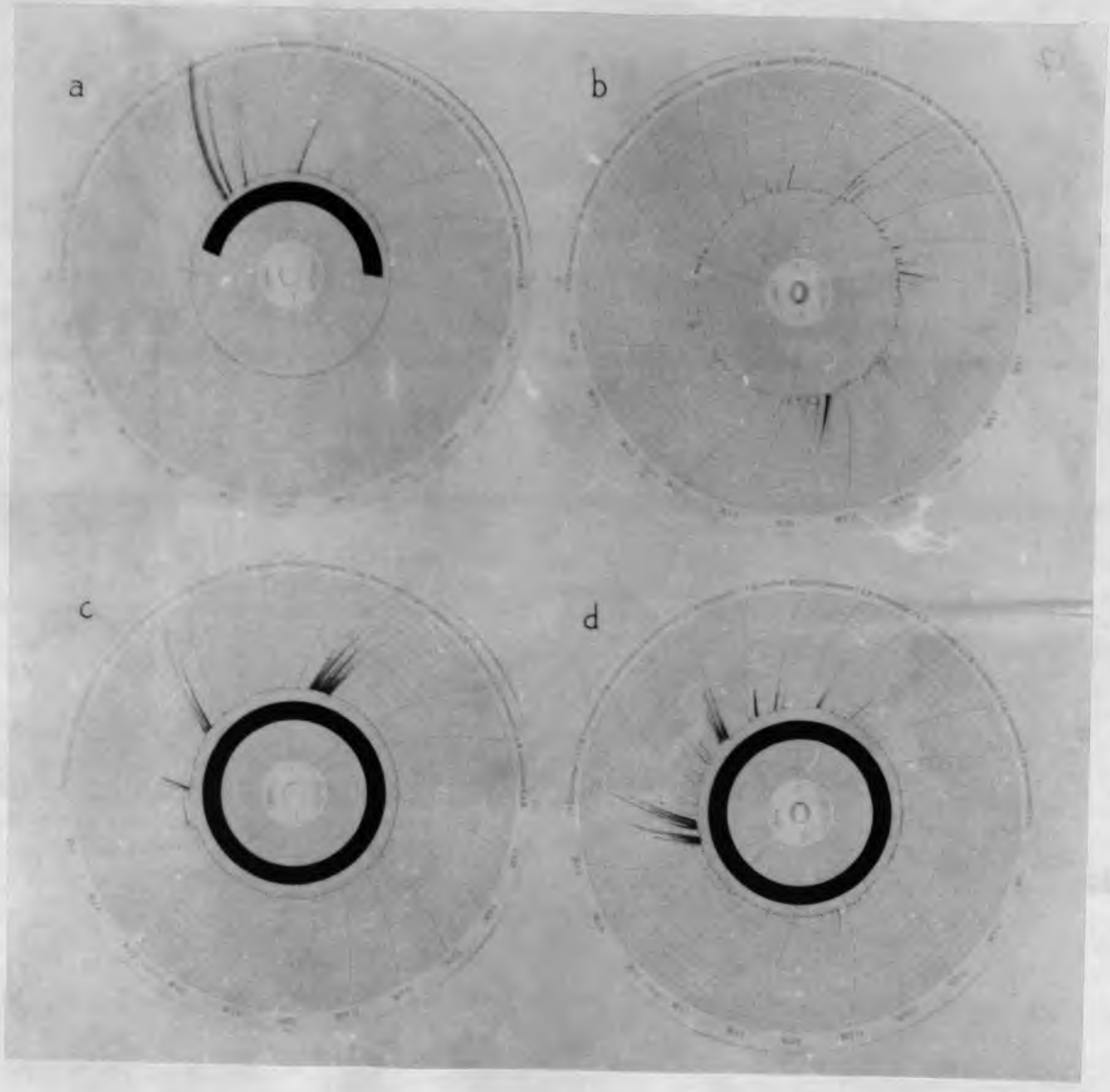

Fig. 17. Activity of T. pronuba. a. Normal scotophase. b. Continuous illumination. c. & d. Constant darkness (same moths and consecutive periods of 24 hrs.).

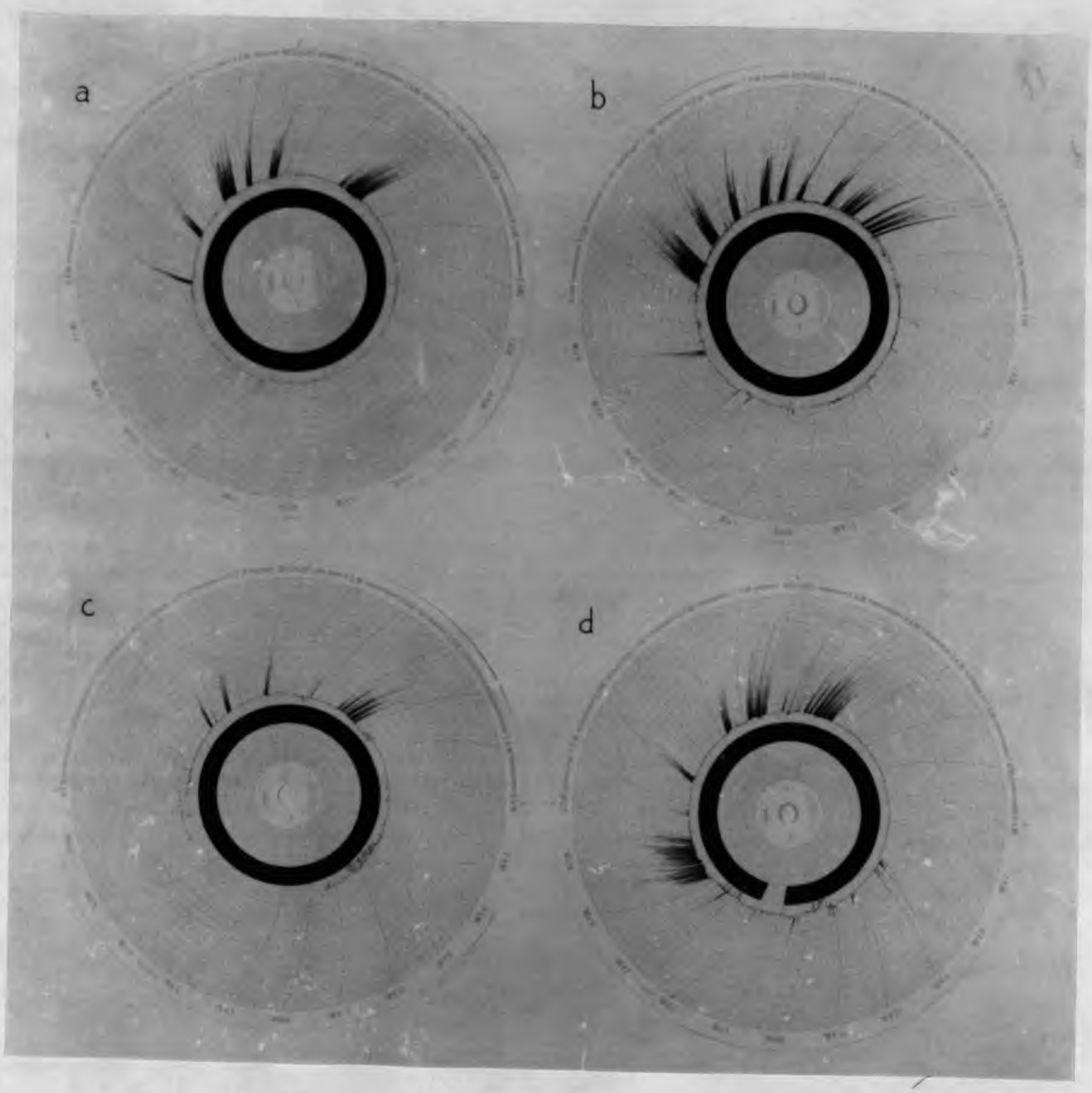

Fig. 18. Continuation of fig. 17. a.-d. consecutive periods of 24 hr.

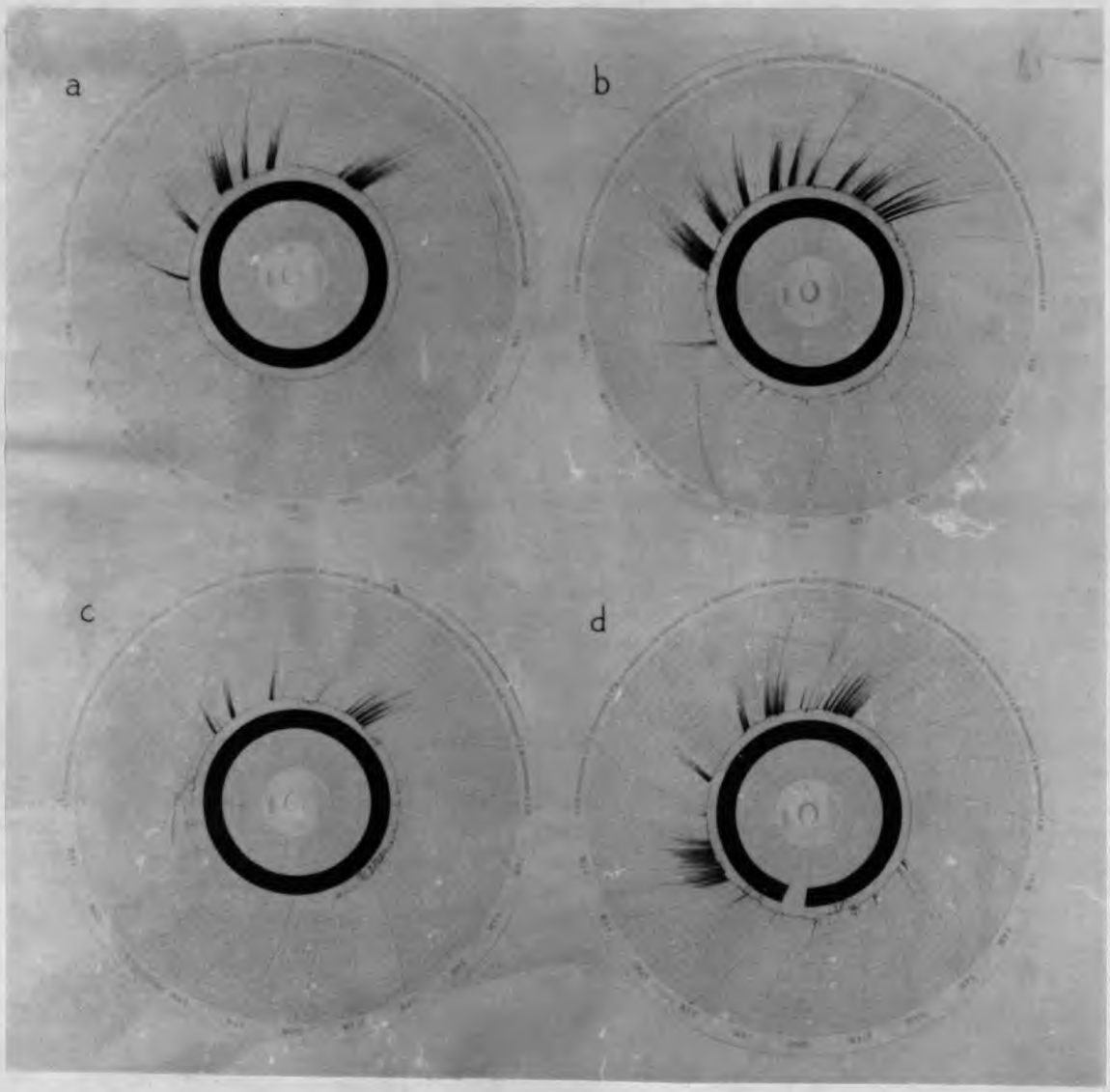

Fig. 19. Effect of reversed scotophase upon flight activity of T. pronuba. a. Scotophase preceded by 4 hr. photophase. b.-d. Activity of the same moths during consecutive periods of 24 hr.

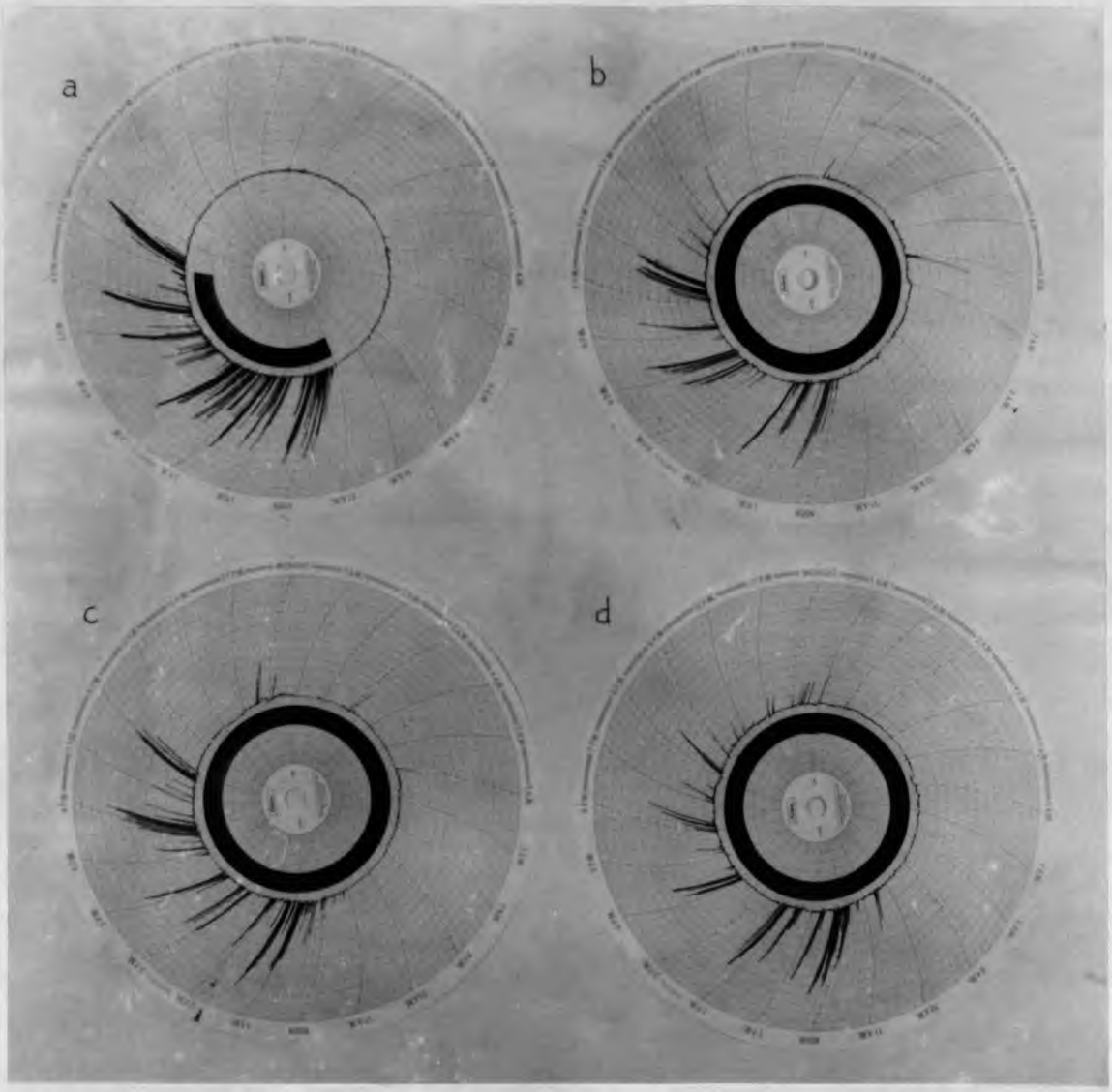

Fig. 20. Effect of reversed scotophase (a.), preceded by 20 hr. scotophase. b.-d. Activity of the same moths during consecutive periods of 24 hrs. in constant darkness.

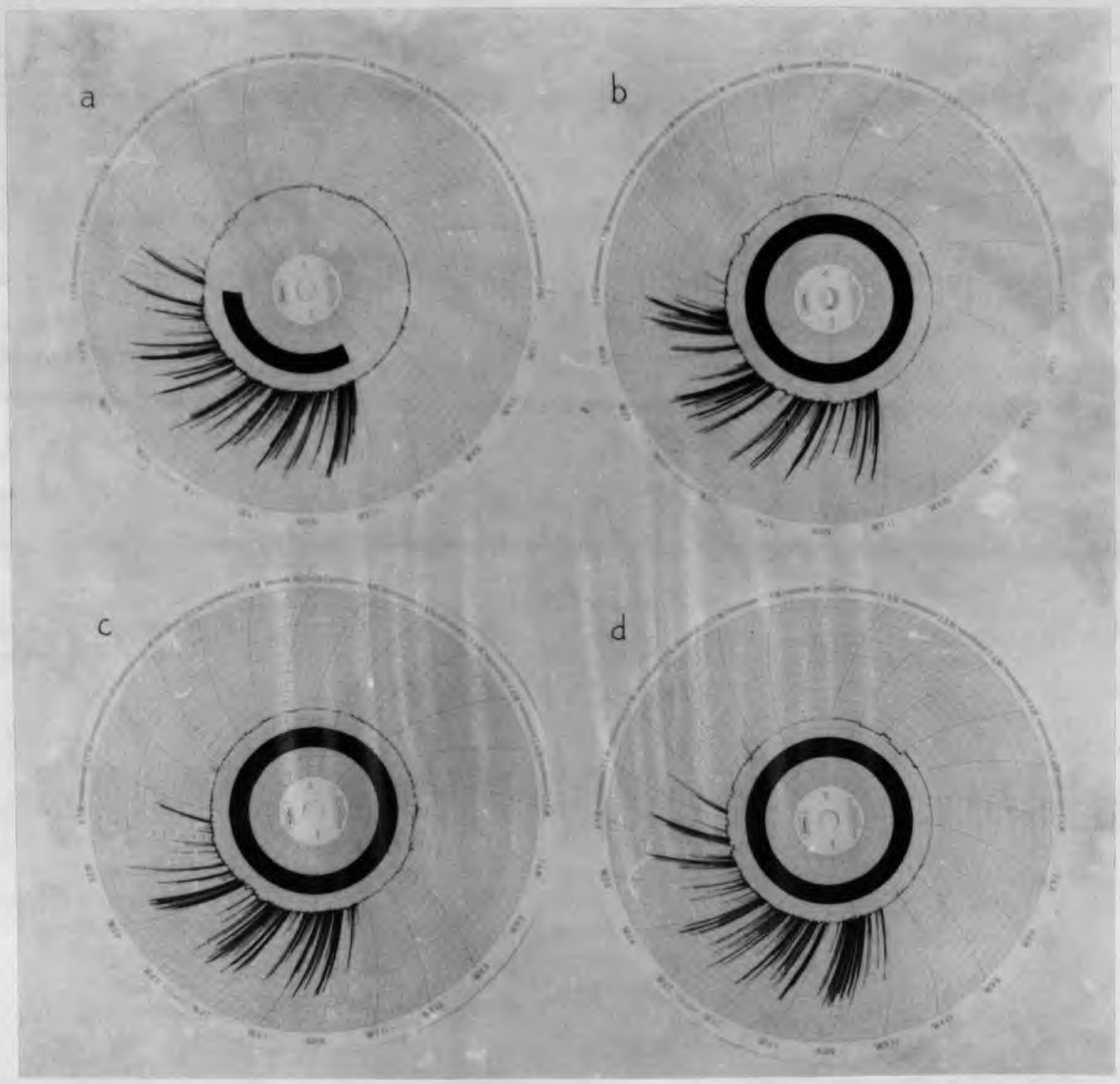

Fig. 21. Effect of reversed scotophase (a.), after chilling moths for 12 hr. b.-d. Activity of the same moths during consecutive periods of 24 hr. (compare with fig. 20).

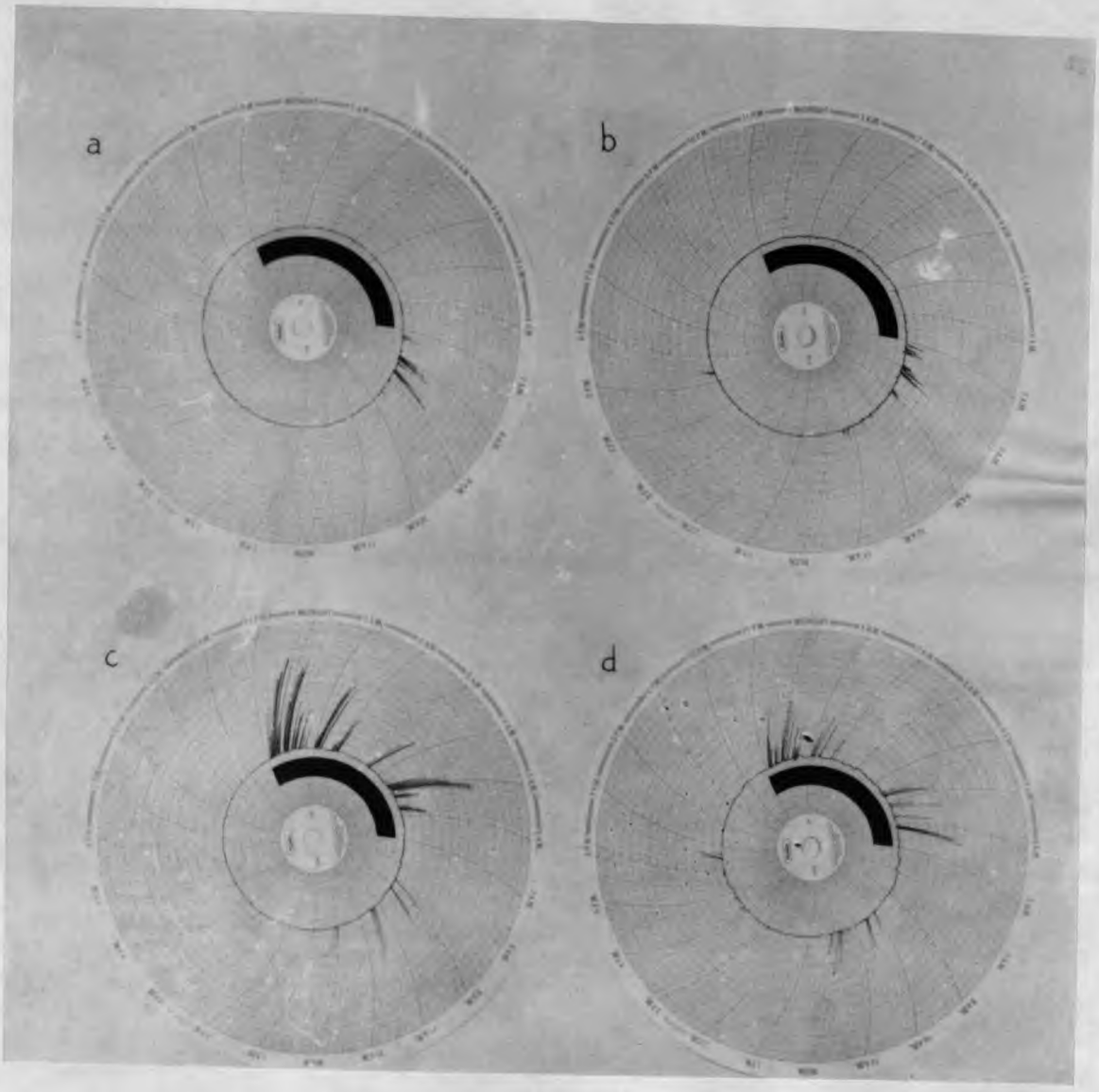

Fig. 22. Effect of ablating medial A-cells (a. & b.). Effect of ablating lateral neurones (c. & d.).

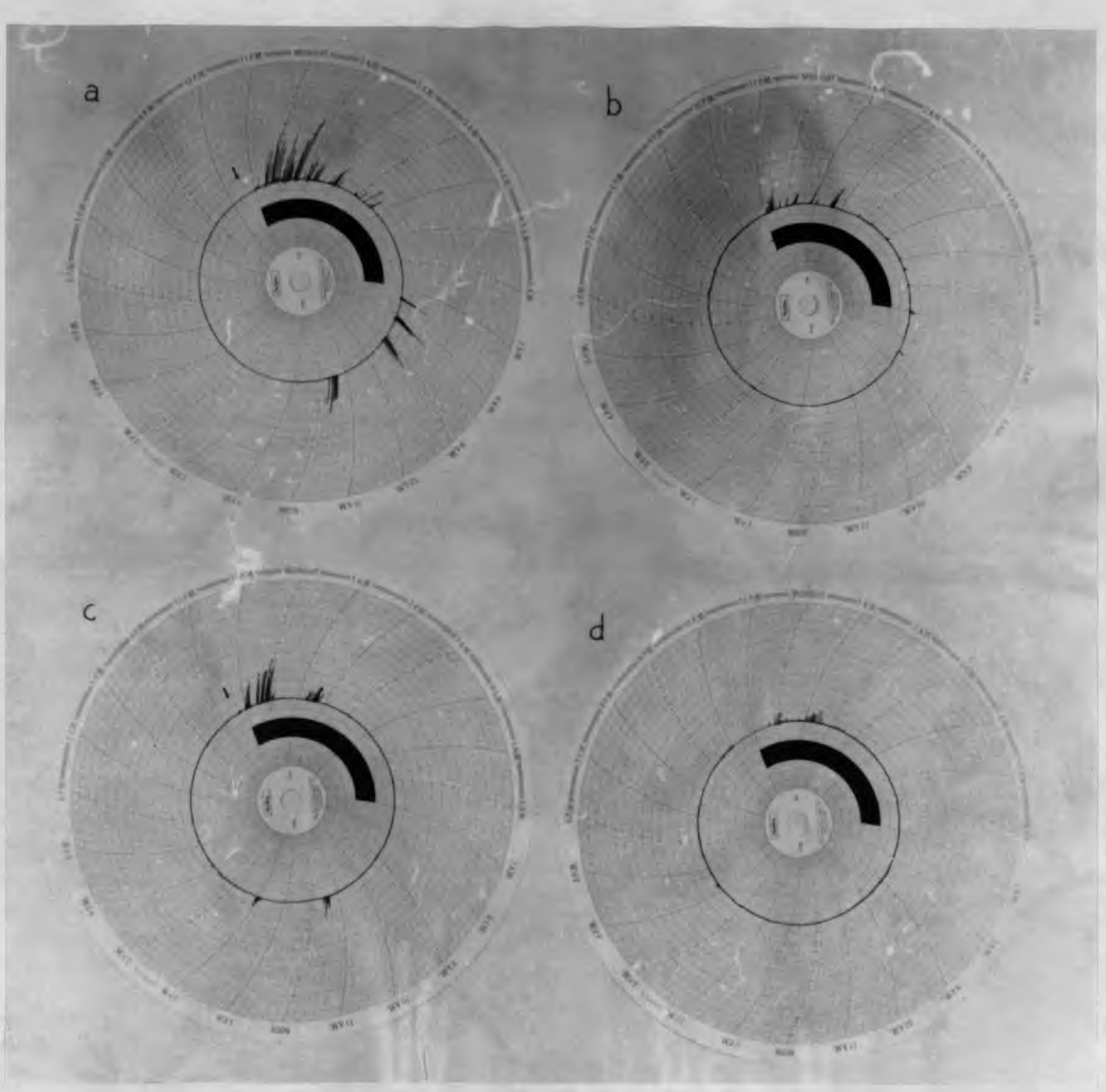

Fig. 23. Effect of ablating medial A-cells, and injecting 1µl, 1 x 10-5 5-HT (arrows in a. & c.).

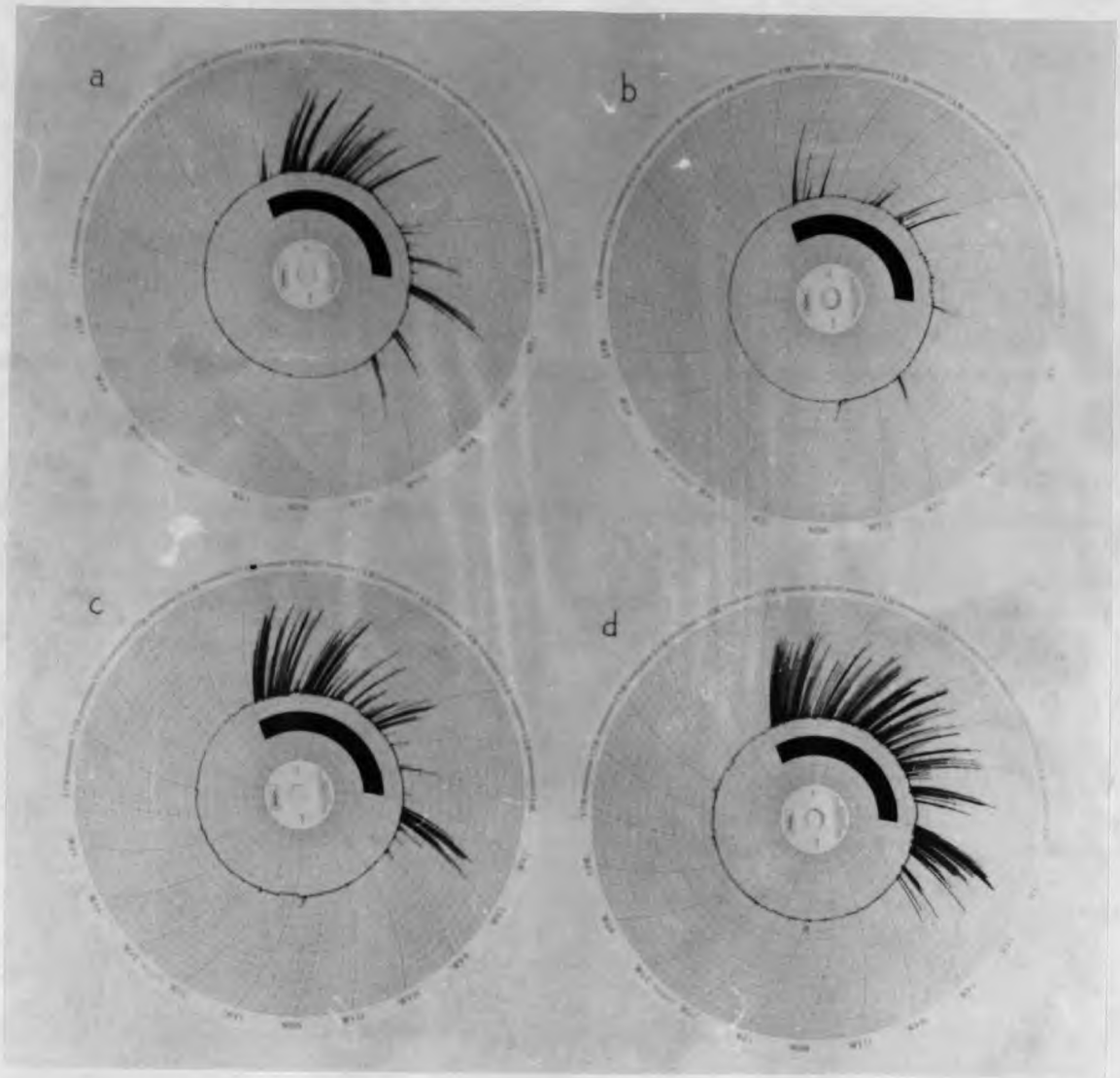

Fig. 24. Effect of 5-HT in unoperated moths (1µl, 1 x 10^{-5}) (moths injected at 10.30 p.m., b.).

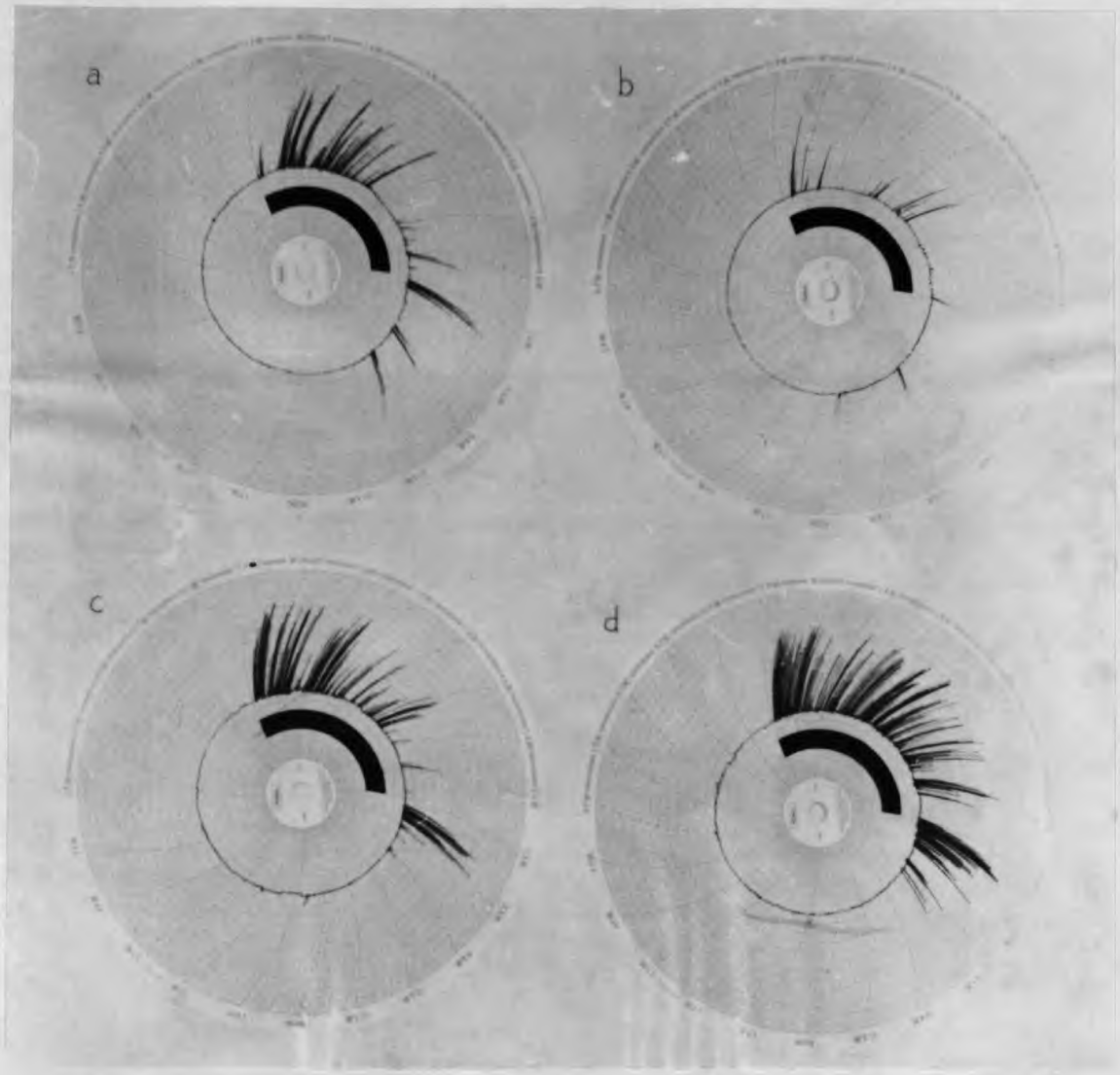

Fig. 25. Effect of 5-HT (1µl, 1 x 10^{-2}) injected into unoperated moths (b.), and reserpine (1µl, 1 x 10^{-6}) injected into unoperated moths (d.). a. & c. Activity before injection.

DISCUSSION.

In this study an endogenous circadian rhythm of flight activity has been clearly demonstrated in the nocturnal moths examined. This phenomenon is well documented for many insects (Reviews by Cloudsley-Thompson, 1961; Harker, 1961, 1964). In contrast strictly diurnal species of Lepidoptera display very weak rhythms, and in these flight activity appears to be an event initiated and maintained by the nervous system, without an endocrine link. In strictly nocturnal species the opposite obtains. Continuous illumination inhibits flight activity, and this is known to occur in other insects, for example in Aeschna nymphs (Serfaty, 1945), in Carausius morosus (Steiniger, 1936; Eidtmann, 1955), in Pseudosmitha aremaria (Remmert, 1955) and in Anopheles gambiae (Jones, Ford and Gillett, 1966).

In the past the influence of extrinsic factors has received most attention, however, during the last decade evidence has accumulated which indicates that the endogenous component involves one or more endocrine centres. In a series of papers Harker (1956, 1958, 1960a, b and c) has postulated a complex endocrine control in P. americana, involving the corpora cardiaca and neurosecretory cells of the sub-oesopageal ganglion. However many of Harker's findings have since been disputed. Roberts (1959, 1965) and Brady (1967, b and c). Were unable to duplicate her results after transplanting sub-oesophageal ganglia and severing the allatal-sub-oesophageal nerves. In an attempt to reconcile the conflicting results of Harker, Robert's and his own work, Brady (1967c) suggests that the brain provides primary

control, which is neural, and the hormonal centres in the sub-oesophageal ganglion and ventral cord ganglia are subordinate, ephemeral and easily upset. Furthermore Brady (1967b) found that cautery of the medial neurosecretory cells failed to abolish the locomotor rhythm. He then dismisses the assumption that similiar operations leading to loss of activity can reveal a vital part of a "biological clock."

It is evident from Harker's (1956) results that P. americana is both diurnally and nocturnally active, indeed she was able to demonstrate a persistent rhythm under continuous illumination; a rhythm that had been entrained by feeding. In other experiments it became apparent that rhythmicity faded after 5-6 days under conditions of constant darkness or continuous illumination. This may help to explain some of the conflicting results that have been obtained with this insect. As I have demonstrated in Lepidoptera, an endogenous rhythm can only be unequivocally demonstrated in strictly nocturnal species. Moreover my evidence from diurnal species suggests that neural (= "electrical" Brady, 1967c) control predominates. It is feasible that in an insect, such as P. americana, which is both diurnally and nocturnally active, the neural mechanism is operative under illumination, but there is a bias towards hormonal control in darkness. If this is true, then an appreciation of this distinction is most important in the interpretation of experimental data.

In T. pronuba and P. cynthia ricini histological studies of the neurosecretory cells of the brain showed differences in content of stainable inclusions at various times during a 24 hour

period. Similiar circadian cycles of secretory activity have been reported in medial neurosecretory cells of Carabus nemoralis (Klug, 1958), and of Drosophila melanogaster (Rensing, 1964, 1965a and b; Rensing, Thach, and Bruce, 1965). In the former, the rhythm is unimodal, and in the latter it is bimodal; in both species it is well correlated with locomotor activity. In addition, Rensing, Thach and Bruce (1965) were able to show cytological circadian rhythms in corpora allata, prothoracic glands and fat body in larvae of D. melanogaster. However, Rensing (1965a) regards the brain as the primary endocrine centre. This view is supported by Roberts (1959, 1965), Eidmann (1956) and Nishiitsutsuji-Uwo (1964), however, Roberts' (1959) evidence is based upon decapitation. Eidmann (1956) showed that surgical removal of the pars intercerebralis abolished locomotor rhythms in C. morosus. A similiar loss of rhythmicity was found in P. americana and Leucophaea maderae (Nishiitsutsuji-Uwo, 1964) after cautery of the pars intercerebralis. In the present study the effect of cauterizing the medial neurosecretory cells leaves no doubt that these cells are intimately connected with the flight rhythm.

As far as P. americana is concerned both Harker and Brady conclude that the endocrine centres involved, lie outside the brain, and are located within the sub-oesophageal ganglion, ventral cord ganglia, or both. In this respect it is worth comparing P. americana and T. pronuba. The latter, like other Lepidoptera, shows a pronounced cephalisation of neurosecretory cells, whereas the former, probably typical of all Blattaria,

have neurosecretory cells in the brain, sub-oesophageal ganglion and throughout the ventral cord ganglia (Goldiay, 1959; Füller, 1960; Scharrer, 1941; de Besse, 1967). Nevertheless the role of non-neurosecretory (neural) elements cannot be dismissed, as they mediate between environmental stimuli and the endocrine centres. Co-ordination between the two in controlling circadian rhythms offer unique opportunities for studying neuro-endocrine integration.

Feeding in T. pronuba profoundly affects the circadian flight rhythm. As it also affects the release of neurosecretory material, an endocrine link between feeding and the observed depression of flight activity can be postulated. In this context it is interesting to compare the results from this study with those of Green (1964). He showed that in Phormia regina stretch receptors in the foregut monitor the presence or absence of food, and the receptors have a nervous connection with the corpus cardiacum. In the presence of food the corpus cardiacum is stimulated to release a locomotory inhibiting factor. Ozbas and Hodgson (1958) found that extracts of corpus cardiaca decreased the frequency of spontaneous nerve impulses in isolated nerve cords from P. americana and Blaberus craniifer. Milburn, Weiant and Roeder (1960) have shown that extracts of corpora cardiaca block inhibitory impulses to the metathoracic ganglia from the sub-oesophageal ganglion. These crude extracts invariably consist of several hormones derived from the brain, plus one or possibly more produced by the intrinsic secretory cells of the corpora cardiaca. This was borne out by the experiments

of Strockova, Sorvit and Novak (1965), who chromatographically separated several fractions from crude cardiaca extracts. Two factors, "C_1" and "D_1" (Gersch, Unger and Fischer, 1957), were tested upon the ventral cord of P. americana; D_1 was found to evoke hyper-autorhythmia and C_2 inhibited existing "electrical" activity. Thus it has become apparent that the corpora cardiaca store stimulatory and inhibitory factors. The histological evidence from this study suggests that as feeding inhibits the release of materials from A_1- and A_2-cells secretion from one or the other type of cell has a stimulatory function. Moreover, histochemical evidence suggests the presence of 5-HT in A_2-cells. Furthermore, when A_2-cells are removed by cauterization, and the moths are injected with 5-HT, nocturnal flight is, to some extent, restored. These lead to an attractive hypothesis that 5-HT has a central role in the initiation and maintenance of the circadian nocturnal flight rhythm.

Most of the catecholamines indoleathylamines and histamines known to occur in vertebrates, have been identified in extracts of insects tissues; nor adrenaline and dopamine (Ostlund, 1954), 5-HT (Gersch, Fischer, Unger and Kapitzer, 1961; Jaques and Schachter, 1954; Collier, 1957; Welsh and Moorhead, 1960; Colhoun, 1963; Brown, 1965; and Bertaccini, Neviani and Roseghini, 1965), an ortho-diphenol (Cameron, 1953), unidentified adrenergic substance (Barton-Brown, Dodson and Hodgson, 1961), histamine and N-acetylhistamine (Bertaccini et al 1965). The findings of adrenaline-like substances in corpora cardiaca extracts (Barton-Brown et al 1961) and of 5-HT (Gersch et al, 1961; Colhoun, 1963;

Brown, 1965) are of particular interest. Even more relevant, 5-HT has been extracted from the brain (Gersch et al 1961), and adrenergic fibres have been demonstrated in the brain by fluorescent microscopy (Frontali and Norberg, 1966). These substances variously stimulate or depress spontaneous contractions in visceral muscles of insects (Davey, 1964). However, as Davey (1964) points out the insect heart, which is frequently used, shows a marked lack of specificity in its response; the effects of the biogenic amines upon intact insects has received little attention. In this respect the stimulatory effect of 5-HT, and the inhibitory effects of adrenaline and nor-adrenaline upon flight activity are especially interesting. Obviously caution is necessary in interpreting these results, Brown (1965) quite justifiably draws attention to the instability and extra-cardiac inefficiency of these "small molecules." If indeed 5-HT is involved in triggering and maintaining nocturnal flight activity it is by no means clear how this is achieved. It is possible that it functions as a synaptic transmitter or "potentiator," rather than as a hormone in the classical sense; with its effector site at the neurosecretory cell axon terminals in the corpora cardiaca, and associated with other neurosecretory cell fibres in the ventral cord. Instead of having a stimulatory action per se, it is possible that it antagonises inhibitory fibres extending from the brain to the sub-oesophageal ganglion. In this connection it is worth noting that the lateral nerves (NCCL) provide direct anatomical connections between the brain, corpora cardiaca and sub-oesophageal ganglion. However this is

purely speculative and much further work is needed to clarify these important points.

The effects of reserpine injection is of interest for two reasons; firstly it has been shown that when it is fed to female insects it drastically reduces fecundity (Huot, Corrivault and Bourbeau, 1960; Hays, 1965; Bonschoter 1966; Wicht and Hays, 1967; Hays and Amerson, 1967), secondly a tranquillizing effect was reported by Hays (1965), and noted in this study when administered in relatively high concentrations. There is substantial evidence that the tranquillizing effect in vertebrates is due to induced hypersecretion of 5-HT in the brain. As 5-HT injected in high doses also depresses activity a mechanism analogous to that known to occur in vertebrates is quite feasible in insects. Nevertheless the situation may well be more complex than this, as reserpine also induces release of other biogenic amines (Shore, 1962; Axelrod, 1964) and also ACTH invertebrates (Meickel, Westermann and Brodie, 1961). However it is likely that the effect upon fecundity is due to endocrine dysfunction rather than a direct effect upon the ovaries; Huot et al (1960), drew attention to the similiarity between reserpine administration and allatectomy.

Insect pharmacology is at present in its infancy, but it is likely to be a fruitful field of research in the future, and may well provide the much needed information on co-ordination between nervous and endocrine functions.

SUMMARY

1. Comparison is made of the neurosecretory cells occurring in the brain of adults of 23 species of Lepidoptera, representing 10 families.

2. Using four staining techniques specific for neurosecretory materials, 10 distinct types of cells are differentiated, and classified as: A_1-, A_2-, A_3-, B_1-, C_1- and D_1- in the medial group; and A_4-, B_2-, C_2- and D_2- in each lateral group of the protocerebrum. Six or more types of cells occur in all 23 species, 7 or more in 20 species; 8 or more in 14 species; 9 or more in 5 species, and all 10 in a single species.

3. There is a striking uniformity in the numbers of A_1-, A_2-, A_4-, and C_1-cells in every species (8, 8, 10 and 2 respectively). Medial B-cells, previously undescribed from the lepidopteran brain, vary in number, from 25 in geometrids to 180 in Herse convolvuli.

4. The outogeny, and secretory activity of each type of cell are described during postembryonic development of Philosamia cynthia ricini. With the exception of B_1-cells which are differentiated during the pupal stage, the remaining types are present from the 2nd instar onwards and in the same numbers that occurs in the adult. Cycles of secretion in A_1-, A_4-, B_2- and C_1-cells are correlated with moulting, but in the 5th instar they become asynchronous; their activity in the last instar is associated with the 'critical period', and the physiology of the pre-pupa.

5. The cytoplasmic volume is calculated for each type of cell during development; rates of growth and maximum volumes are used

as indicators of functional demand.

6. Comparison is made of neurosecretory activity in adults of P. cynthia ricini and T. pronuba. In the former there is a gradual decline in the density of inclusions in all but B_2-cells during the 8-day period of adult life, whereas in the latter there is gradual accumulation of inclusions in most types of cells. No secretory activity is apparent in B_1-cells of P. cynthia ricini, but marked cycles of secretion occur in T. pronuba; in this species as in other noctuids these cells exist in two distinct phases, and undergo independent cycles of secretion.

7. The release of inclusions from A_1- and A_2-cells occurs at night in both species. This is followed by rapid re-synthesis in T. pronuba, and occurs during the day-time. A_1-cells discharge inclusions in P. cynthia ricini in response to injected water. Secretion from A_1-cells occurs later than from A_2-cells in T. pronuba, and is almost completely inhibited after feeding.

8. A detailed histochemical analysis is made of seven types of cells. It is concluded that B_1- and B_2-cell inclusions consist of protein or peptide, and that A- and C-cell inclusions are glycoproteins. The carbohydrate/protein ratio differs from one type of cell to another. Amino acid composition of the protein chain varies, and substituted sugars predominate in all PAF-positive cells; A_2-cells alone contain some unsubstituted sugars, and in addition give a positive reaction for 5-HT. There is no significant sulphydryl concentration in any type of cell.

9. Factors affecting activity are elucidated, using an actograph that records the sounds produced by the insects in flight. In

diurnal species flight is stimulated by light and ultra-violet emmission is important. A persistent endogenous circadian flight rhythm is demonstrated in several nocturnal moths.

10. In T. pronuba, feeding inhibits flight activity for several days. During recovery of activity, bursts of flight still occur in the day-time; this activity which is not endogenous is barely affected by feeding.

11. The effect of shifting the scotophase is described. Re-entrainment readily occurs, but activity during the first scotophase is dependent upon, (i) duration of the intervening photophase, (ii) the time at which the new scotophase commences. There is a tendency to revert to the natural flight time, consequently re-entrainment is most easily accomplished as the natural dusk is approached.

12. Moths chilled for 12 hours, then exposed to reversed phases immediately accept the new regime.

13. Cauterization of the medial A-cells abolishes nocturnal flight but ablation of adjacent areas has no effect.

14. It is concluded that the medial A-cells are intimately connected with the circadian flight rhythm, that the release of neurosecretory material initiates and maintains flight activity.

15. Injection of adrenaline or nor-adrenaline depresses flight activity in unoperated moths; dopamine tryptamine and tyramine have no effect; 5-HT when injected in low concentrations enhances activity but depresses it at higher concentrations. 5-HT alone, to some extent restores nocturnal flight to moths in which A-cells have been ablated. It is postulated that 5-HT has an important role in the control of flight activity.

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Relationship between Serotonin and the Circadian Rhythm in some Nocturnal Moths

THE presence of serotonin has been reported in various insect tissues; mainly in connexion with the venom glands of Hymenoptera[1,2], in head, thorax and abdomen or total insect extracts[2,3] and in the corpora cardiaca[4]. In several cases the corpora cardiaca and corpora allata have shown a relationship to a circadian rhythm[5–8], but the nature of the activating material secreted has remained obscure.

The structure of the corpora cardiaca in the orthopteroid insects, on which attention has largely been centred in the past, differs from that found in the Lepidoptera. In the latter, these organs are considerably smaller, in many species with as few as three intrinsic cells, and it appears in the adult insects to have very little storage capacity (my unpublished work). My histological investigations have confirmed that the neurosecretory material of the median and lateral cells of the pars intercerebralis is accumulated in the cell bodies and along the axons leading to the corpora cardiaca.

In an investigation of the circadian flight activity of various nocturnal moths during the past 2 yr I have produced evidence that serotonin is secreted by certain brain neurosecretory cells, and appears to play a part in raising or lowering the threshold for activation. I was, therefore, interested to note that circadian fluctuation of concentration of serotonin has been demonstrated in extracts of gut and nervous system from the opilionid, *Leiobunum longipes*, and, furthermore, efflux of serotonin from the brain could be correlated with histological changes in the neurosecretory cells[9]. Histochemical investigations of the noctuid moths, *Noctua pronuba* and *Agrotis ipsilon*, revealed a large concentration of tryptophan in the median "A" cells. As tryptophan is the precursor of serotonin, the possible presence and function of this biogenic amine were further investigated.

The brain and retrocerebral complex was dissected from adult male moths in Ringer's solution, fixed for 18 h in Bouin's solution, embedded in wax and sectioned at 5μ. The neurosecretory cells were identified by a modified paraldehyde fuchsin technique. Tryptophan was made visible by the DMAB method[10]. Application of Ninhydrin-Schiff and bromphenol blue indicated a smaller total protein concentration in some "A" cells than in adjacent neurons, all of which are tryptophan negative. These "A" cells alone gave a positive enterochromaffin reaction with diazotized saffranin (Fig. 2). The possibility of the enterochromaffin reaction giving a positive result with

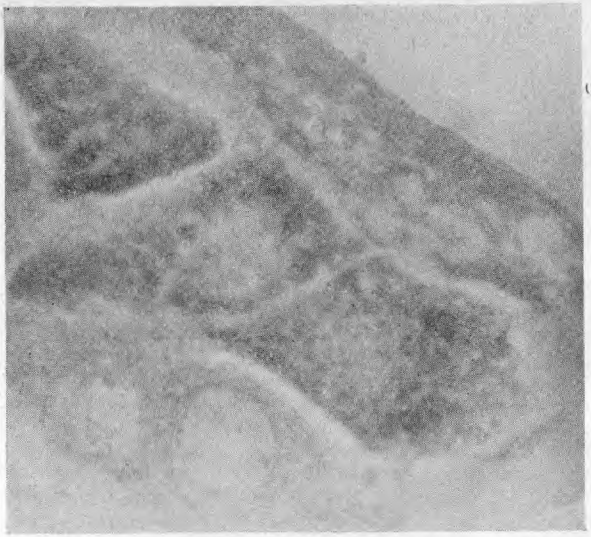

Fig. 1. Tryptophan-rich "A" cells (dark inclusions) in brain of *Noctua pronuba*, DMAB method-phase contrast.

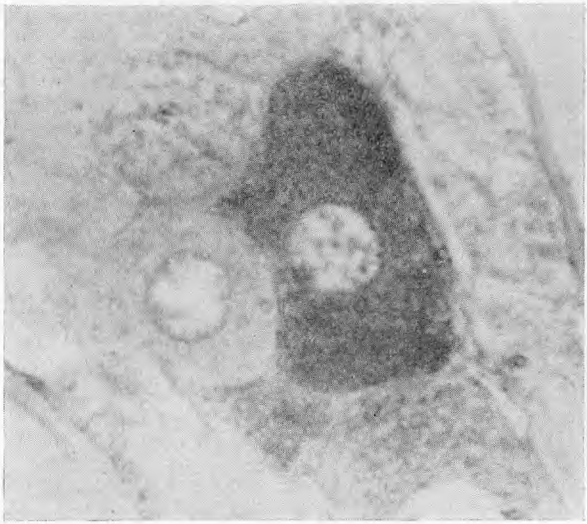

Fig. 2. Enterochromaffin reaction in "A" cell (dark staining) in brain of *Noctua pronuba*.

closely related substances could not be excluded. It seems unlikely, however, that tryptophan is stained, for when sections of the brain of the silk moth, *Philosamia cynthia-ricini*, were similarly treated the "A" cells were tryptophan negative, but strongly positive with the enterochromaffin reaction. An explanation of this dissimilarity might be the difference in longevity of the adult male insects, which is 7–8 days in *Philosamia* and 2–3 months in *Noctua*. Obviously if serotonin is an essential secretion its continued synthesis would be necessary in *Noctua*, whereas *Philosamia* could synthesize and store sufficient serotonin for its comparatively short adult life.

The median "A" cells of *Noctua* exhibit a "Tindall" effect, which renders them visible when the brain is exposed in Ringer's solution. This property is shared by some other noctuid moths and appears to be consistent with observations on *Calliphora erythrocephala*[11]. This feature makes the selective ablation of the "A" cells possible with a microcautery. Experiments in which this operation was performed further support the idea that the material secreted by these cells is connected with nocturnal flight activity.

Operated insects were separated for a period of at least 5 days before being introduced into an aktograph. The post-operative delay was considered necessary because some individuals died after 1 or 2 days, but the survivors lived for several weeks. The result of "A" cell ablation was to abolish the normal night flight, but had little effect on flight activity which characteristically appears from 0·5–1 h after the lights are switched on (="light activation"). As a control to these experiments other moths were subjected to sub-peripheral cautery of the neurones adjacent to the "A" cells. In these, the overall effect was to diminish slightly the flight amplitude, but not to impair either day or night flight. The likelihood of substances other than serotonin affecting the nocturnal circadian rhythm was not overlooked; consequently a number of known biogenic amines of natural occurrence were tested. For these experiments unoperated male moths of known age were injected with 1 µl. samples of serotonin creatinine sulphate, tyramine, tryptamine, adrenaline and L-noradrenaline in concentrations of 1 part in 1×10^{-5}—1 in 1×10^{-8} in both Ringer's solution and distilled water. In all cases adrenaline and noradrenaline suppressed all flight; tryptamine and tyramine had little effect, whereas serotonin usually enhanced the duration and amplitude of the night flight. A description of the aktograph used in these investigations, and details of the flight patterns evinced in the various experimental conditions will be published elsewhere.

On the basis of these results I would like to suggest that "light activation" is mediated directly through the nervous system and that dark activation is mediated or enhanced

by the secretion of serotonin from the "A" cells of the brain. The time of release would, then, comprise the onset of the endogenous component in the circadian rhythm of nocturnal flight.

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C. F. HINKS

Department of Zoology and Applied Entomology,
Imperial College of Science and Technology,
Ashurst Lodge, Sunninghill, Ascot, Berkshire.

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