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MORPHOLOGY AND DEVELOPMENT OF THE SALIVARY GLANDS AND THEIR CHROMOSOMES IN THE LARVAE OF *ANOPHELES STEPHENSI* SENSU STRICTO

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SYNOPSIS

In the larvae of *Anopheles stephensi* s. s. five typically banded salivary chromosomes are present united proximally to a chromocentre. Maps have been constructed to illustrate their constant and characteristic banded pattern. Chromosome IIIR, however, has shown a variation in the form of a heterozygous inversion. The chromocentre is a small, fragile body, closely associated with the large nucleolus.

The development of the salivary glands in the larva is by cell-growth. The chromocentre appears at first as a heteropycnotic " crescent ", which soon gives place to a disc-shaped body. Later on in development the chromocentre loses both its heteropycnotic nature and compactness. The nucleolus persists until the time of histolysis of the glands, which begins at the distal end of the gland and gradually involves the more anterior regions. At the earliest observable stage the homologous chromatin elements constitute a tight coil. Full synapsis is preceded by a process of uncoiling accompanied by a chromomere-to-chromomere fusion. The rate of this process varies among different pairs of homologues and along different regions of the same pair. Synapsis is completed in the late third instar larva.

RNA is localized in the cytoplasm and the nucleolus of the larval salivary gland cells. The chromosomes and the chromocentre include DNA. A faint reaction of the interband areas show that DNA is present in a diffuse state throughout the chromosomes.

Since the year 1933, when the importance of the Dipteran salivary chromosomes in cytogenetical research was demonstrated (Painter, 1933; Heitz & Bauer, 1933), considerable work has been done on the structure, development and functions of these chromosomes of *Drosophila* species and of other Dipteran groups like *Chironomus*, *Sciara*, *Simulium* and the Cecidomyids (see Alfert, 1954; White, 1954; da Cunha, 1955).

The mosquito salivary chromosomes were first studied by Bogojawlensky (1934) in *Anopheles maculipennis*. Later Berger (1936) reported their

presence in *Culex pipiens*, and Sutton (1942) studied them in *Culex pipiens* and *Aëdes aegypti*. Ciaccio (1941) investigated the development of the larval salivary glands in *Anopheles maculipennis*. Frizzi (1947a, 1947b) obtained satisfactory salivary chromosome preparations of *Anopheles maculipennis atroparvus* and succeeded in constructing their maps. He pointed out that a proper rearing of the larvae helped in getting good preparations. Subsequent work of Frizzi (1949, 1951, 1952) proved the existence of distinctive chromosomal rearrangements in the various races of the *Anopheles maculipennis* complex. Magrini (1948, 1951) studied the development of the salivary chromosomes in larvae of *Anopheles maculipennis*, and demonstrated a correspondence between the size of the nuclei and the stage of differentiation of the chromosomes. Kitzmiller & Clark (1952) gave a preliminary account of the salivary chromosomes in the larvae of *Culex pipiens*, and Kitzmiller (1953) followed this up with a review of the previous work on mosquito cytogenetics. Frizzi (1953) revealed the possibility of extending the cytogenetical method of analysis to the American forms of the *Anopheles maculipennis* complex and the *Nyssorhynchus* group of *Anopheles*. Frizzi & Holstein (1956) found a fairly high degree of chromosomal polymorphism in *Anopheles gambiae*, and identified a number of heterozygous inversions affecting all the autosomes. D'Alessandro, Frizzi & Mariani (1956, 1958) pointed out that DDT-resistant strains of *Anopheles maculipennis atroparvus* showed an inversion in chromosome IIIS, the frequency of which rose with increasing DDT-tolerance of the populations.

Practically no work has been done on the larval salivary chromosomes of any of the Indian mosquito species. The present work was started in order to study the morphology and development of the larval salivary glands and their chromosomes of an Indian species—*Anopheles stephensi* s. s. A preliminary note on this has already been published (Rishikesh, 1955).

Material and Methods

Russell & Mohan (1939) first reported the successful colonization of *Anopheles stephensi* s. s. Their technique was followed, with minor modifications, to establish colonies of the species in this laboratory from live eggs procured from the Malaria Institute of India, Coonoor.

The banded pattern of the salivary chromosomes was studied exclusively from aceto-orcein (La Cour, 1941) squash preparations. The glands were dissected out in normal saline under the binocular microscope and fixed in a modified Carnoy's fluid (Nolte, 1948) before squashing. Stain-fixative of the strength of 2% orcein in 60% acetic acid gave good results. The optimum period of fixing and staining was found to vary with each batch of larvae. Larvae were fed on yeast. Special attention was paid to prevent overcrowding of the larvae in the breeding-bowls and pollution of the

water from an excess quantity of yeast. Active, late fourth instar larvae were selected for dissection. A few cells in the proximal lobe of the gland show large chromosomes with a well-defined banded pattern. The separation and removal of the distal lobe of the gland with its secretory products was found to facilitate and improve the squashing and staining processes (see Bertani, 1948). Microphotographs were taken for preference from temporary mounts on about the second day of preparation. The preparations were made permanent according to the method outlined by McClintock (1929), or by remounting in Euparal straight from rectified spirit (Darlington & La Cour, 1942).

The growth of the salivary glands was studied from serial sections of the larval thorax, and whole mounts of the glands. Larvae were fixed in warm alcoholic Bouin for 24 hours and were later cleared in methyl benzoate celloidin (Pantin, 1948). Sections were cut at 8μ thickness, and stained in Heidenhain's iron haematoxylin, with or without eosin. Whole mounts were prepared of glands fixed and stained in aceto-orcein for about 15 minutes. The development of the salivary chromosomes was studied from squash preparations, supplemented by observations on serial sections of the larval thorax and whole mounts of the glands.

To study the qualitative localization of ribonucleic acid and deoxyribonucleic acid in the larval salivary gland cells, the simple pyronin/methyl-green technique of Jordan & Baker (1955) and the Feulgen " nucleal " reaction (De Tomasi's modification; see Pearse, 1954) were employed. A few squash preparations of the larval glands were also stained according to the above techniques.

Observations

Larval salivary chromosomes

The metaphase configuration in dividing brain cells of *Anopheles stephensi* s. s. consists of a subtelocentric pair and two pairs of V-shaped chromosomes (Rishikesh, 1955). The subtelocentric pair represents the sex chromosomes and is heteromorphic in the male.

In the larval salivary gland nuclei five long, typically banded, euchromatic strands are present (Plate II, 1). In some preparations they appear to radiate out from a common point indicating the presence of a chromocentre. The chromocentre, however, is a diffuse structure, and in squash preparations is invariably disrupted, with the result that the euchromatic strands lie separated from one another. The pair of sex chromosomes is represented by a single element in the salivary gland nuclei, and presumably one limb of this subtelocentric pair is almost wholly heterochromatic. This has been designated chromosome I (Plate II, 1). Each pair of V-chromosomes is represented by two elements in the salivary gland nuclei,

corresponding to their right (R) and left (L) limbs. Attenuated, thread-like connexions are occasionally visible between the proximal tips of the euchromatic strands representing the two limbs of the same chromosome. The autosomal elements have been designated chromosomes IIR, IIL, IIIR and IIIL respectively (Plate II, 1). The original numbering of the autosomes (Rishikesh, 1955) has been altered so as to harmonize the nomenclature for this species with that of Frizzi (1947b) for *Anopheles maculipennis atroparvus*.

The individual salivary chromosomes are identifiable on the basis of certain characteristic " land-marks " in their structure. The linear sequence of bands along the five chromosomes has been worked out in detail, and maps constructed (Plate I). The chromosomes have been provisionally divided into a number of sections, and the description of the chromosomes is based on a study of the pattern of bands along successive sections of each chromosome. The banded pattern of each section was copied with the aid of a *camera lucida*, and these " bit " drawings were later consolidated in their normal order to give a complete " map " of the entire chromosome. The sex chromosome has been divided into 5 sections, and the autosomes into 10 sections each. The sections are counted beginning from the left of the " maps ", corresponding to the free tips of the chromosomes.

The bands are disposed so as to conform to a definite linear sequence which is constant for a chromosome. Chromosome IIIR, however, has disclosed in some preparations a rearrangement in the form of an inversion of a long portion of its middle region.

Chromosome I (Plate I, and Plate II, 1) is the shortest element of the salivary chromosome complex. It is comparatively thin and weakly staining in the male. The free end of the chromosome shows five prominent bands which together present a characteristic appearance. Section 3 discloses a thick, curved band, followed by a distinct " puff ". Along the middle of the " puff " is a dark, diffuse band in the form of irregular chromatin clumps from which ramifications spread outwards. Near the proximal tip of the chromosome the homologues fail to synapse for a short distance indicated by a split (Plate II, 2).

The two limbs of chromosome II (Plate I, and Plate II, 1) are more or less equal in length and show relatively inconspicuous free tips. A distinct landmark in the structure of chromosome IIR is constituted by three sets of heavily staining bands in section 9 (Plate II, 3). In between these sets are clear hyaline areas which become stretched in squash preparations, thereby increasing the conspicuousness of the bands. The next section shows a small, globular swelling with a central, well-staining band. Other prominent bands are seen in sections 11, 13 and 14. Section 17 of chromosome IIL (Plate II, 4) discloses three thick, " composite " bands. A " puff " similar to that in section 3 of chromosome I is marked out in section 19 (Plate III, 1). The proximal tip of the chromosome shows some deep

PLATE I. CHROMOSOME MAP OF *ANOPHELES STEPHENSI* S. S.

Consolidated *camera lucida* drawings; X 870.

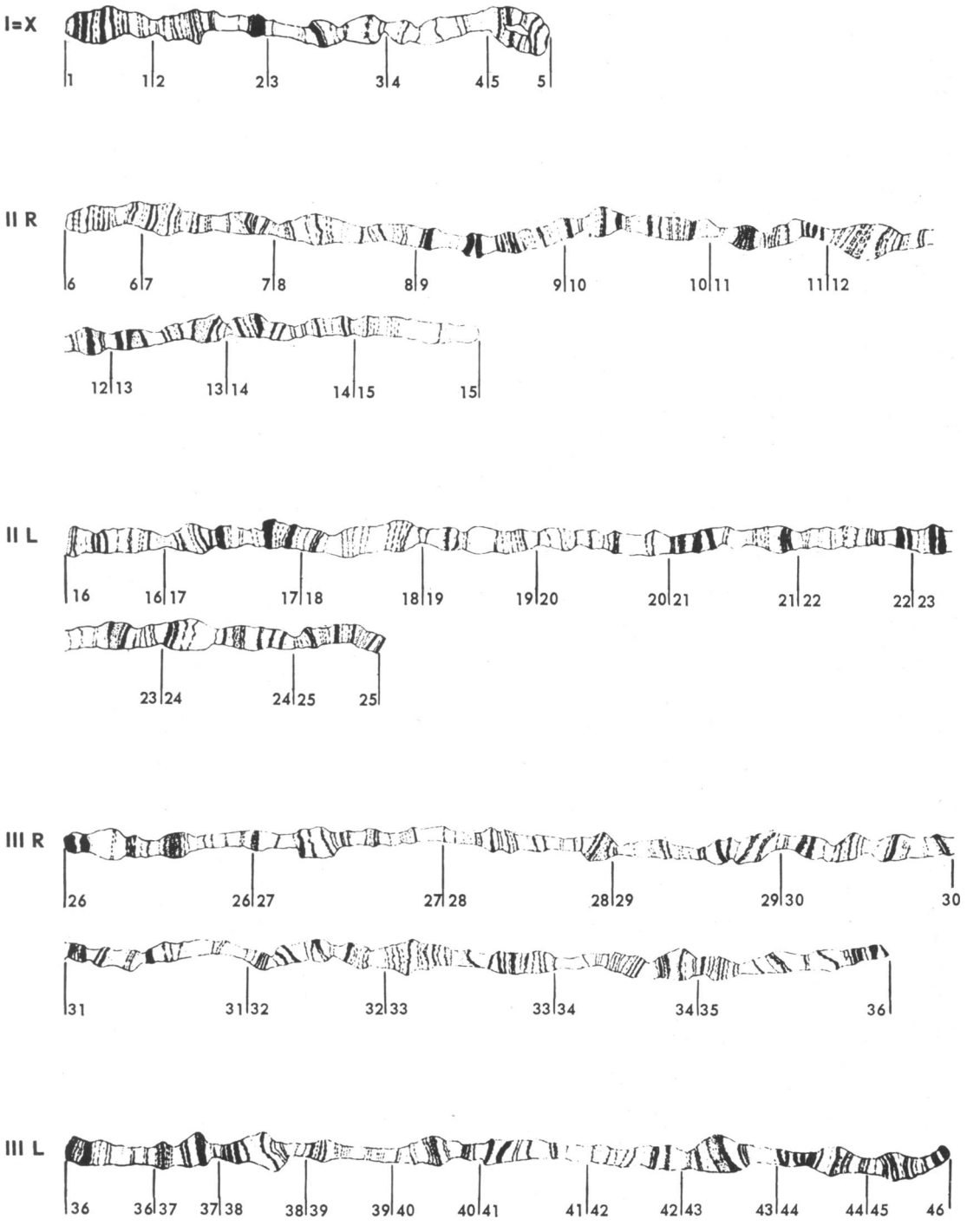
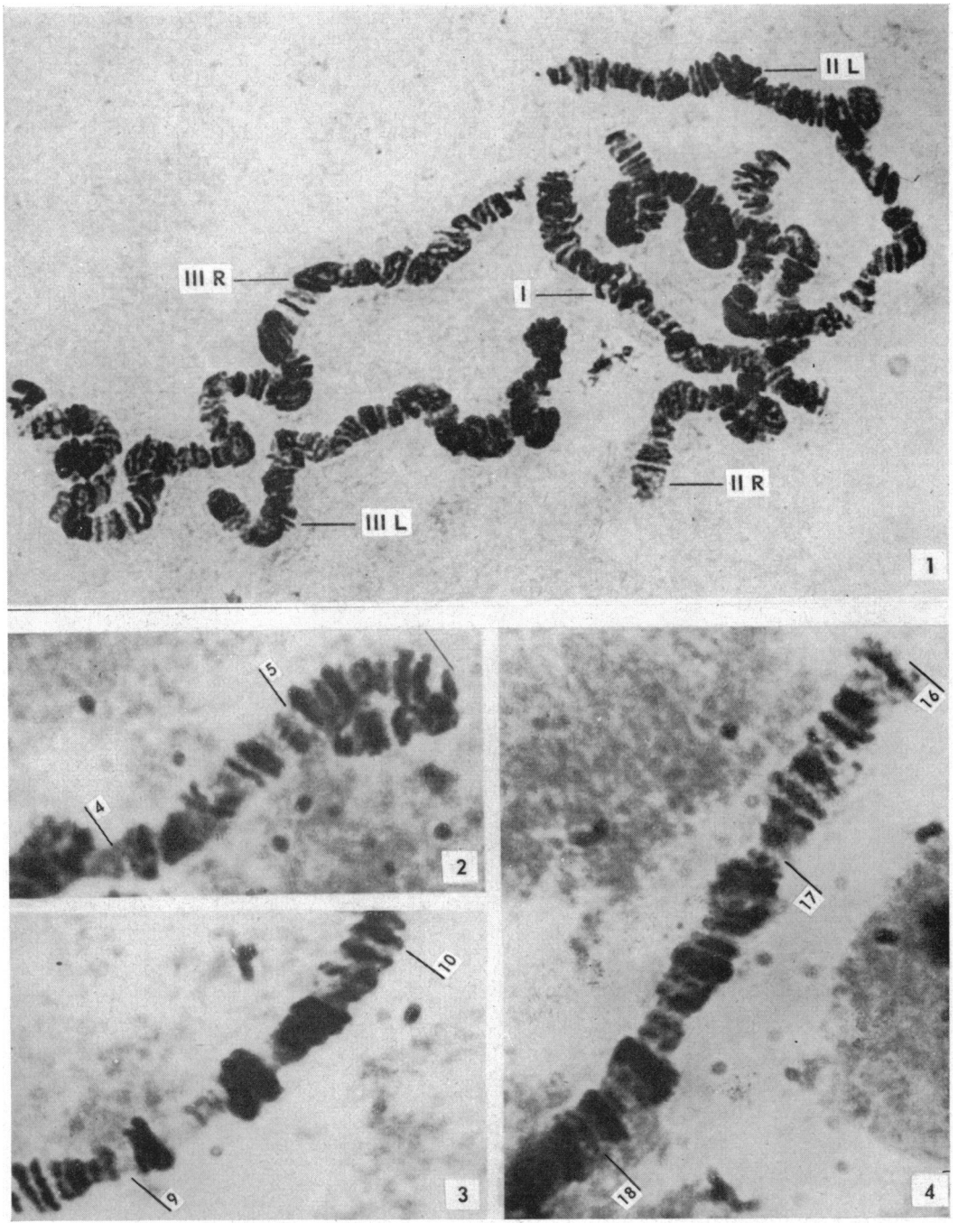
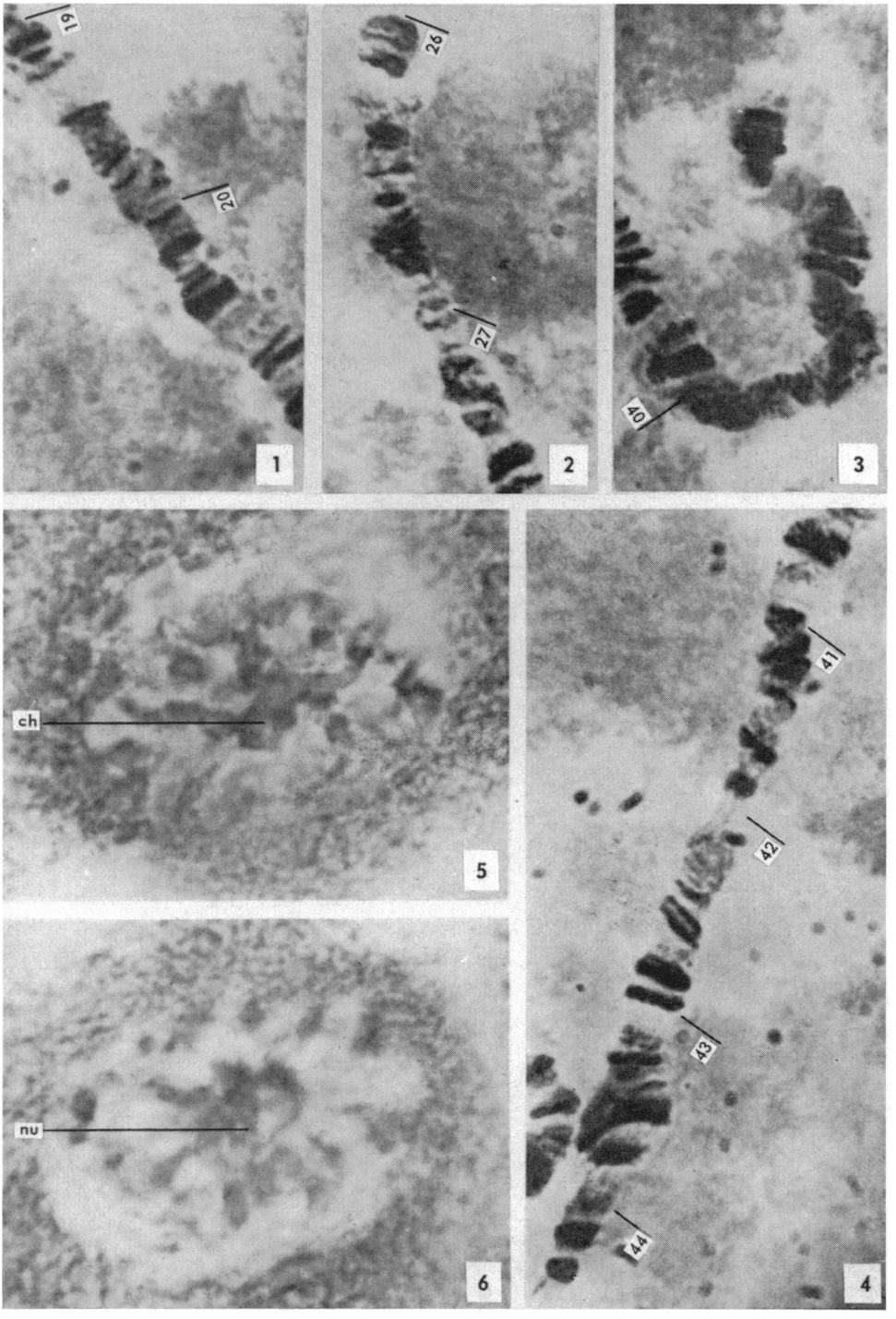


PLATE II. MICROPHOTOGRAPHS FROM ACETO-ORCEIN PREPARATIONS



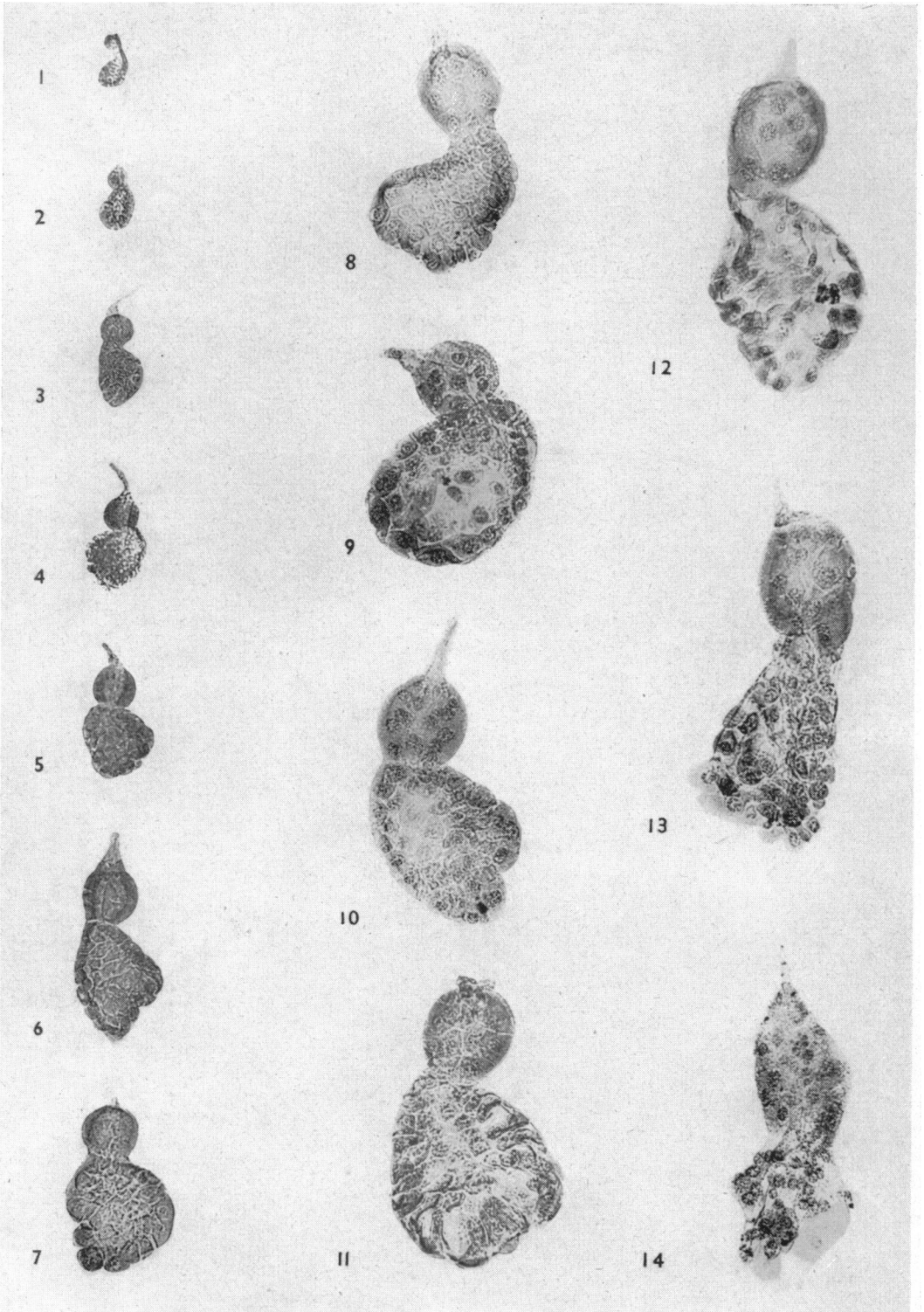
1. Larval salivary gland chromosomes; X 800
2. Proximal portion of the sex chromosome; X 2100
3. Section 9 of chromosome IIR, showing three sets of dark conspicuous bands; X 2100
4. Distal region of chromosome IIL, showing the conspicuous bands in section 17; X 2100

PLATE III. MICROPHOTOGRAPHS FROM ACETO-ORCEIN AND PYRONIN/METHYL-GREEN PREPARATIONS



1. Portion of chromosome IIL; aceto-orcein preparation; X 2000
2. The distinctive free tip of chromosome IIIR; aceto-orcein preparation; X 2000
3. The distinctive free tip of chromosome IIIL; aceto-orcein preparation; X 2000
4. Portion of chromosome IIIL; aceto-orcein preparation; X 2000
5. Larval salivary gland nucleus; section stained according to the pyronin/methyl-green technique; X 1940; ch = chromocentre
6. Same as 5 at a lower focus; X 1940; nu = nucleolus

PLATE IV. MICROPHOTOGRAPHS OF LARVAL SALIVARY GLANDS FROM TIME OF HATCHING TO PUPAL STAGE (× 108)



1-4. First instar 5-6. Second instar 7-9. Third instar 10-13. Fourth instar 14. Early pupal stage

bands interposed among a number of thin dotted lines, and presents a more or less distinctive appearance compared to the corresponding region of chromosome IIR.

The two limbs of chromosome III (Plate I, and Plate II, 1) are unequal in length and both possess distinctive free tips. Chromosome IIIR is the longest element of the salivary chromosome complex. At the extreme free tip (Plate III, 2) are two sets of three bands each, separated by a narrow, clear region. Next appears a rather spherical " swelling ", which presents a clear hyaline structure but for a pair of faint, dotted lines running across its middle. To the right of the " swelling " is a group of three slender, well-staining bands. A pair of elongated S-shaped bands, preceded by two dark bands, constitute a conspicuous feature of section 29. Section 31 starts with a couple of characteristic dark, " oval " bands. A small " swelling " is seen at the right extremity of section 34. The proximal tip of the chromosome bears some moderately staining bands, and ends usually with a short, thick band. Chromosome IIIL is only a little more than half the length of chromosome IIIR. The distinctive free tip of the chromosome (Plate III, 3) shows two pairs of dark, prominent bands followed by an area with scattered granules which sometimes form ill-defined dotted lines. In between sections 37 and 38 is a waist-like constriction which produces a swelling in each of the sections. A small " swelling " in section 40 is easily recognizable in preparations (Plate III, 4). Along its middle are disposed in close approximation three to four slender lines and a fairly discrete band. A larger " swelling " is seen in section 43 (Plate III, 4). Section 44 includes two pairs of thick bands which are usually associated together to form two U-shaped figures (Plate III, 4). Two short thick bands at the extreme proximal tip of the chromosome are prominent.

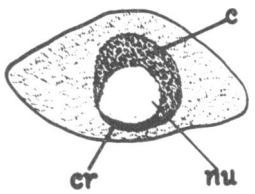
Development of the larval salivary glands and their chromosomes

The salivary glands (Plate IV) are two elongated bodies occupying an anterolateral position in the larval thorax. The gland opens anteriorly into a long, slender duct. The ducts from either side unite medially to form a common salivary duct. The gland consists of two asymmetrical lobes separated from one another by a constriction in the anterior half. The small proximal lobe is more or less spherical in shape and consists of 20-21 large cells, some of which show giant nuclei. The larger, elongated, distal lobe is formed of about 65 relatively small cells with correspondingly small nuclei. The cells are columnar in shape, and present a polygonal surface view. A cross-section through the proximal lobe shows 6-7 large cells surrounding a central lumen, which serves as a passage for the secretory products formed within the distal lobe. The entire gland is enveloped by a thin membrane. A variation in the size of the nuclei is evident in both the lobes, although more marked in the case of the distal lobe. The large

nuclei of the salivary glands show polytene chromosomes, which are seen to radiate out from a chromocentre in favourable preparations. A large nucleolus is present. The cells comprising the ducts are much smaller than those of the gland proper. The duct at its point of union with the gland shows an enlargement formed of a mass of small cells which represent the *Anlage* cells of the imaginal salivary gland. These show elongated nuclei bearing a peripheral basophilic " crescent ". A large fat body is loosely attached to the gland at two places.

The minute salivary gland of the newly hatched out larva shows the full complement of cells and the demarcation into a proximal and a distal lobe (Plate IV, 1). The spherical proximal lobe measures about 24 μ across and the elongated distal lobe measures about 50 $\mu \times 35$ μ. The lumen of the gland is uniformly narrow, and contains very little secretory matter.

FIG. 1. SALIVARY GLAND CELL FROM NEWLY HATCHED OUT LARVA



Semidiagrammatic *camera lucida* drawing from aceto-orcein squash preparation; X 2250.
c = chromatic elements.
cr = crescent.
nu = nucleolus.

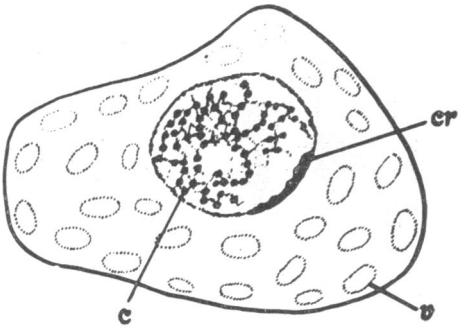
The nuclei from different regions of the gland show a slight variation in size. The larger nuclei measure about 5 μ. The nuclei are more or less refractory to the stain, and owing to their small size do not rupture in squash preparations. In squash preparations the smallest nuclei measure about 5 μ, and show the most primitive nuclear structure noticeable in the larval gland (Fig. 1). A striking feature of these nuclei is a large, clear, vesicular body lying eccentrically within the nuclear cavity and in close contact with the nuclear boundary on one side. At the junction of the nucleolus and the nuclear boundary appears, in side view, a heteropycnotic " crescent ". The rest of the nuclear cavity is filled by a " spireme " consisting of chromatic granules joined by weakly staining intergranular connexions. Squash preparations of glands of the same stage show also larger nuclei measuring about 7.5 μ. In these nuclei the intergranular threads of the spireme are comparatively more prominent.

Plate IV, 2 and 3, shows two developmental stages of the larval gland during the first instar. A lightly staining mass of cells at the junction of the salivary duct with the gland in Plate IV, 3, represents the *Anlage* cells of the imaginal gland.

The proximal lobe of the salivary gland of the late first instar larva remains small and rounded. The distal lobe, on the other hand, shows a disproportionate increase in over-all size owing to the accumulation of secretory matter within its lumen (Plate IV, 4). The cytoplasm of the cells of the distal lobe is vacuolated, indicating secretory activity (Fig. 2 and 3). The nuclei stain moderately, and the variation in their size is more marked.

The ill-defined "crescent" noted above gives place to a prominent heteropycnotic mass in nuclei measuring about 8.5 μ in squash preparations (Fig. 2). The inner margin of this heteropycnotic mass is irregular, and gives rise to thread-like connexions extending to the spireme. Fig. 3 illustrates a larger nucleus, measuring about 12 μ, in which an irregular, disc-shaped chromocentre is noticeable. It appears to have lost its earlier heteropycnotic nature. The chromocentric boundary protrudes in the form of small nodules from which arise the thread-like connexions to the spireme. The nucleolus is closely approximated to the chromocentre. The chromocentre is usually disrupted in nuclei measuring 14 μ and more in squash preparations (Fig. 4). The chromatic elements in these nuclei, however, appear more conspicuous, and envelope the nucleolus completely. Short, favourably exposed regions of the chromatin elements in squashes reveal that the homologous threads are closely coiled together for the greater part of their lengths. These regions do not show an approximation of the homologous chromomeres. A chromomere-to-chromomere fusion, however, is evident along limited lengths of the homologous pairs, where they exhibit a banded structure and little spiralization (Fig. 5 A). This would indicate that full synapsis is preceded by progressive uncoiling of the twisted homologues accompanied by the fusion of the homologous chromomeres, and that the rate of this process varies among different pairs of homologues and along different regions of the same pair.

FIG. 2. SALIVARY GLAND CELL AT EARLY STAGE OF DIFFERENTIATION

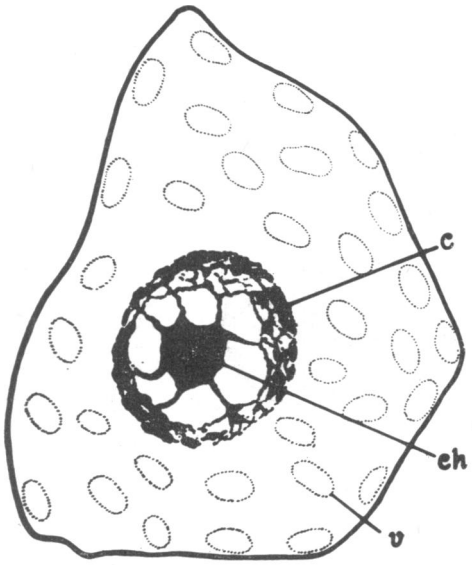


Semidiagrammatic *camera lucida* drawing from aceto-orcein squash preparation ; X 2250.
c = chromatic elements.
cr = crescent.
v = cytoplasmic vacuoles.

The proximal lobe of the salivary gland of the early second instar larva measures about 65 μ, and the distal lobe about 100 $\mu \times 90$ μ (Plate IV, 5). A bend is noticeable beyond the constricted region of the gland on account of which the distal lobe assumes a nearly vertical disposition in the larval thorax. The lumen of the proximal lobe remains narrow. The cells of the distal lobe show secretory vacuoles. In whole mounts of the gland the large nuclei measure nearly 10 μ. The nucleolus is flattened and unrecognizable in squash preparations.

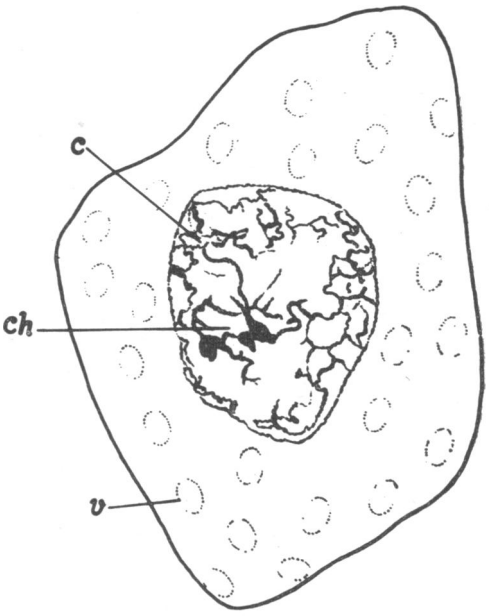
In the gland of the late second instar larva (Plate IV, 6), the *Anlage* cells constitute a thick disc-shaped or cone-shaped mass. The large nuclei

FIG. 3. SALIVARY GLAND CELL AT LATER STAGE THAN FIG. 2



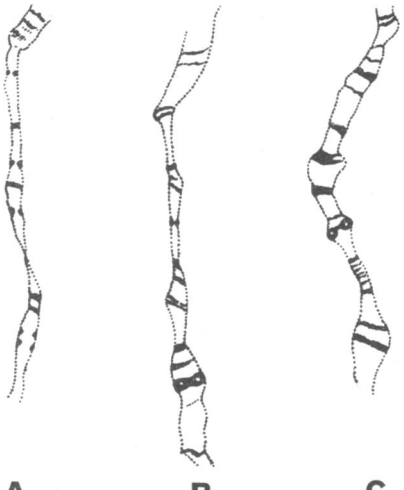
Semidiagrammatic *camera lucida* drawing from aceto-orcein squash preparation ; X 2250.
c = chromatic elements.
ch = chromocentre.
v = cytoplasmic vacuoles.

FIG. 4. SALIVARY GLAND CELL AT LATER STAGE THAN FIG. 2 AND 3



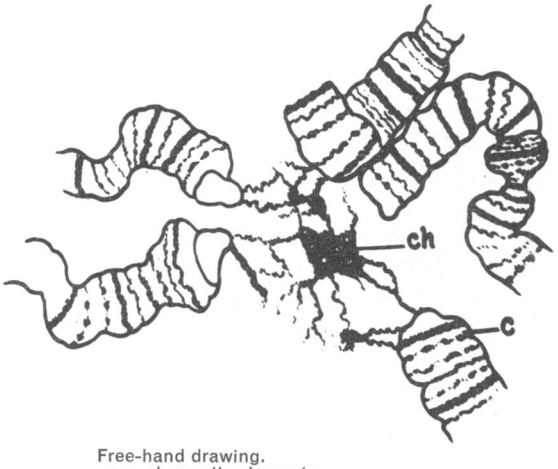
Semidiagrammatic *camera lucida* drawing from aceto-orcein squash preparation; X 2250.
c = chromatic elements.
ch = chromocentre.
v = cytoplasmic vacuoles.

FIG. 5. THREE STAGES IN THE DIFFERENTIATION OF LARVAL SALIVARY GLAND CHROMOSOMES



Aceto-orcein squash preparation.

FIG. 6. MODERATELY SQUASHED NUCLEUS FROM SALIVARY GLAND OF FOURTH INSTAR LARVA, SHOWING PROXIMAL REGIONS OF THE CHROMOSOMES WITH DISRUPTED CHROMOCENTRE IN THE MIDDLE



Free-hand drawing.
c = chromatic elements.
ch = chromocentre.

in the proximal lobe of the gland show an increased rate of growth compared to those of the distal lobe. This distinction has probably a bearing on the difference in secretory function of the cells of the two lobes. The large nuclei measure from 21 μ to 28.5 μ in squashes. In these nuclei portions of the chromosomal threads show a clear banded pattern and represent fully synapsed homologues (Fig. 5 B). Such regions, however, are limited in extent, and along the greater part of their lengths the homologues are in presynaptic stages only.

The proximal lobe of the gland obtained from early third instar larva measures about 80 μ (Plate IV, 7). The distal lobe includes a large quantity of secretory matter. Glands of this stage clearly show the anteroposterior gradation in the size of the nuclei of the distal lobe, and also the disparity in growth-rate between the cells of the two lobes. In whole mounts of the gland the large nuclei of the proximal lobe measure about 14 μ, and those of the distal lobe about 11.5 μ. The nucleolus in the former measure nearly 7 μ. In squash preparations the chromosome bands appear as discrete, uniformly staining, probably " compound " structures, and are separated from one another by clear hyaline areas (Fig. 5 C). The interband areas seem to be " elongated " compared to those of the fully grown chromosomes. Very faint markings are evident in the interband areas, and may represent incipient bands. Synapsis is incomplete along extensive regions of the chromosomes.

Squash preparations of the glands of the late third instar larva show large nuclei measuring nearly 42 μ. The chromosomes spread out better than during earlier stages. Synapsis of the homologues is almost completed, and presynaptic stages are no longer evident. The strands simulate the structure of the fully differentiated chromosomes. In favourable cases the free ends of the chromosomes are recognizable on the basis of their characteristic banded pattern.

The proximal lobe of the salivary gland from the early fourth instar larva measures about 125 μ (Plate IV, 10). The distal lobe often shows a notch which divides its posterior margin into two smaller lobes. In whole mounts of the gland the large nuclei of the proximal lobe measure about 21 μ, and their nucleoli about 9 μ. The latter disclose conspicuous vacuoles. The chromosomes stain well, and show a well-defined banded pattern. Fig. 6 illustrates a disrupted chromocentre in a squash. The proximal ends of the chromosomes are not greatly displaced from their original orientation, and are found disposed in a circle around the chromocentre. The chromocentre appears as an irregular network of diffuse threads. At some of the internodes are evident small chromatic clumps. One particularly large clump in the middle of the network includes small vacuoles.

The proximal lobe of the gland of the late fourth instar larva measures about 150 μ (Plate IV, 12). The giant nuclei in the lobe measure nearly 28.5 μ in whole mounts of the gland, and show chromosomes possessing

optimum stainability and spreading power compared to those obtained from younger or older larvae. The chromosomes are fully differentiated, and the typical banded pattern characteristic of the animal is evident. The interband areas include a certain amount of chromatin, either in a diffuse state or in the form of granules. Some cells of the distal lobe exhibit clear signs of histolysis. The distal lobe shows a certain amount of shrinkage due to loss of secretory matter from inside. The cells are attenuated, and clear spaces are formed in between their inner ends. The nuclei are migrated to the inner ends of the elongated cells, and the cytoplasm of the cells is reduced to a thin film around the nucleus, a slender middle column, and a thick outer layer. The nucleoli are highly vacuolated. The *Anlage* cells constitute a prominent thick mass about the base of the salivary duct.

In the larvae ready to pupate the proximal lobe of the gland remains more or less intact (Plate IV, 13). The chromosome bands, however, have become ill-defined, and the interband areas include a relatively large concentration of granules. The cells of the distal lobe, especially those at the posterior end, are in an advanced state of histolysis. The cell boundaries are lost completely, although the nuclei remain relatively intact. The nucleoli have mostly disappeared. The vacuolated chromosomes remain clumped up in the middle of the nuclear cavity. They stain intensely, while the cytoplasm takes little stain with the result that the distal lobe presents a characteristic appearance.

In the early pupa the proximal lobe of the salivary gland is elongated, and shows marked signs of histolysis (Plate IV, 14). The cell boundaries have become obscure, and the nucleoli have completely disappeared. The distal lobe constitutes a wrinkled sac-like structure trailing behind the proximal lobe. The nuclei in some of the anterior cells retain their compactness, and are held loosely within the outer membrane of the gland. The chromatin clumps are in various degrees of disintegration. The base of the salivary duct is enlarged to form a funnel which includes the considerable mass of *Anlage* cells.

RNA and DNA content of larval salivary gland cells

In sections of the larval salivary glands stained according to the simple pyronin/methyl-green technique, the chromatin stains blue or bluish-green, whereas the cytoplasm and the nucleolus stain red. The cytoplasm bears a number of granules which show an intense red stain. The banded structure of the chromosomes is not clearly noticeable in sectioned nuclei. They appear to consist of heavily staining clumps separated by relatively clear regions. In Plate III, 5, the origin of the chromosome strands from the chromocentre may be noted. The chromocentre showed a blue stain and a few small vacuoles. Plate III, 6, depicts the same nucleus at a slightly lower focus. In this the chromocentre is out of focus, but the nucleolus

can be made out. Some of the sections stained according to the simple pyronin/methyl-green technique showed a variable result in that the chromosomes and the chromocentre appeared to be pyroninophilic.

Possible differences in stainability between the bands and interband areas of the chromosomes were studied from squash preparations. The glands were fixed in Carnoy's fluid, and squashed in a drop or two of 45% acetic acid. The cover-glass and slide were separated from one another, and the material adhering to them stained according to the simple pyronin/methyl-green technique. In most preparations the chromosomes appeared to be pyroninophilic. Only in some did they show an affinity for methyl green. But in either case the bands disclosed an intense stain compared to the interband areas. Substitution of weaker percentages of acetic acid (for instance 20%, 10% and 5%) for squashing resulted in a proportionate increase in the number of preparations in which the chromosomes stained with methyl green.

The distribution of DNA (deoxyribonucleic acid) was studied from whole mounts of the larval salivary glands stained according to the Feulgen technique. Fixation in 10% neutral formol, and acid hydrolysis for 14 minutes gave satisfactory results. The cytoplasm is unstained except for a faint positive colouring of the granules embedded in it. The nucleolus is Feulgen-negative, and appears as a transparent vesicular body. The chromosomes and the chromocentre take a violet stain, and in favourable cases the latter may be clearly observed besides the transparent body of the nucleolus. The chromocentre is apparently diffuse in nature, and may appear as a vacuolated mass, a loose aggregate of granules, or a network of threads. Substitution of Carnoy's fluid for 10% neutral formol gave better fixation, although the staining of the chromosomes appeared to be relatively weak.

In order to procure a detailed understanding of the distribution of DNA in the bands and interband areas of the chromosomes, a few squash preparations were stained according to the Feulgen technique. The bands take an intense stain whereas the interband areas show a slight coloration only. The nucleolus and chromocentre are usually unrecognizable except in some of the smaller nuclei which resist squashing.

Discussion

The salivary gland nuclei of the larvae of *Anopheles stephensi* s. s. show five euchromatic strands united proximally to a chromocentre. The salivary chromosomes of this species are comparable to those of *Anopheles maculipennis atroparvus* (Frizzi, 1947b) in their proportionate lengths. The sex chromosomes of these species show further resemblance in the structure of their free tips, the " puff " about the middle of their lengths, and the short non-synapsed region proximally. A certain similarity is noticeable

between the free tip of chromosome IIIR of *Anopheles stephensi* s. s. and the corresponding region of chromosome IID of *Anopheles gambiae* (Frizzi & Holstein, 1956).

The chromocentre in *Anopheles stephensi* s. s. is small and fragile, unlike that of *Anopheles maculipennis atroparvus* (Frizzi, 1947a). In the possession of a chromocentre these species and *Anopheles gambiae* (Frizzi & Holstein, 1956) differ from *Culex pipiens* (Sutton, 1942; Kitzmiller & Clark, 1952), and many other Diptera such as *Sciara* (Metz, 1935), *Chironomus* (Bauer, 1935), *Simulium* (Painter & Griffen, 1937), *Bibio* (Heitz & Bauer, 1933), and the Cecidomyids (White, 1948). The bulk of the chromocentre is formed of the heterochromatic regions of the sex chromosomes, while the autosomes contribute little or no material. The non-heterochromatic proximal ends of the autosomes are probably united at the chromocentre by virtue of their terminal attraction, as in *Drosophila funebris* and *Drosophila hydei* (Bauer, 1936). Attenuated thread-like connexions are occasionally seen between the proximal ends of the two limbs of the autosomes, similar to those reported in *Drosophila* (Painter, 1934).

The nucleolus is a prominent structure within the nuclei of the salivary gland cells through larval life, and disappears only at the time of histolysis of the glands. Such persisting nucleoli are characteristic of all glandular cells (Gardiner, 1935). The nucleolus and the chromocentre are closely associated, although the nature of the association is not clear. Association of the nucleolus with the chromocentre or with particular chromosomes is common in most of the groups of Diptera (Heitz & Bauer, 1933; Geitler, 1934; Bauer, 1936; Frolowa, 1936; Emmens, 1937; White, 1948).

The distal lobe of the salivary glands in the larvae of *Anopheles stephensi* s. s. is primarily secretory in function while the proximal lobe chiefly functions as a conductive region. Ross (1939) has claimed a similar difference in functional significance between the posterior cells and the anterior cells of the larval salivary glands of *Drosophila*. *Anopheles* species exhibit little variation in the structure of the larval salivary gland and the number of cells comprising it (cf. Ciaccio, 1941; Frizzi, 1947; Magrini, 1948; Frizzi & Holstein, 1956). The gland of the newly hatched out larva shows the full complement of cells, and its development through larval life is by cell-growth, as in many other Diptera (see Ciaccio, 1941).

At the earliest observable stage the homologous chromatin elements constitute a tight coil in the larval salivary gland nuclei. Full synapsis of the homologues is preceded by a process of progressive uncoiling accompanied by a chromomere-to-chromomere fusion, and is more or less similar to the process described in *Anopheles maculipennis* (Magrini, 1948, 1951). In *Anopheles stephensi* s. s. the rate of the process varies among different chromosomes, and along different regions of the same chromosome. This results in the presence of certain " intermediate nuclei " which show a banded structure along some sections of the chromosomes and presynaptic

stages along other sections, as noted also by Sutton (1942) in *Culex pipiens*. Synapsis of the homologues is completed only by the late third instar in all the mosquito species, which is considerably later than in other Dipteran genera like *Sciara* (Buck, 1937), *Simulium* (Painter & Griffen, 1937), and *Chironomus* (Beermann, 1952).

The simple pyronin/methyl-green technique has revealed the presence of RNA (ribonucleic acid) in the cytoplasm and nucleolus of the larval salivary cells, and DNA in the chromosomes and chromocentre. In some preparations the cell constituents have shown complete pyroninophilia, presumably due to the loss of affinity for methyl green by the DNA on depolymerization, as pointed out by Kurnick (1952) and Yakar (1952) among others. Squash preparations using 45% acetic acid show a preponderance of pyroninophilic chromosomes. On substitution of lower concentrations of acetic acid for squashing, a larger number of chromosomes stain with methyl green to the exclusion of pyronin. This suggests that the higher concentrations of acetic acid have a depolymerizing effect on DNA. In squash preparations the interband areas of the chromosomes show a weak staining indicating the presence of some diffuse DNA at these sites also.

Preparations stained according to the Feulgen technique confirm the presence of DNA in the chromosomes and the chromocentre. The interband areas of the chromosome show a weak reaction. The intensity of the stain is greater after fixation in neutral formol than in Carnoy's fluid, as noted also by Swift (1955).

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RÉSUMÉ

L'auteur a étudié de façon approfondie le développement des glandes salivaires et leurs chromosomes, chez *Anopheles stephensi*, première espèce d'anophèle de l'Inde à faire l'objet de telles recherches.

Les chromosomes sont au nombre de cinq, réunis par leurs extrémités proximales en un chromocentre, qui apparaît comme une masse vacuolaire, un agrégat de granules

assez lâche, ou un réseau imprécis. Ces chromosomes sont comparables à ceux qui ont été décrits chez *A. maculipennis atroparvus*. Les chromosomes sexuels de ces deux espèces ont également des similitudes. La présence d'un chromocentre distingue ces deux espèces d'anophèles d'autres espèces ou d'autres genres d'insectes, tels que *Culex pipiens*, *Simulium*, *Bibio*.

L'auteur décrit les cinq chromosomes de *A. stephensi*, leurs bandes caractéristiques, et expose les diverses étapes du développement des glandes salivaires aux divers stades larvaires.

L'acide ribonucléique est présent dans le cytoplasme et le nucléole des cellules des glandes salivaires, et l'acide desoxyribonucléique dans les chromosomes. Cet acide semble exister à l'état diffus dans l'ensemble du chromosome.

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