PREPARATION OF MOSQUITO CHROMOSOMES

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INTRODUCTION. Breland (1959, 1961) ıas provided an outstanding stimulus to he study of mosquito chromosomes. The echniques which he has pioneered have iven immediately useful tools to the ever-rowing numbers of investigators con-erned with the study of mosquito chromo-omes.

Stimulated by his reports, we reiniti-ted our work on mosquito chromosomes nd have developed certain modifications f his technique which may prove useful) other investigators. In addition, in the ourse of several years' study of the sali-ary gland chromosomes of *Culex* and *Anopheles,* we have developed a highly atisfactory method for the preparation of olytene chromosomes from these genera. ince details of these methods are not gen-rally available in the literature, it seems vorthwhile to present them, without pur-uing in detail the many interesting leads ıncovered. The literature has been well eviewed by Breland (1961). This paper, hen, proposes to give details of the spe-ific techniques which have proven useful ı our laboratory.

GENERAL CONSIDERATIONS. Clearly rec-gnizable differences exist in different pop-ılations with respect to the ease with vhich chromosome preparations can be nade. For example, polytene chromo-omes may be easily prepared from *Anoph-les,* less easily from *Culex,* and only with at present) great difficulty from *Aedes.* The reasons for this variation are not lear; they may be simply a function of hromosome length, but numerous and enacious interchromosomal and intra-hromosomal connectives are involved.

Laboratory populations which have been iven optimal care will routinely produce ood salivary chromosomes, but very us-ıble preparations can be made from larvae ollected in the field.

Mosquitoes are excellent animals for cytological studies in many respects. The diploid number is low (2n = 6 in all species thus far studied). Testes and ovaries show all division figures, and in many cases, particularly in the testes, stages from early prophase I to anaphase II may be seen on the same slide. The metaphase chromosomes are relatively large and are easily identifiable. Salivary gland chromosomes from larvae are excellent. Especially in the genus *Anopheles* polytene chromosomes are large and well-banded, with characteristic ends and consistent banding patterns. The small number of chromosomes per nucleus plus the relative ease of spreading, makes relatively simple the study of the numerous aberrations, such as inversions, which are typical of certain populations.

MITOTIC AND MEIOTIC CHROMOSOMES. Good preparations may be routinely made from the brain, testis and ovary (Figs. 4, 5, 6, 7, 9). Mitotic figures may be found in the brains of all larval instars, but the late fourth instar (prepupa) is by far the best (Figs. 4, 7). For testes smears, early pupae are best (Fig. 9). We attempt to get male pupae as soon as they metamorphose. Older pupae, at least 24 hours old, are the best for ovarian chromosomes (Figs. 5, 6).

The key to consistently good preparations in our laboratory has been the use of a colchicine pretreatment which blocks cell divisions and stock-piles abundant figures on the same slide. This pretreatment has made the difference between laborious preparation of poor slides with few figures and easy, rapid preparation of many slides with numerous chromosomes.

COLCHICINE PRETREATMENT. The modified mammalian chromosome technique of Lewis & Riles (1960) has been used with great success. Animals of the right

age are placed in a solution of 0.1 percent colchicine (0.1 gram colchicine alkaloid U.S.P. in 100 c.c. of double distilled water). Successful procedures for brain, testis and ovary are as follows:

Brain: Select late fourth instar larvae. These larvae may be sexed by noting the presence of the blunt, spindle-shaped testes at about the level of the sixth abdominal segment, and by the larval pigmentation in some strains (Jones, 1957). The sex of larvae of earlier instars may be determined by examination of the antennal discs or of the genital discs (Jones, 1957). Certain species such as *Anopheles freeborni* can be sexed more easily than others, but the most certain way is to dissect out the testes or ovaries after the brain has been dissected. The larvae are allowed to remain in the colchicine solution 2 to 4 hours.

Testis: Male pupae are easily identified by the genitalia, but with practice are easily separated from females by their narrower thorax. Best results are obtained in individuals which are collected immediately after pupation and are allowed to remain in the colchicine solution for 24 hours.

Ovary: Female pupae 24 hours old are placed in the colchicine solution for 24 hours. Dissection of ovaries from 48-hour pupae produces good results. If the female emerges, the adult ovaries may be dissected; indeed, ovaries from such females have produced some of the best figures.

DISSECTION. All dissections are made in a small drop of hypotonic sodium citrate solution (1 gram $Na_3 C_6 H_5 O_7 2H_2O$ in 100 c.c. double distilled water). This produces a slight swelling of the chromosomes. Silicone (General Electric SC-87) treated slides are used for all dissections. Breland (1959) describes a satisfactory method for dissecting the brain. A les precise but effective modification is t sever the head from the thorax and pres on the dorsal surface of the head with flattened needle until the brain is exude from the posterior part of the head capsul

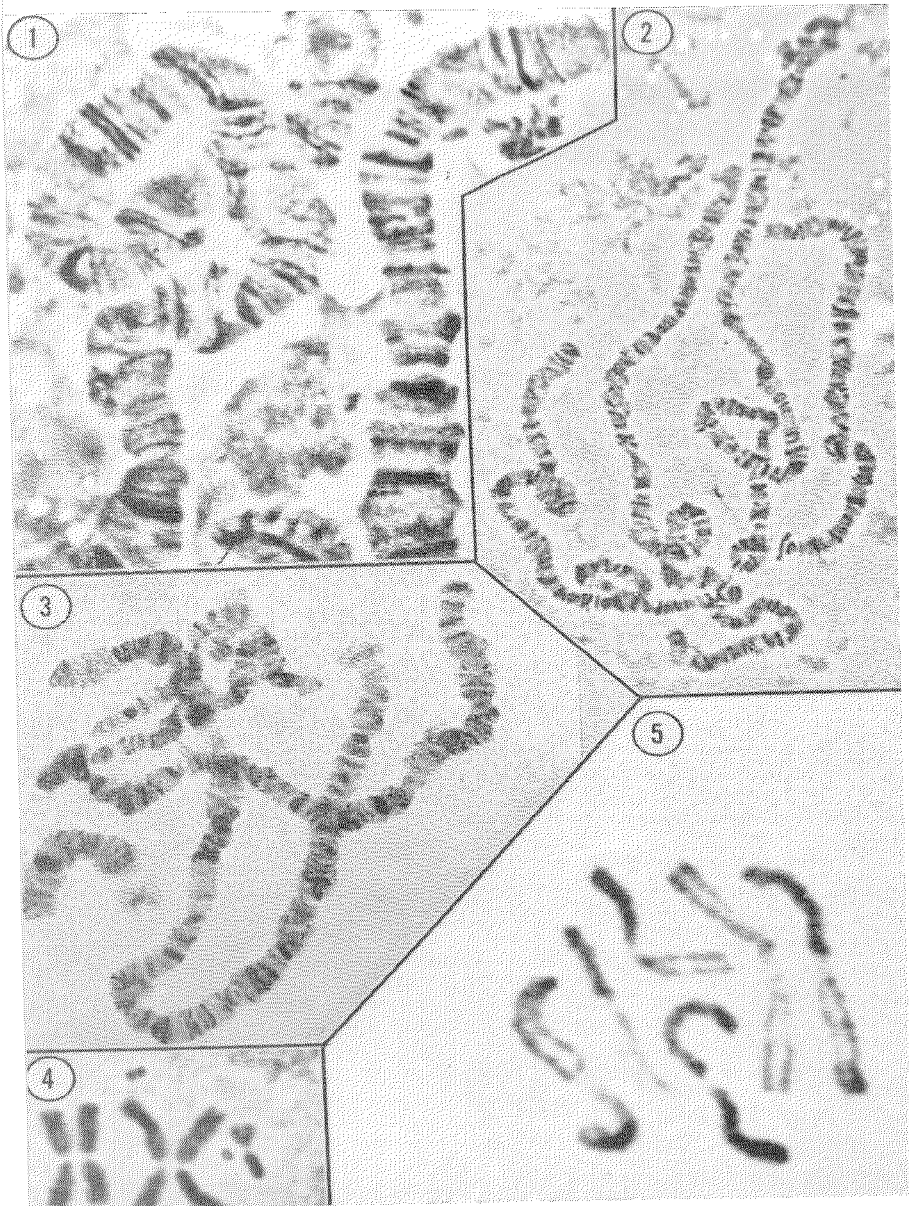
Pupal testes and ovaries are best di sected by severing the abdomen from th thorax, holding the posterior segment c the abdomen with a bent needle, gentl forcing the abdominal contents anteriorl then severing the last abdominal segmen Next, holding the abdomen anteriorl gently press posteriorly with a bent need along the dorsal surface of the abdome until the internal organs are exuded po teriorly. The gut, Malpighian tubules an gonads usually come out together. Th testes may be recognized as white or ye lowish oval organs, usually still attache to their ducts. The ovaries are transluce oval structures which may be most readi recognized by the prominent attached ne work of tracheoles.

Larval gonads are prepared in esse tially the same manner. A quick metho usually successful, is to hold the anteri part of the severed abdomen with a needl lay a bent needle over the last abdomin segment, press and give a sharp pull po teriorly. The internal organs will usual pull out as a group.

FIXATION AND STAINING. Dissected ti sues are transferred, on the tip of a needl to a small drop of modified Carno fixative (equal parts of 45 percent acet acid and 95 percent ethyl alcohol), on siliconized coverslip for 30 seconds. small drop of lacto-aceto-orcein (2 gran Gurr's synthetic orecin, 50 c.c. 85 perce lactic acid, 50 c.c. glacial acetic acid, W shons and Russell, 1959) is placed on t same coverslip, and the tissue transferr to it. (For examinations under phase, t 2 percent stock stain is first diluted

PLATE I

FIG. 1.—*Anopheles quadrimaculatus.* Portion of salivary chromosome III. Larva. Phase, oil. 1500X. FIG. 2.—*Anopheles quadrimaculatus.* Entire salivary chromosome complement. Larva. Pha ca. 400X. FIG. 3.—*Anopheles quadrimaculatus.* Entire chromosome complement from malpighi tubule. Larva. Bright field. ca. 450X. FIG. 4.—*Anopheles quadrimaculatus.* Metaphase chrom somes, male brain. Pupa. Phase, oil. ca. 3000X. FIG. 5.—*Aedes taeniorhynchus.* Ovarian nurse c chromosomes. Adult. Phase, oil. ca. 3000X.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0.5 percent with equal parts lactic acid and glacial acetic acid and the tissues are stained in a small drop of this dilute stain for 30 seconds.) Staining time will vary according to the batch of stain used and whether the slides are to be examined under phase or with a light microscope.

After staining for 30 seconds, add a small drop of 45 percent acetic acid. Move the coverslip to a piece of filter paper, wipe off the drop of fixative, then lower a clean, non-siliconized slide on top of the coverslip and press gently. The amount of pressure necessary to spread the chromosomes is best gained by experience. We have found that a series of sharp taps is better for breaking the cell and nuclear membranes than steady pressure. The exact technique is again best acquired by practice.

For temporary mounts, we ring the coverslip with dental wax. Thus prepared, the slide may be used for a period of several weeks. In practice, we seldom make temporary mounts, but make permanent preparations as described below.

PERMANENT MOUNTS. (Conger and Fairchild, 1953.)

Dip the slide in liquid nitrogen for 30 seconds. Remove, and snap off coverslip with a razor blade. The tissue should now be affixed to the non-siliconized slide. Immerse the slide immediately (before it dries) in 95 percent ethyl alcohol and leave for five minutes. Transfer to 100 percent ethyl alcohol for one minute. Remove, and cover the tissue immediately with a drop of mounting medium (Zeiss Einschlussmittel L-15 is best). Dip a clean coverslip in 100 percent ethyl alcohol, touch one edge momentarily to filter paper, and lower gently over the preparation.

The slide is then placed upon a slide warmer (40° C.), covered with a small piece of filter paper, and weighted. A system which works well for us includes a shell vial glued to a microscope slide with epoxyresin glue, and weighted with 50–100 grams of shot. The uniform distribution of the weight helps maintain the spread and facilitates drying. For best results, slides should dry for at least 48 hours, and should be kept level for at least six weeks, although they may be used in the meantime.

If wax-ringed slides are prepared by this method, dip the slide in liquid nitrogen remove the wax by rubbing or scraping with a needle or razor blade, and immerse immediately in liquid nitrogen. The slide may then be prepared by the method described above.

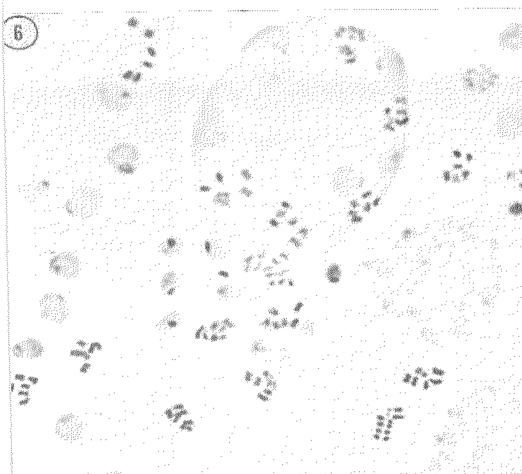
In some cases the tissue may adhere to the coverslip rather than to the slide. In these cases run the coverslip through the alcohols, mount in Einschlussmittel as before.

MODIFICATIONS. The methods described above might be called our "standard" methods. A modification, now used routinely, helps prevent loss of tissue from the slide, the most critical part of the whole process. This modification is as follows After removal of the slide from the liquid nitrogen, place it, coverslip up, on a slab of dry ice for 30–45 seconds, then snap off the coverslip. We are not certain why this is effective, but it works. The differential thawing of the slide and coverslip is a possible reason. In any event loss of tissue from the slide is now an unusual event.

Another modification involves dissection and staining. Dissection on a silicone treated microscope slide placed on a cold surface apparently enhances spreading and staining. A convenient cold surface is a synthetic sponge (about 1″ x 3″ x 5″) which has been soaked in water, wrapped in plastic, then frozen. Several of these sponges may be prepared in advance and stored in the freezing compartment of a refrigerator. As one thaws out, replace it in the freezer and use a fresh one.

PLATE II

FIG. 6.—*Anopheles freeborni.* Ovarian nurse cell chromosomes. Phase. ca. 700x. FIG. 7.—*Anopheles freeborni.* Metaphase, male brain. Larva. Phase, oil. ca. 1500x. FIG. 8.—*Anopheles freeborni.* Entire salivary chromosome complement. Larva. Phase, ca. 500x. FIG. 9.—*Anopheles freeborni.* Meiotic division I, testis. Pupa. Phase, oil. ca. 1500x.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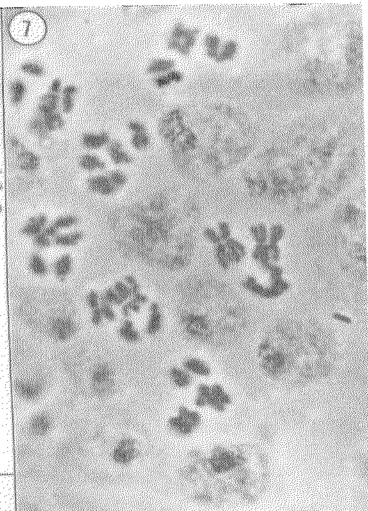
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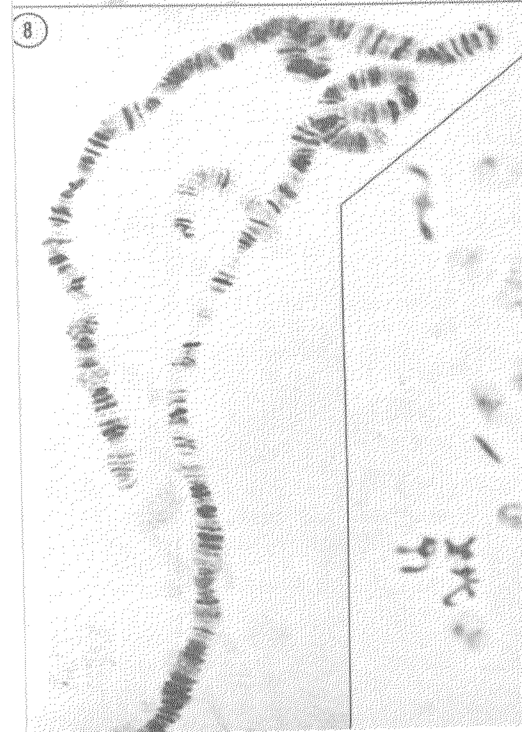
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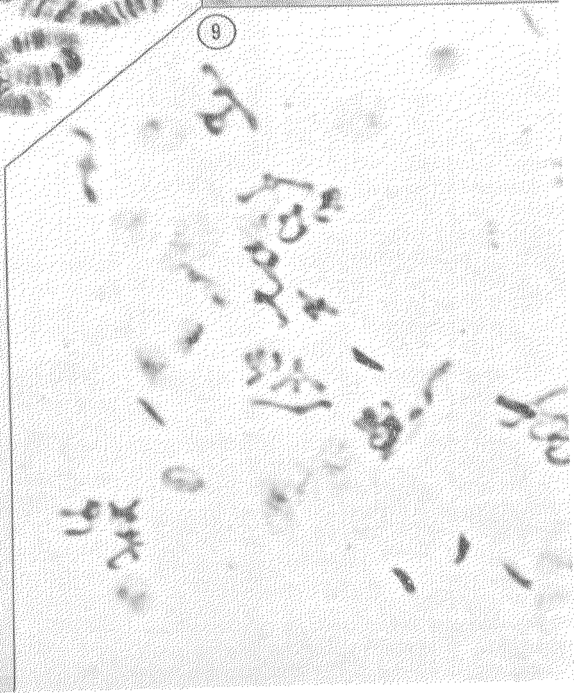
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After dissection and fixation the tissue is removed to a very small drop of stain (0.5%) on the same siliconized coverslip, still on the cold surface. The tissue is macerated with needles. This helps spread the cells and enhances the spreading of the chromosomes. The cold surface helps prevent the evaporation of the very small drop of stain.

As in the standard procedure, a non-siliconized slide is touched against the drop of stain, the slide turned over, and four small drops of destain (45 c.c. glacial acetic acid: 55 c.c. distilled water) applied to the edges of the coverslip. The destain helps stop the staining action and also helps prevent excessive dispersion of the tissue. Often this procedure is enough to rupture the cells and spread the chromosomes; slight pressure or tapping may be necessary.

A nicety which may be gained by practice is the removal of the testis sheath in the fixative. After the testis is placed in the modified Carnoy's fixative, the sheath which remains attached to the sperm duct usually becomes loosened from the cells of the testis. Very gentle teasing with fine needles will separate the cells from the sheath. The improved fixation, staining, and spreading is well worth this extra effort.

SALIVARY CHROMOSOMES. The best salivary gland preparations are made from large and active larvae of the early fourth instar (Figs. 1, 2, 8). These larvae are removed from the rearing pan with a small pipette, rinsed in clean distilled water and dropped onto a piece of clean, dry, filter paper to remove excess moisture. The larva is removed from the filter paper by means of a fine forceps by grasping it firmly between the thorax and abdomen. It is then placed upon a clean, dry, silicone-treated slide. Slides and coverslips are dipped into G.E. SC-87 Dri-Film, then into distilled water, then dried with clean, lint-free paper tissues to remove excess water and silicone.

The thorax is severed from the abdomen by a scissors-like cut with a pair of dissecting needles. The abdomen is removed to another area of the slide for subsequent removal of the Malpighian tubules and for dissection of the gonads to determine sex.

A small drop of dilute Carnoy's fixative (one part Carnoy's: 19 parts distilled water) is placed on the thorax as a dissecting fluid. A dissecting needle bent to about 120° from the shaft about 2 mm from the tip is inserted from the posterior under the dorsal surface of the thorax and pushed forward into the head capsule. Rubbing a second needle along the inserted one opens the thorax completely along the mid-dorsal line. The gut is then lifted at its posterior end and folded back over the head of the larva. The gut and head are then severed from the thorax. The bilobed salivary glands (*Anopheles*) are usually evident in the antero-lateral region of the thorax (Rioux *et al.*, 1959). The edges of the mid-dorsal incision are spread apart, the tissue overlying the glands is pushed aside, and the glands are lifted out with a needle.

The glands are placed in a small drop of Carnoy's fixative (1 part glacial acetic acid: 3 parts 100 percent ethyl alcohol) and fixed from one to two minutes.

During fixation, a clean, silicone treated coverslip is placed on the slide. In the center of the coverslip is placed a small drop of stain (2 percent orcein in equal parts 85 percent lactic acid and glacial acetic acid, diluted 1 part to 3 with 45 percent acetic acid).

After fixation, the glands are transferred to the stain and allowed to remain for 30 seconds to 2 minutes, depending on the intensity of the stain desired. The coverslip is then removed to a flat surface, preferably white filter paper, then a clean non-siliconized slide placed over the coverslip, gently pressed to spread the stain and tissue, then turned over. Our best spreads have been obtained by holding the coverslip firmly in place with a small square of filter paper, then tapping the coverslip sharply several times with a finger. The amount of spread will be determined by the number of taps and the force exerted. Mosquito chromosomes cannot be pressed with great force as can

hose of *Drosophila;* the tapping seems to ›roduce the best results.

The coverslip may be ringed with wax or temporary mounts, or the slide may be nade permanent by the liquid nitrogen echnique exactly as described above for nitotic and meiotic chromosomes.

MODIFICATIONS. We have found that he best salivary preparations result from arrying out the entire process in the cold. Ve now routinely dissect, fix and stain n a cold surface (see above, frozen ponges). The fixative and stain are stored n the freezing compartment of the re-rigerator and used cold. Several advan-ages of this cold treatment are immediately pparent. Mass production of several slides s possible due to reduced evaporation and ubsequent economy of time. Maceration nd teasing of tissues proceeds better with-ut evaporation, resulting in better pene-ration and more uniform staining. The hromosomes thus prepared seem more lastic, spread more easily and are less ubject to breakage. This is especially nportant in the preparation of chromo-omes showing inversions or other aberra-ions. Finally, the slower penetration of he stain produces, we believe, better stain-ng (Bridges 1938).

Slides of the polytene chromosomes of he Malpighian tubules are very easily repared, and offer excellent material for tudy of inversions and other chromosomal berrations; they can be obtained from hird and fourth instar larvae, from pupae nd from adults. The banding patterns n these chromosomes, however, are not lear and distinct and are inferior to the hromosomes of the salivary glands for etailed cytological study (Fig. 3).

After the thorax has been severed from he abdomen as described in the prepara-ion of salivary gland chromosomes, the bdomen is moved to another area of the lide and dissected as follows. The distal egment of the abdomen is first removed vtih dissecting needles and the gut which rotrudes (teasing may be required) from he anterior end of the abdomen is pulled orward with a probe while another probe s used to hold the rest of the abdomen in lace.

No dissecting fluid is used except the body fluid present in the larvae; fixation (Carnoy's fluid) is accomplished immedi-ately upon removal of the gut and attached Malpighian tubules from the rest of the abdominal material. The Malpighian tubules are dissected from the gut in the fixing fluid.

After fixation the preparation of poly-tene chromosomes from the Malpighian tubules follows exactly the procedure de-scribed for salivary chromosomes.

In preparing pupal and adult Malpighian tubule chromosomes the same technique is used except that in adults a dissecting fluid (dilute Carnoy's) is often helpful.

SUMMARY. Consistently good prepara-tions of mosquito chromosomes may be prepared quickly and easily by using col-chicine pretreatment and a modified mam-malian chromosome technique. Brain, testis and ovary thus treated and stained with lacto-aceto-orecin produce up to a 100-fold increase in usable figures per slide.

Salivary gland chromosomes, especially of *Anopheles,* are routinely prepared by dissection, fixation and staining on a cold surface. The details of this process are given.

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