THE STRUCTURE AND THE DEVELOPMENT OF THE SALIVARY GLAND CHROMOSOMES OF SIMULIUM

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INTRODUCTION

THE discovery that individual genes are associated with definite chromatic "bands" along the giant salivary gland chromosomes of *Drosophila melanogaster* (PAINTER 1934) focussed our attention on the nature of these bands and on the structure of these chromosomes in general. At the present time two very different interpretations are given to the observed phenomena. On the one hand we have the concept, suggested independently by BRIDGES (1935) and by KOLTZOFF (1934), that these giant chromosomes represent prophase elements which have undergone a number of divisions. In effect each chromosome is a bundle of chromonemata; the "bands" are rows or discs of homologous chromomeres, the number of which will depend on how many times the original chromonema is split; and the achromatic spaces between the bands contain the threads which connect the chromomeres of the separate chromonemata. This view has been widely accepted, alike by cytologists and geneticists, and a considerable amount of work has appeared supporting it, the most outstanding being the studies of BAUER (1935, 1936) dealing with the chromosomes of Chironomus larvae. On the other hand, METZ (and his students) does not believe that the original prophase chromosome has divided a number of times; instead, he thinks that the giant chromosomes have a honeycomb structure and are made up of little droplets of material. When the chromosome is stretched these droplets elongate forming "alveoli"; and the side walls of these alveoli, where they touch, have been mistakenly interpreted by others as chromonema threads. Between the abutting ends of alveoli, as a sort of chinking, chromatin is deposited forming a continuous disc or band across the chromosome. Thus the threads and chromomeres of the chromonema interpretation are regarded, by METZ, as different aspects of a system of discontinuous alveoli (in a longitudinal direction) and continuous discs or plates of chromatin.

Needless to say, the admitted correlation between certain visible elements and gene loci makes it highly desirable that we understand the physical structure of the giant chromosomes as completely as possible. Both of the interpretations referred to above are based on essentially similar observations on fully differentiated salivary chromosomes, and while more study of the facts is necessary, a moment's consideration will

indicate that fully as important for an understanding of the adult structure is the ontogenetic history of the elements. This was recognized early in the development of the salivary chromosome work, at this laboratory, but unfortunately *D. melanogaster* has not proved favorable for this type of study. Some eighteen months ago we began working on Simulium, principally *S. virgatum*, the larvae of which are common about Austin most of the year. In this form the chromosomes are about three times as broad as in the fruit fly; and what is far more important, somatic synapsis does not lead to such an intimate association of the component parts, so that one can generally distinguish individual chromomeres and the connecting strands, and from a study of early stages determine how the various type of bands are formed.

MATERIAL AND METHODS

Larvae collected in the streams about Austin can be held alive in the laboratory until they pupate, provided they are kept in a cool room in a stream of fast flowing water.

For a study of living nuclei larvae were placed on a clean slide, carefully dried and covered with paraffin oil; in this medium the salivary glands were dissected out and, surrounded by a drop of body fluid, were mounted under a coverslip. At no time were the glands exposed to the air or to salt solutions. If the effects of fixatives were to be studied the coverslip was sealed except for a narrow space at each end which allowed us to draw new fluids under the cover with filter paper.

A number of different fixation methods and stains were tried but none proved more useful than either temporary or permanent aceto-carmine preparations. Wratten filters 61 and 62 are indispensable for the study of the latter with artificial light, and oblique illumination brings out many fine details not ordinarily seen.

A few observers have described and given photomicrographs of living salivary chromosomes showing that they have essentially the same form before and after fixation with aceto-carmine. DOYLE and METZ (1935), however, report that the unaltered living chromosomes from active larvae of *Sciara* appear as optically homogeneous cylinders within the nucleus and show no sign of the bands until after fixation. They also state that after fixation with aceto-carmine there is a shrinkage in the chromosome diameter of from 50 to 70 percent. Such a serious indictment of the method so extensively used for staining requires a careful checking.

In Simulium the unaltered living chromosomes appear very much as they do in a well fixed and stained aceto-carmine preparation. The various types of bands are visible; chromomeric vesicles, both large and small, are clearly apparent and in the spread-out region the longitudinal fibers

are visible. We have carefully measured the diameters of chromosomes before and immediately after fixation, on photomicrographs, and find a shrinkage of chromosome diameter of from 10 to 17 percent. Fixation alters the image of the chromosome somewhat in that some of the chromomeric vesicles appear to swell a little but on the whole the images before and after preservation are remarkably similar. This indicates that in Simulium we may accept the validity of the fixation image as far as major details are concerned.

In somatic equatorial plates in Simulium there are six chromosomes with medial or sub-medial spindle fiber attachments; in the salivary gland nuclei we find three pairs of more or less intimately synapsed elements. There is no chromocenter in this form, and somatic synapsis[1] appears to be more intimate than in the European form studied by GEITLER (1935). For the most part the salivary chromosomes exhibit throughout their lengths the typical banded form, and there is the strictest sort of regimentation of the chromomeres. We shall call this type of area "euchromatic." However, in the longest chromosome (pair) sub-medially there is a region where homologues do not unite in intimate synapsis, and even within one homologue, or one chromatid, the chromomeres do not form compact bands. This is accompanied by a spreading of the chromosome constituents which makes this region favorable for the study of certain details. For the present we call this the "spread-out" region, although it may be related to the heterochromatic areas of other species of Diptera.

The twisting of homologous chromosomes about each other, which accompanies somatic synapsis, is easily seen in Simulium and in euchromatic areas amounts to about one full turn to each four diameters of the synapsed chromosomes. In the spread-out region the twisting is less pronounced; in younger ontogenetic stages the mates often lie parallel for a relatively long distance, and we observe that each homologue is split (fig. 4). We have then, in reality, within each giant chromosome four chromosomes or chromatids and as one focusses carefully on any band four parts are generally apparent. Similarly, in the achromatic spaces there are four bundles of "fibers" running at somewhat different angles. It is highly important that this tetrapartite nature of the giant chromosome be kept in mind because when the chromosome is greatly flattened by coverglass pressure, a single band may be separated into four sections and the fibers of each chromatid, running at different angles, may give the misleading impression of a net-work.

[1] Some authors use the term "somatic synapsis" to include not only the close apposition of homologous chromomeres, the sense in which Painter uses this term, but also the more general phenomenon long known as "somatic pairing." It may well be that both phenomena are basically due to the same force or forces, but their objective expressions are so different that it seems wise to use both terms in their original senses.

THE STRUCTURE OF THE EUCHROMATIC AREA

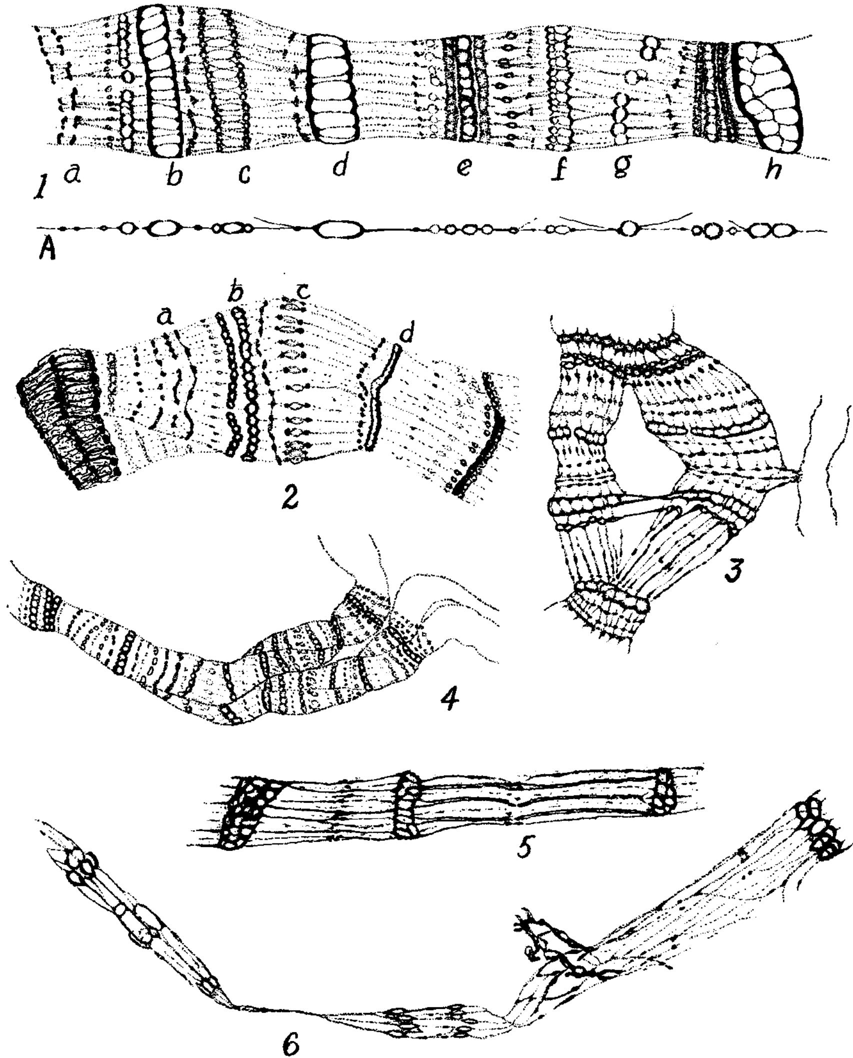
As one examines a series of well preserved and stained aceto-carmine preparations he finds three different expressions of the same chromosome organization. The first, which we shall call the "large vesicle" type, is shown in figure 1. The enlarged chromomeric vesicles can be readily observed in living nuclei and hence this form is assuredly "normal" in the sense that it appears in the unaltered living chromosome. The "small vesicle" form is shown in figure 2 and differs essentially from the first only in that the vesicles are small and, as a consequence, the chromomeres appear more clearly as separate entities. We regard this second expression as "normal" because in many living nuclei enlarged vesicles do not appear and yet the bands are double. In some preparations the chromomeric vesicles are scarcely visible as separated spaces; in such chromosomes the chromatic hulls remain intact, however, and we have coarse granular bands or lines running transversely across and through the element. Whether or not this form is "normal" we are unable to say because such details can not be made out in living chromosomes. METZ and GAY (1935) claim that in Sciara the character of the image given by a band is greatly affected by the nature of the dissecting medium; but since according to DOYLE and METZ the unaltered living chromosome is optically structureless, the basis for this conclusion is not clear. In Simulium we have used throughout this study the same methods of handling, fixing and staining the glands; and since in any given larva one expression of form dominates, we are inclined to think that the source of variation is extrinsic to the technique.

Figure 1 is a camera lucida drawing made from a permanent aceto-carmine mount. So far as possible we have drawn in only those details which show on the surface of the upper half of the chromosome. But since the chromomeres form a sort of plate or disc transversely through the chromosome, as one focusses downward superficial units disappear and deeper lying ones come into view and we may have included a few of the deeper lying features in the surface view. Likewise, while it is clear enough that the longitudinal fibers connect with the chromomeres of the compact bands, such as at *d*, we cannot be sure that the fiber drawn goes to the exact chromomere illustrated in the figure.

Figure 1A is a semi-diagrammatic representation of the types of chromomeres observed in figures 1 and 2, and of the ways in which these are connected.

Beginning at *a* there are two rows of dot-like chromomeres which tend to associate in pairs. Individually these are about the same size and stain deeply with no visible cavity or space inside the chromomere; there are about 16 in each row, across the surface of the chromosome. Fine lines arise

from each of these chromomeres and pass to the next row on the right which contains 15 or 16 elements. The chromomeres of the third row are much larger and there is a rim or hull of chromatin surrounding a non-staining center which we call the vesicle. The chromatin in the vesicular



FIGURES 1–6. Euchromatic region. Figure 1. A camera lucida drawing showing the details which appear on the upper surface of a fully developed salivary chromosome of the large vesicle type. Figure 1A is a semi-diagrammatic sketch of the types of chromomeres which make up the bands of figure 1. Figure 2. A chromosome of the small vesicle type showing the same region as figure 1 *a* to *e*. Figure 3 was made from a chromosome after treatment with KOH to remove part of the chromatin. Figure 4, drawn at a lower magnification, shows that each homologue is split prior to somatic synapsis. Figure 5 shows how unusually heavy fibers are formed when the chromomeres of a band are displaced. Figure 6 shows single and compound fibers in a young chromosome in about the 8-strand stage.

wall tends to lie on the sides next to the fibers giving a clam-shell effect, to use BRIDGES' figure of speech (1935).

The band labelled *b* is made up of 15 or 16 large vesiculate chromomeres closely pressed together. The chromatin is largely confined to the free surfaces or ends of the chromomeres, where it forms two deeply staining saw-tooth lines across the chromosome. The resulting double band effect is what is so commonly observed in all types of salivary chromosomes. Between the double band the adjoining walls of the chromomeric vesicles appear as fine lines. Here it should be remarked that the localization of the chromatin may be, in part, the result of fixation; for while in the living chromosome many bands appear double, showing that in life there is asymmetry in the distribution of chromatin, it is also true that after fixation the bands appear to be somewhat broader, as though the acetic acid caused the vesicles to swell a little. The vesicles of band *b* are all about the same size but it is not uncommon to observe, even in living chromosomes, a band with one or two vesicles which are considerably larger than the rest. The explanation for this will be given later.

To the right of the band *b* there is a row of non-vesiculate chromomeres which are rather large for this type. These are connected to the chromomeres of adjoining rows by fine parallel threads.

The band at *c* merits careful study, for this type is very common. Superficially it appears as a "double" band; however, the edges are not sharply delimited, as at *b*, but are fuzzy. Close inspection shows that there are present three rows of vesiculate chromomeres which are closely joined in a linear direction. A similar type of band is shown at *f;* here at one end the chromomeres are not vesiculate.

The band at *d* is much like *b* except that the chromatic edges are thicker, stain more densely, there are fewer vesicles, and the chromomeres are larger. Instead of 15 or 18 units at the surface, there are about 8 at *d*. On close inspection, especially with oblique illumination, it will be observed that many fine lines arising from the chromomeres at *c* join in pairs before they connect with the dot-like chromomeres just to the left of *d*. While we realize that chromomere counts are open to error when we try to enumerate the number lying at the upper surface, it is evident that the number at *d* and a few more bands to the right is much less than at the left in the figure. Such differences in adjoining bands are not uncommon and ontogenetic stages indicate that the cause (or causes) lies within the chromomeres themselves. Some seem to undergo fission more readily than others.

At *e* there are five rows of chromomeres rather closely associated; in small or less perfectly preserved chromosomes this would be classed as a "broad and fuzzy" band. Between *e* and *f* the threads seem to bifurcate and in the latter band there are 17 or more chromomeres. At *g* the number

is lower again and once more we see the threads associating in pairs. The band at *h* is somewhat unusual for there are two rows of large vesiculate chromomeres closely pressed together, the chromatin appearing on the free edges.

Figure 2 is a camera lucida drawing taken from a chromosome of the small vesicle type, showing the same region as figure 1 *a* to *e*. One has no difficulty in recognizing corresponding bands, in the two figures; they are made up of the same kinds of chromomeres, in the same sequence and with about the same number of elements in the rows. In general, in figure 1 the chromomeres are larger than in figure 2, and in the case of bands *b* and especially *d* the difference is very great. The amount of hypertrophy which the chromomeres of a band undergo seems to depend on some intrinsic factor and not on its position. Thus the chromomeres in the row just to the left of *b*, are perhaps twice as large in figure 1 as in figure 2, while the difference in band *b* itself must be of the order of 4 or 5 to 1. Similarly, in the row just to the left of band *d*, the chromomeres are not greatly different in size, while in *d* the ratio must be 6 or 8 to 1. Going hand in hand with these size differences in the vesicles we note differences in the way the chromatin is laid down. In figure 2, in the band *b* the chromatin more or less surrounds each vesicle giving a scalloped effect, while in figure 1, the chromatin is confined to the free edges. At *d*, in figure 2, the chromatin in the right hand edge is much heavier than on the left, but in figure 1 there is no sensible difference between the two sides.

The granular form of chromosome is not shown, but it differs from figure 2 in two ways. First, the chromatin appears to be more localized at two sides of the chromomere, just as in band *g* of figure 1 and in the absence of visible vesicular walls we get a scalloped and coarsely granular double band across and through the chromosome. In the second place, we are usually unable to detect any longitudinal fibers in these granular chromosomes; at any rate, there are no prominent threads. This absence of discrete chromomeres and of connecting fibers in granular chromosomes seems to warrant the conclusion that such elements must be regarded as aberrant in type though this may be a normal stage in living chromosomes.

Figure 3 was taken from a slide in which the chromosomes were first preserved in 45 percent acetic acid then spread and treated for about an hour with caustic potash to remove some of the chromatin. The stain used was Delafield's haematoxylin followed by eosin.[2] In the area illustrated the

[2] Salivary glands are fixed for about 10 minutes in 45 percent acetic acid and then placed on a slide and crushed under a coverslip, just as in making an aceto-carmine mount. The coverslip is now removed and after rinsing in water the slide and cover are placed in a .1 percent solution of potassium hydroxide where they are left for 10 minutes to 1½ hours. After washing well, the tissue is stained in Delafield's haematoxylin followed by eosin and mounted in euparal from 95 percent alcohol.

coverglass pressure has separated the homologues for a space but the chromomeres of two bands have stuck together so that those on the edge are greatly drawn out. It should be remembered that when the nucleus was preserved it was intact and that the spreading came afterwards. We do not know, therefore, whether the cohesion between the chromomeres of a band obtains in life, but it is very strong in preserved material. In general, figure 3 gives us the same picture of chromosome organization as figure 1; but the removal of part of the chromatin enables us to see somewhat more clearly the individual chromomeres which go to make up the bands.

In this study we have constantly looked for evidence which would indicate that each of the four chromatids was encased in some sort of pellicle and that lines of tension in this covering produces part of the longitudinal "fibers." It is true that in greatly stretched areas, or areas where the rows of chromomeres are displaced, we find fibers which are not ordinarily seen; but the explanation for this is very simple and obvious, and at no time have we found any evidence for a separate covering.

In figure 1 the threads which appear to connect the chromomeres of adjoining rows are extremely fine and are not easily seen except in subdued light or better still, with oblique illumination. They do not stain appreciably with aceto-carmine, or any of the other stains employed, and their visibility seems to depend more on their refractive properties than on anything else. The fibers shown are only those which lie at the surface; but as one focusses through the chromosome, more come into view until the lower edge of the chromosome is reached. When the chromomeres are in regular rows the fibers are smooth in outline, are about the same thickness, as far as we can tell, and run parallel from one band to the next when the chromomeres of adjacent rows are equal in number. If one examines greatly stretched chromosomes, however, he will find areas where some threads are much thicker than others. We have spent a good deal of time in determining how these thick threads are formed. As it turns out the explanation is simple; stretching or pressing on the chromosomes often displaces the chromomeres of a row so that several of the fine connecting fibers are brought into close apposition, forming a thicker thread.

Figure 5 is a drawing of a portion of a chromosome which was greatly stretched when the nucleus was crushed by coverglass pressure. Note that the chromomeres in the band to the right are not arranged in a regular transverse row but have been crowded into an irregular heap. From this heap three prominent lines arise and pass on the left to another broken-up row of non-vesiculate chromomeres. Many of the latter have been pulled out of line so that several may lie in one longitudinal plane (only surface features are drawn in). Farther to the left the rows of chromomeres are

more regularly arranged and the usual fine parallel fibers predominate. It appears then that when chromomeres are pressed or pulled out of line several of their fibers come into close physical contact and produce thick compound threads not usually seen in undistorted chromosomes.

A similar condition is shown in figure 6. On the right there are two homologues, but these separate and one passes upwards (only the base of it is drawn). The other chromosome runs to the left and is greatly stretched. At the extreme left of the figure we have a heap (disarranged band) of about eight chromomeres. From these arise apparently five fibers which pass to the next disarranged band on the right. One of the fibers bifurcates just before it reaches the latter. From this second clump of chromomeres we see both fine and thicker fibers running on to the right. About the middle of the figure is a short section where the chromomeres are more regularly disposed, and at the extreme right fine lines predominate.

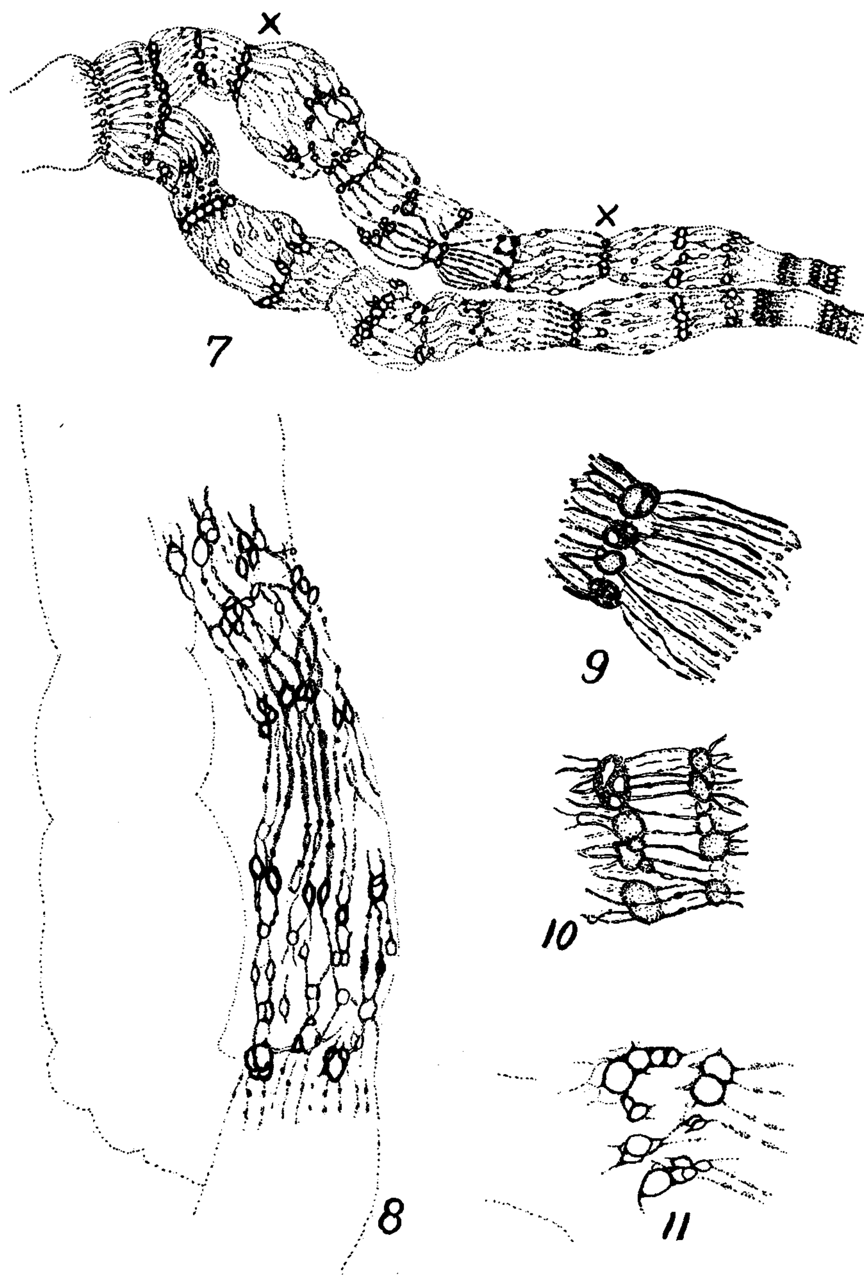
There is another cause for thick fibers, most conspicuously seen in the spread-out region, but occurring here and there in euchromatic areas. It may happen that from some cause a chromomere does not divide (or two or more fuse). From such compound elements small bundles of fibers are seen to arise.

THE STRUCTURE OF THE "SPREAD-OUT" REGION

In the spread-out region of the longest chromosome pair there appears to be little attraction between homologous chromomeres, a fact that is indicated both by a failure of the two homologues to unite in an intimate synapsis at a time when the distal euchromatic parts are closely joined, and by a lack of precision in the arrangement of the parts within a single chromatid. Here like chromomeres often form clumps instead of bands, or they may lie singly and irregularly spaced with regard to the transverse plane. This loose organization is most pronounced in a few segments; but in younger and less differentiated stages the absence of synapsis and of twisting prevents the union of the adjacent euchromatic areas so that it is only in very large elements that this region is sharply delimited.

Figure 7 is drawn at a lower magnification than most of the illustrations and represents a "half-grown" chromosome. One cannot say just where the loose organization of the spread-out area begins and ends but it is pronounced in the region lying between the X's.

Figure 8, drawn at a higher magnification than figure 7, and taken from a fully differentiated chromosome, shows surface details of one chromatid. (The other chromatid is illustrated in figure 18). At the top of figure 8 there are large, more or less oblong chromomeres with prominent vesicles and with chromatic hulls of different thicknesses. These units are inter-



FIGURES 7–11. Spread-out region. Figure 7 is drawn at a low magnification showing how the spread-out region—between the x's—is joined to the euchromatic areas. Figure 8 shows surface details of one chromatid. Figures 9 and 10 illustrate characteristic areas. Figure 11 is a drawing of part of a band which is mashed out.

connected by threads, some very fine, others quite thick and obviously compound. Some of these details can be seen in living chromosomes. About the middle of figure 8 there are thick deeply staining fibers arising from a loose band of thick-walled chromomeres above and passing downward to a more scattered transverse row of thick-walled chromomeres. These fibers seem to be made up of two or more strands, some of which bifurcate at the lower end. Below this conspicuous fibrous area we have again rounded chromomeres, many showing connections with four threads. In spite of the irregular distribution of the parts, one has no difficulty in identifying homologous elements in any general transverse plane.

The heavy fibers illustrated in figure 8 are a prominent feature of the spread-out area and, in well preserved material, at least, seem to possess considerable rigidity. The chromosome sometimes breaks just where the threads bifurcate; then the fibers stand out stiffly, like bristles in a brush. Figure 9 is a drawing illustrating such a case.

Another characteristic area of this general region is shown in figure 10. One notes that homologous chromomeres associate in clumps and vary considerably in size. Thick fibers connect these clumps in a longitudinal plane.

From the foregoing description and the figures it is evident that in the spread-out region we have the same structural elements as in euchromatic parts, namely, chromomeres and connecting fibers. But the association of homologous chromomeres in irregular clumps rather than in regular transverse rows and the apparent irregularity in the fibers, give a different and often confusing picture. Indeed, in very large chromosomes which are not stretched this region sometimes looks as if it were made up of soap bubbles or droplets of material and METZ's concept of the structure would seem to be well founded. But when the same area is elongated—the stretching of a dead and stained chromosome can do nothing more than make it easier to observe the relation of parts—the source of the confusion becomes apparent. In contrast to the euchromatic areas (where the chromomeres of a band are usually about the same size, each with its connecting thread) in the spread-out region there is a great variation in the size of homologous chromomeres. Figure 11 is part of a "band" which was mashed out by coverglass pressure. Note the differences in size of the chromomeres. The same variation can be seen at the bottom of figure 8 and in other parts, as well as in figures 9 and 10. The presence of both large and small units within one clump makes it very probable that the former are essentially compound. This might result from the fusion of two or more chromomeres, or from a failure of a chromomere to undergo fission; or possibly the union is induced by fixation. In any event, assuming that each chromomere unit lies on or is attached to a fiber, we can understand now why some fibers

are very thin while others are very thick and why a number of thin and thick fibers may appear to arise from one giant (compound) chromomere. Add to this condition an irregularity in the placement of homologous chromomeres, due to a lack of attraction perhaps, and we have all the elements needed for an understanding of the images seen under a microscope.

One more point should be stressed. When a clump of homologous chromomeres is preserved, there seems to be a tendency for the chromatin to collect at the periphery of the clump, just as it does in the compact rows in the euchromatic area. Under these conditions the contiguous walls of the vesicles are not easily made out and we observe one enormous vesicle. Sometimes tiny chromatic dots appear within these large vesicles, as BAUER (1935) has noted in the heterochromomeres of Chironomus; but if such heterochromomeres in Chironomus are essentially compound as they are in Simulium, these dots may well be either minute chromatic inclusions between the vesicular walls or portions of these walls viewed from the end.

Thus far we have made no statement about the total number of chromomeres in the bands of fully developed chromosomes, and hence the total number of chromonemata involved. Probably the type of band on which it is safest to make such counts is that containing tiny dot-like chromomeres; yet to enumerate these dots accurately is extremely difficult. From time to time we have estimated the number of chromomeres and have concluded that generally we have in the neighborhood of from 64 to 128 elements.

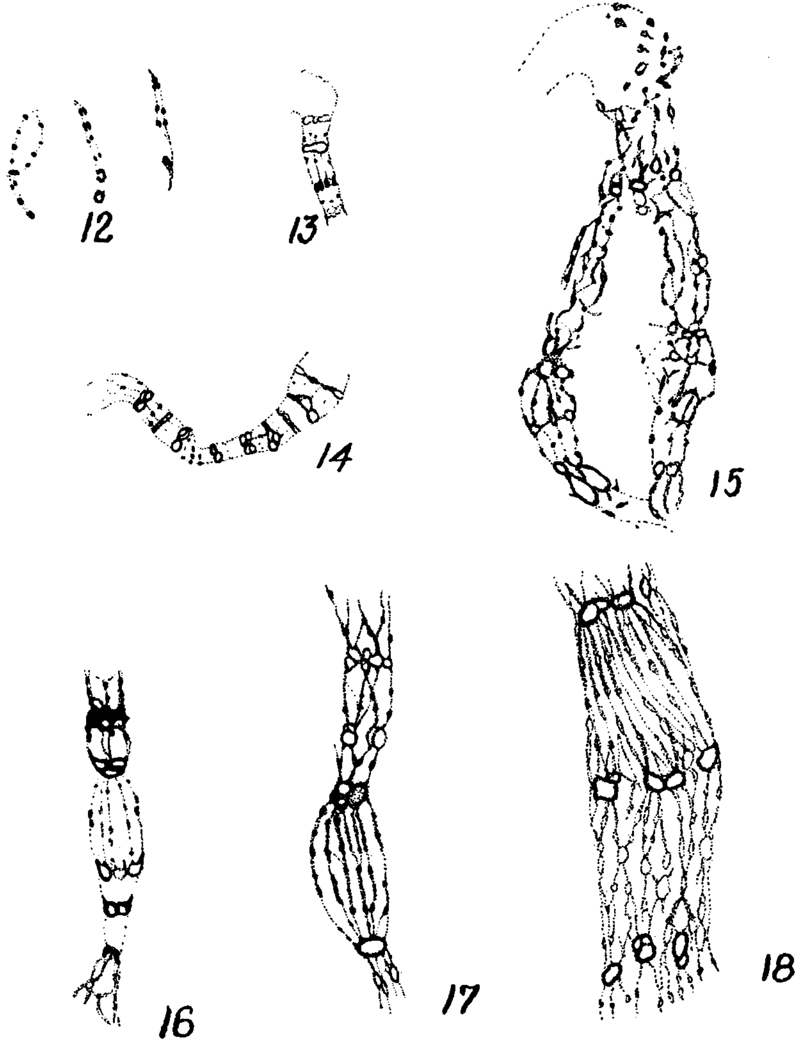
THE DEVELOPMENT OF SALIVARY CHROMOSOMES

Within a single gland there is a considerable variation in nuclear size and in chromosome differentiation, and in the glands of young larvae the differentiation can be followed from the reticular stage. For convenience we will describe the euchromatic and spread-out regions separately.

Euchromatic Area.—In the earliest stage at which we can recognize individual chromosomes, apart from the general reticulum, the nucleus is very small and usually does not rupture with coverglass pressure; hence one can follow single elements only for short distances. Figure 12 shows short sections of such young chromosomes taken from a single nucleus. We observe a series of pairs of chromomeres, part of which are connected linearly by a very fine thread. Most of the chromomeres appear as solid granules and the range in size is not very great. Since we can not recognize and follow all the individual chromosomes we do not know whether we have, at this time, single (split) elements, or two homologues which have united in synapsis. We believe the first is the case because later the twisting of the two sister chromatids is independent of the twisting of the homo-

logues (fig. 4); this is most easily understood if we assume that sister strands unite first.

As the nucleus increases in diameter the chromosomes become broader and longer (figs. 13 and 14), a condition which is obviously due, for the



FIGURES 12–18. Developmental stages. Figure 12 is the earliest stage found for incipient giant chromosomes. Figures 13 and 14 represent a 4-strand stage. Figure 15 is the spread-out region from the same nucleus as that from which figure 14 was taken. Figures 16 to 18 represent stages in the development of the same area.

most part, to an hypertrophy of most of the chromomeres and to the wider separation of the incipient bands. In figure 13 there is a maximum of four chromomeres per row and four threads; although the nucleus from which figure 14 was taken was much larger than that of figure 13, and the chromo-

somes much broader, we still find a maximum of four chromomeres per row. The hypertrophy of the chromomeres and the wider separation of the bands account for the difference in size, as the figures show. In figure 14 we note the further illuminating fact that while there are generally four chromomeres in each row there may be only three when one chromomere is much larger than the other two, or two. An example of the latter is seen in the row just below the bend in the figure. Thus it is apparent that there is some irregularity in the time of chromomeric division and certain chromomeres divide less often or less readily than others. This accounts for the condition observed in the fully grown elements, such as figure 1 or 2, where the band *d* has about half as many chromomeres as band *b* or *c*.

As we have explained, we cannot be absolutely certain that in figures 12 to 14 we are dealing with only one (split) homologue, but we think this is the case. If this is true, then between the stage of figure 12 and figure 13 or 14 there has been only one division of the chromomeres and their associated threads. If, on the other hand, somatic synapsis occurred between figures 12 and 13, no division has intervened.

In figure 6 we have a single homologue (left side of the figure) with about eight chromomeres in each row or band. Note, however, how large many of the chromomeres have become. Obviously the hypertrophy of the chromomeres has continued. Later stages show higher chromomere numbers per row and in the fully differentiated elements, such as figures 1 and 2, we estimate an average of about 64. Since we start with four chromomeric elements (the 4 chromatids) it is necessary to assume only four division cycles to account for the chromonemata observed. Most of the increase in chromosome size and nuclear volume must be due to the great hypertrophy of the chromomeres and to some extent to their greater separation.

Spread-out region.—Figure 15 is taken from the same nucleus as figure 14, and from another part of the same chromosome. Note that the two homologues are widely separated. It is difficult to make out all the fine details in the upper part of the figure, but the lower section represents all the visible elements. Here we are able to observe four chromomeres and threads, the maximum number shown in the euchromatic region (fig. 14); yet the upper part of the right hand homologue suggests that more strands may be present, a possibility which agrees with our general observation that in the spread-out region more elements appear than in euchromatic areas. Figures 16, 17 and 18 represent different stages in the development of the conspicuous fibrous area, which we can recognize very early. In the middle part of figure 16 we observe five longitudinal strands and this probably represents a transition from the four to the eight strand stage. In figure 17 we note an increase in both the length and thickness of the fibers. We can observe only six heavy fibers; but these may be compound,

for one of them bifurcates. At the top of the figure there are at least six chromomeres. Since two are larger than the others, we are safe in assuming that we have about an eight strand stage.

The end stage is represented by figure 18, which is a surface view of a single chromatid. We estimate that there must be in this one chromatid at least sixteen heavy fibers, perhaps more, which would give a total of thirty-two for a single homologue and sixty-four for the pair.

This study of development clearly shows that the great size of the fully "grown" chromosome is due to the reduplication of the original chromomeric gene string three or four times, together with a differential hypertrophy of the chromomeres and some linear extension of the threads (compare threads in figures 16 to 18).

GENERAL DISCUSSION

The bands.—From this study of developmental stages and fully differentiated chromosomes we are now in a position to understand the nature and structure of the bands seen in all salivary gland chromosomes, and to point out the bearing our findings have on cytogenetics.

During the multiplication and hypertrophy of the chromomeres within each of the chromatids, the individual sorts of chromomeres exhibit differences in behavior which account for the various types of bands observed.

1. Some types of chromomeres, usually those which show the greatest hypertrophy, do not separate completely after division, and as the number increases they become packed in transverse rows, the chromatin coming to lie mostly at the free edges where it forms a "double band" as at *b* or *d* in figure 1. There is no evidence that these chromomeres lie only on the periphery of the chromatids; on the contrary, BAUER is entirely right in his contention that the chromomeres extend through the entire diameter of the chromosome. This is plainly seen when in a mashed chromosome the "band" lies at or near a right angle to the plane of observation. The rounded surfaces of the chromomeres remind one of the surface of a honeycomb or a dish of soap bubbles.

Some workers have attempted to correlate somatic synapsis with a two by two pairing of the chromonemata, but there is no need for this assumption and no evidence for it in development. The individual homologues are split before somatic synapsis occurs and afterwards the chromomeres are held in place by the twisted condition of the separate bundles.

2. Some chromomeres divide once or twice and separate completely; then on further multiplication, the progeny of single units adhere forming a cluster of homologous chromomeres. Under these conditions the chromatin tends to lie on the periphery of the cluster, forming one "giant chromo-

mere," when we are unable to distinguish the separate vesicles (as a result of indifferent fixation or great hypertrophy). The best examples of this type of behavior are seen in the spread-out region of Simulium (figs. 8 to 10). When this occurs in the euchromatic area, there result the heavy dash-like bands which are so conspicuous in *D. melanogaster*. Needless to say, by counting these clusters we can arrive at no accurate notion of the number of chromonemata present in an element unless we know how many chromomeres are in each group.

We would interpret the "heterochromomeres" which BAUER finds in Chironomus as such homologous chromomere clusters.

3. Very small chromomeres usually separate completely giving the familiar dot-like bands which may stain deep or faintly depending on the amount of chromatin present in each unit.

Whether a given row of chromomeres will appear as a double, dash- or dot-like band will depend largely on the relative size of the units. It must be remembered that the two sister chromatids are twisted together before hypertrophy has progressed very far and there are longitudinal fibers which would tend to hold the chromomeres in place. After somatic synapsis and the twisting of homologues which accompanies it, there would be further reinforcement so that the opportunity for a movement of the units would be very limited. As growth in the size of the chromomeres and their division goes forward, the largest elements would be tightly pressed together, while the smaller ones would lie separated. If, for some reason, the usual hypertrophy did not occur in a row, the appearance of the band would be greatly altered without any further change.

Most of the bands in Simulium are compound in the sense that more than one row of chromomeres is usually present. That the same condition obtains in other Diptera is not to be doubted. PAINTER recognized the complex nature of the bands of Drosophila in his first studies and later workers have confirmed this. Thus ELLENHORN, PROKOFIEVA and MULLER (1935) were able to show, using ultra-violet light, that the second heavy band in the X chromosome of *D. melanogaster* is composed of at least four rows (discs) of chromomeres. This complex nature of the bands has, of course, a very direct bearing upon a number of the current problems in cytogenetics.

Every one who has worked on *D. melanogaster* salivary chromosomes has doubtless encountered difficulty, at times, in identifying in a given region of a chromosome the bands shown on the chromosome maps of PAINTER or of BRIDGES. Aside from variations due to fixing and staining and incomplete or poor illustrations, different degrees of stretching often entirely alter the appearance of a familiar and a generally conspicuous band. Prolonged study of any restricted area, with many preparations, invariably

shows more bands than we first thought present and, for this reason, the exact localization of gene loci is not as easy as it first appeared. Earlier estimates about the number of genes carried by the species, on the assumption that each band represents a gene, have required revision. Thus BRIDGES' first estimate of some twenty-six hundred has given way to five or six thousand (cited from BAUER 1937) and MULLER and PROKOFIEVA (1936) reckon the number now around ten thousand. Unless we know how many genes are represented, it is hazardous to calculate the size of the individual gene from measurements on salivary chromosomes. In studies dealing with the patterns of related species it is worth while to remember that should a break come between the elements of a compound hand, a familiar landmark would disappear. Also, the failure of similar chromomeres to undergo the same degree of hypertrophy, in two species might be very misleading.

Our observations indicate that one cannot judge the composition of a band unless the individual chromomeres can be seen. A double band may represent one row, as at *b* or *d* in figure 1, or three rows as at *c*, *f* and elsewhere in this figure. In a recent paper, METZ (1937) has pointed out that in a stock of Sciara which has a deficiency in one homologue, only one half of a "double band" is missing; he uses this as an argument against the chromonema theory on the implied assumption that this double band is made up of one row of chromomeres. Needless to say his evidence can not be considered critical until he shows that only one row of chromomeres is involved.

The Chromomeres.—In the youngest stage, the vast majority of the chromomeres appear as solid chromatic granules, not very different in size. But as the chromomeres begin their differential growth or hypertrophy the range of sizes may become very great especially in chromosomes of the large vesicle type. Figure 1A shows, in a semi-diagrammatic way, the apparent structure of twenty-eight different chromomeres. Here we note that all but the smallest are vesiculate, and there is no reason to suppose that they are different in structure. The chromatic hull may be thick or thin, evenly or asymmetrically distributed about the vesicle. The non-staining vesicles, in turn, may be relatively large or small, and when the chromatic hull is very thin, as often obtains in parts of the spread-out region, it may be difficult to distinguish between vesicular and interchromomeric spaces. Since a given chromomere, under the same conditions, generally gives the same form expression, the variations observed must be due to intrinsic and not external causes. The relative amount of chromatin in the vesicular wall, the way it is distributed (aside from external pressure), whether the hypertrophy is limited or marked, all are properties of the particular chromomere concerned. We now turn to discuss some questions which are raised by our observations.

Foremost stands the question, how are the chromomeres observed at very early stages (for example, in figure 12) related to those seen in fully differentiated chromosomes. To answer this question with finality, it would be necessary to make detailed comparisons between whole chromosomes, at the initial and the final stages. This has not proved possible so far. We regard it as very probable, however, that the chromomeres seen at first (fig. 12) are compound, in the sense in which BELLING (1928) used this term, that is, are made up of several different kinds of chromomeres. The simplest way would be to regard the early chromomeres as corresponding to all of the elements of several closely associated bands. Thus in figure 1, there are seven rows of chromomeres closely associated in the region of *b*, and no doubt these would all appear as one in the early stage. Likewise all the chromomeres clustered about rows *e* and *f* would probably look like one large chromomere. As the individual chromomeres of these aggregates begin to grow, they cause the chromosome to increase in length as well as in diameter and the component parts become visible. At this point it is well to remember that in figure 1, the fixed chromosome is stretched and in the living state the bands would be much closer together.

If we have correctly interpreted the facts, the chromomeres of single rows would correspond to the "ultimate" chromomeres which BELLING saw in the liliaceous plants and it is interesting to note that there are several points of similarity in their fundamental structure. The "gene chromatin" is the chromatic hull, and while BELLING does not mention it he shows a nonchromatic space, corresponding to our vesicles, between the rind and the submicroscopic dot which he thought might be a "naked gene." BAUER (1936) has described and figured a number of tiny chromatic dots in the heterochromomeres of Chironomus and we have seen them in large chromomeres (figs. 9 and 10) of Simulium; but these large chromomeres are almost certainly homologous chromomere aggregates and the dots may be simply explained as points where the abutting walls of vesicles enclose bits of chromatin, most of which is pressed to the periphery of the cluster. We may well ask whether the dots seen by BELLING may not be explained in the same way. Not all cytologists will agree that the leptotene threads are single; indeed, there is much cogent evidence to the contrary (NEBEL and RUTTLE 1937).

Another interesting feature of this study is the demonstration that the simple longitudinal division of the original (4) chromatids four times does not account for the enormous increase in chromosome size and nuclear volume. Most of this increase is the result of the hypertrophy of the individual chromomeres. That this hypertrophy is associated with the great secretory activity of these gland cells seems probable but how is the increase in the chromatin and vesicular material brought about? Several possibilities suggest themselves. One might suppose that the large size of

many of the chromomeres was due to the accumulation of some sort of secretory material elaborated by the genes. Or, as seems more probable at present we may say that the hypertrophy is due to a growth or reduplication of the chromomeric substance without a visible separation into smaller units. Several lines of evidence indicate that this may be the explanation. (1) In spite of the great size of Simulium chromosomes, there is evidence for only about 64 chromonemata. In contrast to this, in Chironomus BAUER finds evidence for 350–400 strands. And in *D. melanogaster*, where the number of chromonemata is variously estimated as 16, 32 or even 64, HERTWIG (1935) has calculated that on the basis of nuclear volume, there should be at least 512 strands. These discrepancies can be readily explained if we assume that the chromomeres seen in many forms are really homologous chromomere aggregates and have often high genic valences. In Simulium, in young stages there may be four, three or two chromomeres in adjacent rows of the same chromosome. If there are three, one is characteristically larger than the other two, indicating a growth of the chromomeric substance without a division. And elsewhere we find abundant evidence that many of the chromomeres have higher valences than one. Furthermore, the longitudinal threads seen connecting the chromomeres in fully developed chromosomes generally appear tubular, a fact which we can well understand if they really represent bundles of thinner strands.

We do not propose to discuss, in detail, METZ's ideas of chromosome organization and the objections he has raised to the chromonema concept. Many points have been covered in the body of this paper, and the evidence from the ontogenetic stages leaves little room for doubting the correctness of the original suggestion of BRIDGES and of KOLTZOFF. We follow the current practice of calling the separate longitudinal elements, chromonemata, not in the first sense of the word "chromonema," implying as it does a more or less uniformly thick thread, but rather as the separate units (daughter gene strings) which are found within one apparently single chromosome. In reality, these gene strings have a chromomeric structure and we believe this must be considered the fundamental structural organization of all chromosomes, other conditions being derived from this.

Morphologically a chromosome consists of chromomeres which are held together by a longitudinal filamentous strand of protoplasm; the chromomeres consist of an outer covering of chromatin and an inner space filled with achromatic material. This concept is not new; the thread, the chromatic rind and the vesicle have all been described and figured repeatedly. Neither the thread nor the vesicular material stains appreciably with aceto-carmine, and whether the thread is really separate from, or a prolongation of, the vesicle must remain an open question.

New light has recently been thrown on the physical and chemical nature

of chromomeric chromosomes by CASPERSSON (1936), who has approached the subject from a different angle. CASPERSSON developed and applied to the salivary chromosomes of Chironomus a technique for the study of the chromatin, involving the use of ultra-violet absorption curves and a special enzyme which digests away the proteins, leaving the chromatin as an insoluble lanthanum salt. He finds that the chromatin is in the form of nucleic acid. When the proteolytic enzyme is used, the first part of the salivary chromosome to be affected is the thread region which quickly dissolves, indicating that these strands are protein in nature. Initially the vesicular material of the chromomeres is unaffected, but on prolonged digestion this material also dissolves indicating that it too is protein. On the basis of CASPERSSON's work it appears that we have three types of substances in the salivary gland chromosomes, a conclusion which is in complete agreement with the cytological picture in Drosophila (BRIDGES and others), Chironomus (BAUER) and Simulium. Of course it is possible that the thread and vesicular material may be one and the same substance, for naturally an extremely thin thread of protein would be more quickly affected than the relatively massive vesicles.

We must now consider the question "Where is the locus of the gene?"—a problem necessarily bound up with the ultimate structure of the chromosome. Some progress may be claimed for the present study, not in that the individual facts are new, but rather because Simulium chromosomes are so favorable for study that the chromomeric nature of chromosomes rests on a broader and firmer foundation, the essential morphological parts or components standing out, perhaps, in sharper focus. With one notable exception (METZ) most cytogeneticists who have recently considered the gene's locus have assumed a chromomeric organization, and we find expression of the opinion that (a) the genes are represented by the chromomeres in some way; (b) that the interchromomeric threads contain the genes; and (c) that the whole chromosome is made up of genes end to end, there being no intergenic material; in short, that both the thread and the chromomere may represent a gene complex. A brief review of these ideas will serve to give the status of the question at present.

While many cytologists have doubtless thought of the chromomeres as representing the genes, BELLING brought this view into the foreground, from his analysis of liliaceous plants and his concept of an "ultimate chromomere." Of course, BELLING had no real evidence that the chromomeres contained the genes except that the genetic linear order of the genes had a striking counterpart in the linear differentiations of the chromosome. When later it was found that definite chromomeres along the salivary gland chromosomes of *D. melanogaster* could be associated with definite genes, PAINTER regarded the chromomeres as the probable locus of the

genes, a view subsequently shared by many Drosophila workers. But it was, and is, realized that the evidence is not unequivocal because the absence of a band, and with it a gene, does not exclude the possibility that the gene lies outside of the chromomere and on the thread, part of which is removed with the band. In view of our experience with Simulium we may well question whether any of the deletions studied by Drosophila workers consist of a single row of chromomeres. Rather it is the fact that the chromomeres are arranged in a linear order and that they alone show great qualitative differences, such as one might expect from the varied nature of the genes, which has caused the Drosophila workers to look to the chromomere as the gene's home. It is interesting to note that CASPERSSON has been led to the same general point of view, for while he thinks that the protein of the thread might represent enough varieties of molecules to account for the genes, he points out that the compounds which could be formed within the chromomeres, between the protein and the nucleic acid, would give a much wider range and variety of chemical combinations; for this reason the chromomere might be regarded as the more probable locus of the gene.

The view that it may be the achromatic portion of the chromosome which carries the gene has been advocated by KOLTZOFF (1934) and seems to be favored by METZ. Here let it be noted that KOLTZOFF referred to the interchromomeric threads, while METZ, who envisions another type of chromosome structure than that accepted here, would include both the thread and the achromatic portion of the vesicles, in terms of the present paper. We think that cytogeneticists generally recognize the possibility that the genes might lie between the chromomeres, but at present this seems less probable.

From a theoretical genetic standpoint MULLER has pointed out that a chromosome might be made up of a chain of genes linked end to end without any intergenic substance or thread. The same sort of structure is implied by WRINCH'S (1935) theoretical concept of salivary gland chromosomes. The latter writer approaches the problem from the standpoint of molecular structure and pictures the chromosome as made up of amino acid units linked in linear order. Some of these units are basic; and to these the nucleic acid molecules become attached, forming the chromatic deposits or bands. When the amino acid unit is not basic, no nucleic acid will be deposited. The genes, being specified as amino acid units, may lie either in the banded or in the achromatic (thread) region.

From this brief review, it is evident that while there is as yet no unanimity of opinion about the gene's locus, nevertheless we are passing from the theoretical speculative stage to a vigorous practical attack upon

the problem from a number of different angles, an attack made possible by a better understanding of chromosome structure.

LITERATURE CITED

BAUER, H., 1935 Der Aufbau der Chromosomen aus den Speicheldrüsen von *Chironomous thummi* Kieffer. Zeit. f. Zellforsch. u. mikr. Anat., **23**: 280–313

1936 Beiträge zur vergleichenden Morphologie der Speicheldrüsenchromosomen. Zool. Jahrb., **56**: 239–276.

1937 Cytogenetik. Fortsch. d. Zool. **1**: 521–538.

BELLING, J., 1928 The ultimate chromomeres in *Lilium aloe* with regard to the number of genes. Univ. of Cal. Publ. in Bot., **14**: 307–318.

1931 Chromomeres of Liliaceous plants. Univ. of Cal. Publ. **16**: 153–170.

BRIDGES, C. B., 1934 The structure of salivary chromosomes and the relation of the banding to the genes. Amer. Nat., **69**: 59.

1935 Salivary chromosome maps. J. Hered. **26**: 60–64.

CASPERSSON, T., 1936 Ueber den chemischen Aufbau der Strukturen des Zellkernes. Skand. Archiv f. Phys. Suppl. to Vol. **73**: 1–151.

DOYLE, W. L., AND METZ, C. W., 1935 Structure of the chromosomes in the salivary glands of *Sciara*. Biol. Bull. **69**: 126–135.

ELLENHORN, J., PROKOFIEVA, A., AND MULLER, H. J., 1935 The optical dissociation of Drosophila chromomeres by means of ultraviolet light. C. R. Acad. Sci., U.S.S.R. **1**: 238–241.

GEITLER, L., 1934 Die Schleifenkerne von Simulium. Zool. Jahrb. **54**: 237–248

1935 Neuste Forschungen ueber der Chromosomenbau. Der Züchter, **7**: 294–304.

HERTWIG, G., 1935 Die Vielwertigkeit der Speicheldrüsenkerne und-chromosomen bei *Drosophila melanogaster*. Z.i.A.V., **70**: 496–501.

KOLTZOFF, N., 1934 The structure of the chromosomes in the salivary glands of Drosophila. Science **80**: 312.

METZ, C. W., 1935 Structure of the salivary gland chromosomes in *Sciara*. J. Hered. **26**: 177–188.

1937 Deficiencies and structural variation within the giant chromosomes in relation to the problem of gene structure. Proc. Nat. Acad. Sci., **80**: 595–596.

METZ, C. W., AND GAY, E. H., 1934 Organization of salivary gland chromosomes in Sciara in relation to genes. Proc. Nat. Acad. Sci., **20**: 617–621.

METZ, C. W., AND LAWRENCE, E. G., 1937 Studies on the organization of the giant gland chromosomes of Diptera. Quart. Jour. Biol. **12**: 135–151.

MULLER, H. J., AND PROKOFIEVA, A., 1936 The individual gene in relation to the chromomere and the chromosome. Proc. Nat. Acad. Sci., **21**: 16.

NEBEL, B. R., AND RUTTLE, M. L., 1937 Chromosome structure XIII. Zeit. f. Zellforsch. u. mikr. Anat., **26**: 281–292.

PAINTER, T. S., 1934 A new method for the study of chromosome aberrations and the plotting of chromosome maps in *Drosophila melanogaster*. Genetics **19**: 175–188.

1934 Salivary chromosomes and the attack on the gene. J. Hered. **25**: 465–476.

PAINTER, T. S., AND GRIFFEN, ALLEN B., 1937 The origin and structure of the salivary gland chromosomes in *Simulium virgatum*. (abstract). Genetics **21**: 202–203.

WRINCH, D. M., 1935 Chromosomes and molecular aggregates. Proc. Internat. Congr. Bot., Amsterdam, **2**: 24–26.