The Chromosomes in the Nurse-cells of *Drosophila melanogaster*

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In the course of a study on the vitellogenesis in *Drosophila*, one of us noticed certain conditions in the nurse-cell chromosomes which appeared at variance with those reported by Painter and Reindorp ('39), and which would therefore lead to a different conclusion from that put forward by them. We have since made slides with many different methods. Observations made on these slides have convinced us that neither endomitosis as first defined by Geitler ('38), nor simple reduplication of chromosomes occurs in the trophocytes of *Drosophila* ovary. We believe our observations warrant a brief report.

Material and methods

The flies used in this work were of the same stock and were raised under the same cultural conditions as those used by one of us in tracing the history of the cytoplasmic elements during vitellogenesis (Hsu, '52).

Ovaries of young flies not older than 24 hours after hatching were dissected out in a drop of physiological saline and immediately immersed in various fixatives or a drop of aceto-carmin or aceto-orcin. The ovaries were fixed for 3/4 of an hour, hardened in 1% chromic acid for 2 hours, and washed in running tap-water for 4 hours. Following all fixatives, N-butyl alcohol was used for dehydration and clearing. The fixatives used were Nawashin, San Felice, and Flemming and Bouin and their various modifications. But of them all, the mixture of La Cour known as "La Cour 2B" gave the best fixation of the chromosomes at all stages of development. Apparently, the osmotic pressure of the fixing fluid is a very important factor in obtaining good fixation in our material. The virtue of La Cour 2B as a fixative became unmistakable when materials treated in it were compared with those fixed in other fluids. The true chromosome condition such as illustrated in figures 2, 6, 7a, and 12 simply could not be observed with any reasonable degree of certainty in cells fixed in fluids other than La Cour 2B. Nawashin's mixture, for instance, shares with the others the troublesome effect of causing the nuclear content as a whole to contract, drawing away from the nuclear membrane. In material thus affected, it is difficult to observe the minute details in the chromosomes.

For staining, Fe-haematoxyline proved almost useless for this work because of the intense basophilily of the cytoplasm. This stain did not turn out satisfactorily for us even after treating the sections in turpentine and ammonium persulphate as recommended by Guyénot and Naville ('33). Feulgen reaction technique, however, has yielded excellent slides. For sections 5 or 12 μ thick, 15–20 minutes hydrolysis in HCl and 1–1/2 hours in Schiff's reagent gave the best result. If slides were then counter-stained in lightgreen, nucleoli could be clearly demonstrated.

It was difficult to obtain good slides by means of the aceto-carmine or aceto-orcein squash technique. But since it would be definitely desirable to have for examination whole nuclei and to have the chromosome or nuclei somewhat swollen and, as a result of squashing, more spread out than possible in paraffin sections, many aceto-carmine and aceto-orcein squash slides were made. Ovaries were left in a drop of either aceto-carmine or aceto-orcein for about 1 hour and then squashed under the flat and smooth end of a brass rod. After applying the cover-glass, more pressure was exerted on the material. If upon immediate examination a slide was found satisfactory, it was made permanent according to McClintock's method. For those nuclei having attained the size of more than 25 μ , squashed material is useless because the thickness of the cytoplasm of the nurse-cells themselves and of the follicle cells interferes with accurate observation. If violent pressure were used to squeeze the nuclei out of the cells, they invariably become injured to such an extent as to be useless for study.

The nurse-cell chromosomes seem to be capricious in their reaction to aceto-carmine and aceto-orcein. But from a large number of slides made, many were obtained in which chromosomes did come out reasonably well-stained. In such slides the early stages of chromosome change could be easily observed in the smaller cells, especially if a green filter was employed.

Observations

In most of the youngest nurse-cells we could find, the chromosomes appeared as 5 threads, each connected to the single nucleolus by a very fine segment (fig. 1). The nucleolus at this stage of development was always seen appressed to the inner wall of the nuclear membrane. In a number of nuclei, however, a sixth and comparatively much shorter thread could be seen (fig. 2).

Early in our investigation we were led to the belief, which later observations have confirmed, that the 6 threads, all connected to the single nucleolus, are not just 6 simple chromosomes but are compound structures. It seemed to us clear that the members of each homologous pair in the very young nurses are in synaptic union. We also have

evidence, which will be presented in the following paragraph, to believe that the synaptic mates even at such an early stage as shown in figure 3 are relationally coiled around each other. The 6 threads as shown in figure 2 can therefore be identified with the 4 pairs of chromosomes in *D. melanogaster* as follows: the very short thread is the synapsed 4th pair; one of the 5 longer threads is a pair of x-chromosomes; and the

Figs. 1-20. Camera lucida drawings of the nuclei of nurse-cells: 7a, 8a, and 9a are free-hand enlargements of a single unit in figs. 7, 8, and 9 respectively; 1, 2, 3, 4, 5, 6, 7, 12, 13, 18, 19, and 20 from La Cour-Feulgen material; 8 and 11 from Acetoorcein material; 9, 10, 14, 15, 16 and 17 from aceto-carmin material. 20, portion of a nucleus showing chromosomes, Feulgen-positive material (heavy dots), fuzzy fibres, and nucleoli (stippled areas). For explanation of the other figures, see text.

remaining 4 threads are the right and left arms of the synapsed 2nd and 3rd pairs. The thinner segment of each thread, though that of the short one has never been actually seen, is the heteropycnotic segment known to exist near the centromere. That it is thinner than the euchromatic section may be accounted for by its being relatively slower in nucleination and splitting (Schultz, '41). The centromeres of the chromosomes are all either embedded in the single nucleolus or in very close contact with it. We are inclined to believe that the relative position of the synapsed chromosomes shown in figures 2 and 3 is the one in which they found themselves when they reached the pole in the previous telophase (Smith, '42).

In stages such as illustrated in figures 1 and 2, it is difficult to see evidence of duality in each thread. But in a slightly older stage, the duality in each is unmistakable. Figure 3 shows the two arms of a pair of synapsed long chromosomes. The heterochromatic section of one of the arms reveals particularly well its two components. And the duality of one arm of a pair of long chromosomes and that of both arms of another pair are clearly shown in figures 4 and 5 respectively. In stages shown in figures 1, 2, and 3, it is difficult to say definitely if the two chromosomes of a pair form a relational coil or are simply held together parallel to each other throughout their length. But that the homologous chromosomes do very early in the development of the nurses coil around each other relationally is shown in the two pairs of chromosomes separately illustrated in figures 6 and 7a. The relational coiling in the thin part of the pair is very clear in figure 6; while in the pair represented in figure 7a, it is in evidence in both the hetero- and euchromatic sections. Figure 7a has additional interest because we believe it to be a pair of x-chromosomes.

So long as the threads appear as elongated structures, the conditions as shown in figure 5 may be considered as the last step of this stage of development. Here the homologues remain synapsed, and each arm is about twice as thick as it appears in the earlier stages. It will be presently shown that this thickness is probably due to the individual chromosomes having split into two chromatids, and probably also due to the fact that there has occurred by this time a considerable growth in length on the part of the chromatids.

In the next phase of development, we find 6 bulb-like structures instead of the 6 threads seen in earlier stages (figs. 8 and 9). Four of the bulbs occur in 2 pairs. They are the 2 pairs of long chromosomes. The other two bulbs are single units—one big and one small: they are the x's and the 4th pair respectively. In most material, whether of paraffin or squash technique, the true chromosome condition in such bulbs is difficult to make out. In most of the fairly good squashed material,

however, a bulb would appear to have a vacuolated structure (fig. 9a); in others and in all paraffin sections, each bulb seems to be composed of a ball of chromatic threads embedded in an achromatic matrix (fig. 10). In very favorable material, however, the true situation in each bulb is seen as shown in figures 8a, 11 and 12. Figure 11 is from an aceto-orcein slide. Due to the fixative and the extreme flattening, one of the bulbs shows four separate wavy lines embedded in, as far as could be determined, a common matrix. We take this to mean that each homologous chromosome has split into two chromatids. Figure 8a brings out the situation even more instructively. In this bulb, it is very evident that it contains the segment of two pairs of chromatids, the bulb being one arm of a pair of synapsed homologues. Figure 12 shows only four of the six "bundles" or loose balls of threads, each of which would appear like a "bulb", if the fine inner structure were not revealed. The one which we believe to be a pair of x-chromosomes, deserves a more detailed description. The nucleus which contains it was found in La Cour-Feulgen material. Its chromosomes are in a later stage of development than those shown in figure 8a, but this pair of x-chromosomes was clearly seen as opening out in the same way as the chromosome arm shown in figure 8a, i. e., it is the section between the distal ends and the centromeres of the two homologues that open out first. We wish to emphasize the fact that from figures 8a and 12 it is clear that the transformation into a bulb-like structure from an elongated one is largely the result of the particular way of opening out of the whole homologous chromosomes (fig. 12) or the homologous arms of a pair of them (fig 8a); a bulb-like structure is not the result of repeated chromosome reduplication. It will be noticed also that each x-chromosome in figure 12 does not reveal duality, probably due to the method of preparation. In view of the fact that we have seen chromosomes already split into two chromatids in stages much earlier (fig. 11) than this, we assume that each chromosome of the pair under discussion must also have two chromatids, though this condition was not visible. Two more points about the chromosomes at this stage of development are interesting and both are convincingly evident in this pair of x-chromosomes. First, the chromosomes have increased considerably in length, and second, this increase in length seems to be largely confined to what we regard as the euchromatic section: the heterochromatic section of the two chromosomes remain thin, short, and visibly free from any twisting or coiling. At least, it does not appear from our slides that the increase in length, if any, in the heterochromatic segment is anywhere comparable to that which occurs in the euchromatic segment.

We mentioned in the foregoing paragraph that the heterochromatic segments do not appear to increase in length—at least, not as much as the euchromatic section. This conclusion is based on the observation that

the heterochromatic segments show hardly any coiling and twisting: they remain short and thin. The comparative thinness of the heterochromatic section suggests the question that, if it does not increase in length as fast or as early as the euchromatic section, does it also remain unsplit up to this time? In figures 8a, 11 and 12, the heterochromatic sections do not seem to have divided, though in figures 8a and 11 the euchromatic sections do show duality. However, we did often observe duality also in the heterochromatic segments very early in development (fig. 10). It would seem then that up to this stage of development, the only observable difference in changes between the hetero- and euchromatic segments of a chromosome seems to be one of increase in length, with the euchromatic outdoing the heterochromatic segment.

After the chromosomes have reached such a stage of development as shown in figure 12, they seem to be capable of following either one of two ways in reaching a state of reticulation. These two ways involve the same series of changes on the part of chromosomes, but differ from each other in the order in which these changes take place. These changes are: 1) the chromosomes continue to acquire length; 2) concurrent with this increase in length, the homologous chromosomes become farther and farther separated from each other and spread out more and more evenly within the nucleus; and 3) anastomoses appear between chromosomes, which, together with the distribution of the chromosomes, give to the nucleus a reticular appearance.

In the first way, which may be called a short-cut, the production of the anastomoses and, to a less extent, the separation and the spreading out of homologous chromosomes occur before a certain degree of growth in length on the part of the chromosome has been obtained, so that a nucleus may acquire a reticular structure when it is yet comparatively small. Figures 13 and 14 illustrate the conditions of two nuclei taking the short way to reticulation. From a condition shown in figure 12, the chromosomes immediately uncoil and untwist so as to form six loose balls or bundles of threads, and very soon realize a typical reticular condition by developing anastomoses and by further separation and spreading out of the whole homologous chromosomes, each possessing two chromatids. Figure 14 taken from a carmine-squash slide shows six units—one small and five big more or less connected bundles. A nucleus in a reticular condition may be seen, though rarely, in a very young cyst in which all other nuclei are yet in the condition represented in figure 12. In fact, figures 12 and 13 were taken from two nuclei lying side-by-side in the same cyst. So far as we could ascertain, the reticular nuclei developed thus early would remain in this condition; and further changes would presumably consist in enlargement only as a result, at least in part, of further proportional increase in length of the chromosomes.

The second and longer route which a nucleus in the condition shown in figure 12 can take to attain reticulation differs from the first in that here the anastomoses are not developed until the chromosomes have gone past the stage wherein the very much elongated chromosomes appear to be already fairly evenly distributed within the nucleus. Figures 15, 16 and 17 are all from carmine-squash material, and may be taken to represent three successive stages of growth and separation and spreading out of chromosomes. It will be noticed that no anastomoses have yet developed in any of the nuclei. Since no more than four bundles could be made out in any of them we must mention the fact that at this stage of development it is difficult to separate each individual group from its near neighbor. The significant thing is that we have never encountered any nucleus with more than six bundles. This is to assume, however, that each pair of the long chromosomes, being yet held tight at the centromeres, would form two groups. But it is also possible that the heterochromatic sections have already advanced sufficiently in nucleination so that each pair of long chromosomes appears as one group of threads. Then each group would simply represent one pair of chromosomes (fig. 16). It should be emphasized that careful examination of many nuclei at the stages represented by figures 15, 16 and 17 has not yielded any evidence that these bundles are made up of many short individual chromosomes. On the contrary, we are forced to conclude that they are just a few very long chromosomes coiled upon their own length like ropes. By careful focussing we could usually follow out a segment of one of the threads to a length two or three times that of the chromosome depicted in figure 4. The length which we could thus trace with confidence on one of the threads at this stage was probably not the whole length of it, since we often ran into a spot where it was difficult for us to decide whether we had come to the end of a chromosome or whether it represented in reality a point where one chromosome was in contact with another in such a way as to make it unsafe to consider the section farther on from there as a part of the chromosome under examination. What to us is significant is that we could often trace a thread to a length much greater than that of the one illustrated in figure 6, for instance; and if we consider the length which we could trace with confidence as belonging to one chromosome, we just could not see enough thread-length in the nucleus to make up for what would be required for more than eight chromosomes, even though two of the eight are very short ones. These observations were done on squashed material, and therefore we were studying whole nuclei and not sections of one. We feel confident that no considerable number of threads could have escaped our attention. The nucleus depicted in figure 16 is from carmine-squash material and measures about 24μ in diameter. If we take its diameter to be roughly only 12μ , had it gone

through ordinary fixation and paraffin embedding, then according to Painter and Reindorp's calculation based upon their interpretation, each "aggregation" should have 32 chromatids. But among the many whole nuclei of this size studied we never met with a single one in which such a high number of chromatids was anywhere near being reasonably evident. On the contrary, we could only trace out about eight separate lengths in each nucleus. The chromosomes depicted in figure 16 are interesting also because they clearly show duality. This degree of distinctness in duality in each thread was not observed in every nucleus, though we have seen it sufficient times to be certain that each chromosome possesses two chromatids all the way from the bulb stage of development to what we must consider the peak of development.

Figure 18 shows a nucleus from La Cour-Feulgen material. It measures about 13μ in diameter. For this one also, 32 chromatids would be required if chromosome number doubling is to account for the nuclear volume increase. But it was difficult to make out even 16 separate units. In this figure not all the length of each chromosome is represented. It is interesting in that it shows that homologous members have completely parted company from each other and that all the chromosomes are evenly distributed within the nucleus, though no anastomoses could yet be seen. So far as even distribution of chromosomes is concerned, this nucleus is of a stage later in development than that shown in figure 17. The smaller size of figure 18, as compared to figure 17, must be partly due to the method of preparation of the original; but it should also be borne in mind that there is really no absolute correlation between the size of the nucleus and the stage of development of its chromosomes. The next stage is shown in figure 19 in which anastomoses are shown to have developed between chromosomes. This figure reproduces only an optical section of the nucleus, and no attempt was made to include all the length of any chromosome which could be observed. The individual segments in the figure represent only sections of much longer structures. In thus simplifying the drawing, it was easier to bring out the anastomoses. Further development from here would lead to a reticular condition shown in figure 20. When a nucleus has attained such a stage of development, chromosome threads are no longer stainable by Feulgen technique; but Feulgen-positive material in the form of lumps or droplets may be seen within the meshes of the reticulum or on the surface of the nucleolar masses.

Whether a nucleus follows the short or the long route to reach the reticular stage of development, it seems that growth in volume of the nucleus does not stop with the attainment of that condition. The reticular nucleus of the size of about 40μ represented in figure 20, for instance, would keep on increasing in volume until it measures about 50μ in diameter.

Long before a nucleus of La Cour-Feulgen material has attained its full growth, the chromosomes in most cases show very clear duality, and the anastomoses are no longer visible. But at this stage, very fuzzy fibers could be seen in the nuclear sap (fig. 20). They are much smaller in diameter than the chromatids and reveal no visible evidence of duality, and they are therefore easily distinguishable from the chromatids. Their origin and possible rôle in vitellogenesis are now under investigation.

Discussion

Painter and Reindorp ('39) reported findings regarding the chromosomes in the nurse-cells of *D. melanogaster* which led them to the conclusion that the nuclei of these cells in the course of their development from the youngest phase to maturity go through a number of endomitotic cycles. Relying upon volume studies of their own and those by Hertwig ('35), they believed that the number of endomitotic divisions would probably be eight in order to account for the difference in size between the oogonial and the largest nurse nuclei observed, and therefore, "that the largest nuclei are at least 512-ploid".

Probably due to the fixative, Nawashin's fluid, which they used, and due to the lack of aceto-carmin and aceto-orcein squash material, they either entirely missed or obtained only more or less distorted pictures of such phases of chromosome changes as are represented in our figures 1-14. Since they missed the crucial facts that the homologous chromosomes in the young nurses are in very close synaptic union and that the centromeres are either embedded in, or more probably, in close contact with the single nucleolus, they interpreted each unit in their figures as one single chromosome or a bundle of chromatids from one original chromosome. We have determined, however, that each unit represents either a pair of homologous chromosomes or one arm of a pair. They estimated the coiled threads seen in each of the units in their figure 2 to be about eight. According to them then, each chromosome at that phase of development has already split three times, counting the one which occurred in the previous telophase. Even if the number of coiled threads is accepted to be eight, our finding that each unit seen in their figure is really a pair of chromosomes or one arm of a synapsed pair would reduce the number of time of splitting at the stage of development represented in their figure 2 from three to two. But we have also established the fact that even at the "bulb" stage (figs. 8 and 11),—a stage later than that represented in their figure 2 and probably corresponding to the stage of their figure 9—each unit has only four threads. Since each unit is really a synapsed pair, each single chromosome therefore has split, up to the "bulb" stage, only once. If this doubling is regarded as having taken place during the telophase of the previous division cycle, we may say

that up to the time of development under discussion, no splitting of chromosome has occurred in the nurse-cells. Yet on the strength of volume increase from the oogonial stage to the time shown in their figure 9, Painter and Reindorp concluded that there had been four division cycles and that there should be a maximum of 32 chromatids in each "aggregation" in that figure. Since we could see only four chromatids to each of their "aggregations", which we believe to correspond to our "bulbs", we cannot help doubting that the largest nuclei are 512-ploid even if we assume that endomitosis does take place from that stage on (their figure 9) to the largest nuclei as many times as the volume differences would require, since the doubling would have to begin with four instead of thirty two chromatids for each aggregation. But the chromosome changes observed by us in cells of later growth also do not yield evidence of endomitosis or simple reduplication. The changes seen in the nurses, from the youngest to the oldest, form a linear series, although a nucleus, in realizing a reticular condition from one such as shown in figure 12 may take a short-cut somewhere between stages represented by figure 12 and figure 17 respectively.

We have not seen any evidence of chromosome reduplication such as the falling apart of polytene complexes preceding metaphase as reported by Berger ('38) and by Grell ('46) in *Culex*, or as reported by Gentcheff and Gustafson ('39) in *Spinacia*. We have also failed in observing any evidence of endomitosis such as reported by Geitler ('38) in *Gerris*, by Witkus ('45) in the tapetal cells in *Spinacia oleracea*, and by Lipp ('53) in the trichogen cells in *Corixa punctata*. Bauer ('38) reported haploid number of giant chromosomes in the nurse-cells of the diptera, *Lacilia caesar*, *Pollenia atramentaria*, *Musca domestica*. These giant chromosomes later separate into a number of rod-like chromosomes which despiralize into fine threads, and these, through their confused spreading out and disposition within the nucleus, give to it a reticular structure. He also pointed out that reticulation may not be necessarily preceded by rod-like chromosome formation. It may take place directly through the loosening of the giant chromosomes at the stage of maximum despiralization, or during any of the condensing phases. In our material, we have confirmed Baur's report on two points: 1) synapsis of the homologues, and 2) that reticulation may start from many different points of chromosome development. But we cannot say we have seen anything which could be called "giant chromosomes", nor have we seen any cross-lines on any of the chromosomes. Likewise, we have seen nothing that could be interpreted as a chromosome falling apart into many little ones. We can only believe, on the strength of our own findings, that as the nucleus grows in volume, there is a proportionally enormous increase in length on the part of the chromosomes, each of which is composed of two chromatids. The course of development

is linear but not cyclic, though a short-cut may be taken. An additional support of the linear development idea is found in the fact that the nucleolus in the nurse-cells persists in nuclei of all ages and, in fact, increases in mass as the nucleus grows instead of waxes and wanes as would be expected if there were endomitosis and as Geitler ('38) did report in his study.

We feel that the nurse-cells in *Drosophila*, instead of being examples of the result of endomitosis or simple reduplication of the original diploid number of chromosomes, are really cells of a nature intermediate between that of a germinal cell and a somatic one. Due to their immediate origin and very probably also their immediate environment, the nurse-cells, being sisters to the oocyte, must remain for sometime under the influence of whatever factors which may operate to make the oocyte an oocyte. The homologues not only synapsize, as they would in meiotic cells, but the relational coiling about each other, the splitting of each into two chromatids, and the opening out of the homologous pair, are also reproduced in such cells. Although we cannot say we have seen any chiasma, the peculiar opening out of the two homologues as shown in figures 8a and 12 is very interesting. This type of opening out of either two whole homologues, two x-chromosomes, for instance, or homologous segments of a pair of long chromosomes is very characteristic in our material. It reminds one very much of a meiotic bivalent in which the chiasmata have been terminalized. In a bonafide oocyte, meiotic division I would follow this stage. But in the nurse-cells the meiotic processes seem to be shunted off their course at this point. As a consequence, the homologous chromosomes just fall apart and spread out evenly in the nucleus, and, by developing anastomoses, give to the nucleus a reticular structure. The double or apparently single threads shown in figure 20 we interpret to be segments of enormously elongated chromosomes. In nuclei even larger in diameter than the one illustrated in figure 20, the chromosome condition remains unchanged except that the space between the double or sometimes apparently single threads are much wider and that the segments can be followed to a much greater length.

We are inclined to attribute the enormous increase in nuclear volume, at least in part, to the tremendous increases in chromosome length; the very great increase in the nucleolar mass as the nurse-cells grow in size must also be taken into account. Increase in chromosome number either by way of endomitosis or by simple chromosome reduplication is perhaps not the only concomitant to an increase in nuclear volume. Schrader and Leuchtenberger ('50) have shown that the total protein increase in the nuclei of various sizes characterizing the different lobes of the testes of *Arvelius albopunctatus* corresponds to the increase in their volume, but DNA remain approximately constant in all. We are tempted to quote

Ris ('51); "In cells with intense metabolic activity the nonhistone protein fraction of the chromosomes increases and DNA remains unchanged. This is accompanied by a lengthwise growth of the chromonemata, an increase in nuclear volumes, and, because of the dilution of DNA, a decrease in the intensity of the Feulgen reaction and basic staining". In this connection, it may also be mentioned that just what is the relation between this tremendous increase in length of the chromosomes in the *Drosophila* nurse-cells and the enormous increase in volume of the chromosome and the profuse development of loops by the chromonemata in the growing eggs of *Amphibia* poses a very interesting and important question.

Summary

1. Chromosome changes observed in the nurse-cells of *Drosophila* ovary do not indicate that endomitosis or simple chromosome reduplication occurs in these cells. The increase in volume which their nuclei exhibit is probably due to an increase in length rather than in number of chromosomes.
2. It is evident that up to a certain stage of development of the nurse-cells their chromosomes behave quite like those in meiotic cells: the homologues synapsize and relationally coil about each other, and then each split into two chromatids. It is suspected that chiasma-formation may also occur. Nothing like an actual meiotic division I, however, has been observed. After the homologues have freed from each other, reticulation of the nucleus follows sooner or later. An increase in nucleolar mass accompanies all these changes.

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