DISTRIBUTION OF DNA IN HETEROCHROMATIN AND EUCHROMATIN OF POLYTENE NUCLEI OF *DROSOPHILA HYDEI*

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THE concept that all cells of an organism contain the same quantity of DNA or a geometric multiple of it has been demonstrated in a large number of cases (SWIFT 1950; MIRSKY and RIS 1951). Many exceptions to this concept have been observed, some recent ones being those described by BEERMANN (1959, 1966) and GEYER-DUSZYNSKA (1966). For some organisms, it was demonstrated that the exception to the concept of DNA constancy could be explained by a different replication behavior of the euchromatin and heterochromatin (NUR 1966; KEYL and HÄGELE 1966).

In other organisms the validity of the concept is also in doubt. As long ago as 1934, HEITZ indicated that polytene nuclei of *Drosophila virilis* showed less heterochromatin than would be expected on the basis of visible heterochromatic structures present in diploid mitotically active nuclei. It is generally accepted now that the absence of visible sex chromosomes or parts of them in polytene cells is mainly responsible for this deficit. These chromosomes show a heteropycnotic behavior during the mitotic cycle (KAUFMANN 1934; COOPER 1959).

Recently, RUDKIN (1963) suggested three possibilities to explain such a shift of the euchromatin-heterochromatin ratio during the process of polytenization. RUDKIN proposed that either a slow replication of the heterochromatin in relation to the euchromatin, a delayed replication of the heterochromatin, or a partial replication of the heterochromatin caused such a shift. His observations supported the second possibility. The data indicated that the centric heterochromatin stops its replication soon after the process of polytenization begins (RUDKIN 1965).

The present study deals with the replication of euchromatin and heterochromatin in polytene nuclei of the brain ganglion of *Drosophila hydei*. In this tissue, the centric heterochromatin shows a replication behavior during the first stages of polytenization that is different from that observed by RUDKIN in the salivary glands of *Drosophila melanogaster*.

MATERIAL AND METHODS

A laboratory stock of *Drosophila hydei* was used in this investigation. The brain ganglion of Drosophila consists mainly of diploid cells (Figures 1, 2). However, a large number of polytene nuclei can be observed in this tissue during all stages of larval development. Polytene cells are situated in groups at the proximal side of the hemispheres, between the hemispheres and the ventral ganglion. Polytene cells can also be observed within the hemispheres (Figure 1). Studies
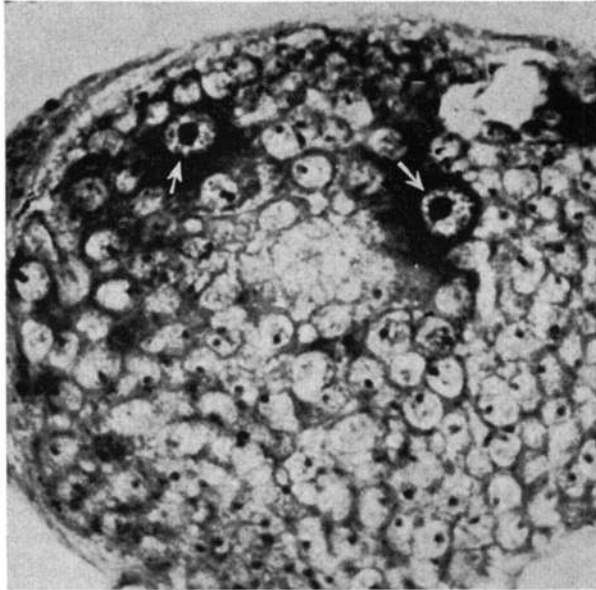
FIGURE 1.—Section of a brain ganglion hemisphere of a late third instar larva of *D. hydei*. Within the mass of diploid nuclei some polytene nuclei can be distinguished (arrows). ×1000.

on other Dipteran species, Chironomus and Musca, revealed that the presence of polytene cells in the brain ganglia is a common feature of this tissue.

In all preparations used for autoradiography and for cytophotometric DNA measurements, the brain ganglia were isolated from late third instar larvae and the adhering ring gland and eye imaginal discs were carefully removed. Because of the low degree of polyteny and the presence of different polytenic stages together with diploid nuclei, the brain ganglia provide almost ideal material for the study of replication of euchromatin and heterochromatin during the early stages of polyteny. The heterochromatin is present in most of these nuclei as a single dark staining body, presumably representing the chromocenter (arrows in Figure 2). It is certain that this chromocenter consists mainly of the heterochromatic arm of the X chromosomes in the females and of this element and the complete heterochromatic Y chromosome in the males.

Autoradiographic procedure: Brain ganglia were isolated from late third instar larvae and incubated for 15 min in a Ringer's solution containing 20 μc of thymidine-H^3 (Specific Activity 14.5 c/mmole). After squashing and processing the slides as described by BERENDES (1966), Kodak AR 10 stripping film was applied and the slides were exposed for 10 days. The films were developed in Kodak D19b for 5 min (20°C), fixed, rinsed, and the tissue was post-stained through the film with a gallocyanin-chromalum solution.

Cytophotometric procedure: Slides with squashed brain ganglia prepared from larvae of the same sample used for autoradiographic experiments, were processed through a series of formalde-hyde-methanol mixtures with increasing formaldehyde concentration. The preparations were stored overnight in isopropanol at 4°C and stained according to the Feulgen procedure (see KEYL 1965). For preparation of the Schiff reagent, pararosanilin (Merck) was used. Embedding was performed with Caedax. Extinction measurements of total nuclei were made with the Universal-Mikro-Spektral-Photometer (UMSP, Zeiss). All measurements were made according to the scanning principle at a wave length of 550 mμ. All measurements were performed on one slide containing brain ganglia of five larvae. A detailed description of the possible systematical errors inherent to the method is given by KEYL (1965). The material used in the present investigation is less favorable than that used by KEYL (1965). Unspecific absorption produced by protein
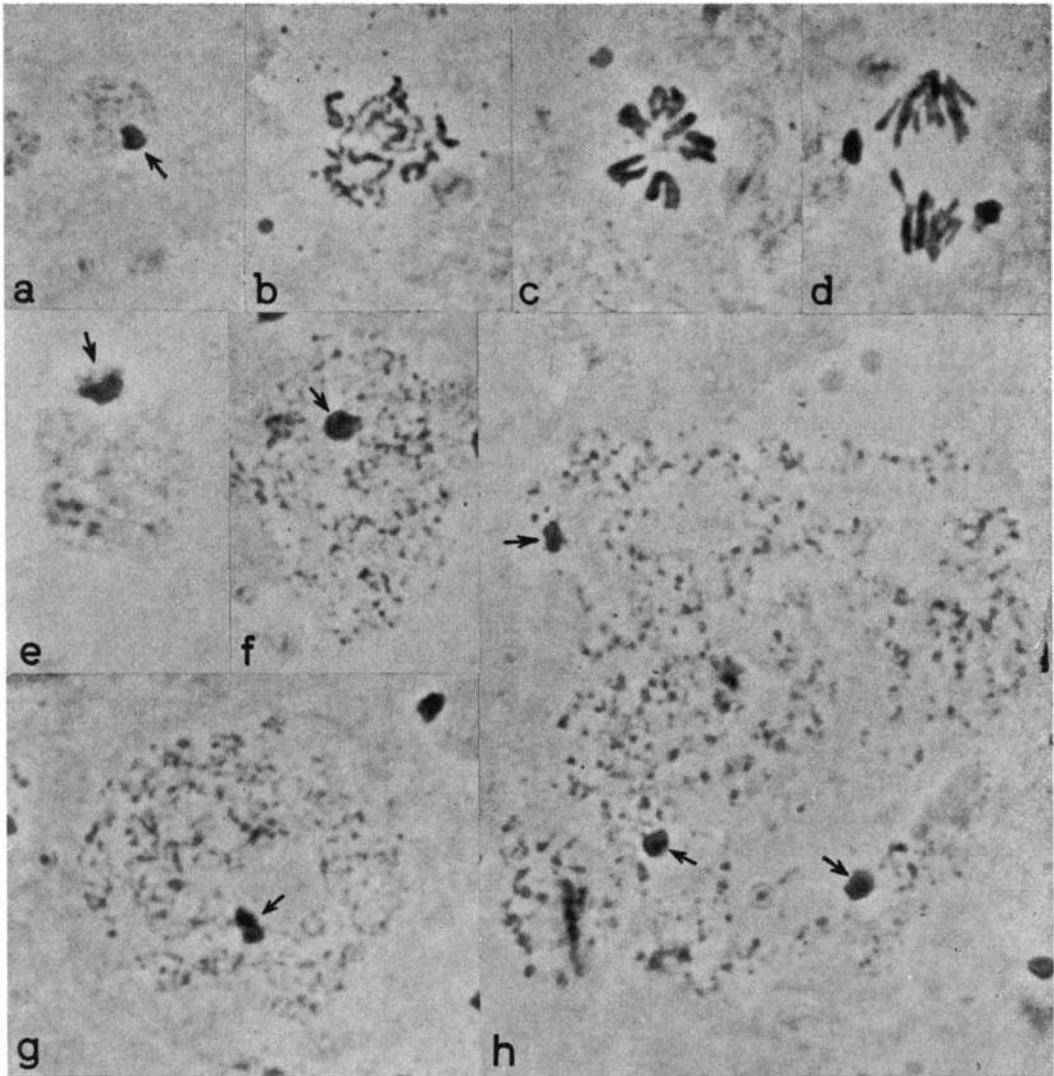
FIGURE 2.—Photograph of some nuclei from a squashed late third instar brain ganglion of *D. hydei*, stained with aceto-orcein. a, b, c, d, mitotic stages of diploid nuclei. It is difficult to decide whether the nucleus in e contains polytene chromosomes or a polyploid set of interphase chromosomes. All other nuclei (f, g and h) contain polytene chromosomes. The sincere contamination of these nuclei with structures not containing DNA, which should lead to high unspecific absorption values, are completely absent in appropriate Feulgen stained preparations (see Figure 3). Arrows indicate the chromocenter. ×2600.

structures can be neglected in the strongy squashed nuclei (See Figure 3). Moreover, the use of a mounting medium with appropriate refractive index (Caedax) reduces the contribution of these structures to the total absorbancy of a nucleus almost completely. The highest absorption values presented may have a standard error of ±15%. For values lower than 50 (see text) a maximal standard error of 10% was calculated. The variation of absorbancy values in the high polytenic
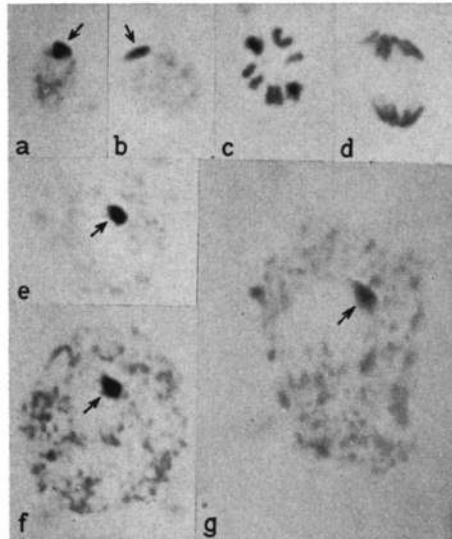
FIGURE 3.—Photograph of some nuclei in a Feulgen stained squash preparation of the brain ganglion. a, b, c, and d, mitotic stages of diploid nuclei. e—g, large nuclei. Arrows indicate chromocenter. ×1700.

nuclei does not depend on unspecific absorption, but is concomitantly due to the use of the scanning method applied to fine structures with inhomogeneous distribution. All absorbancy integrals recorded in this investigation are instrument readings and are mutually comparable.

RESULTS

Autoradiography: Brain ganglia were exposed to thymidine-H^3 in order to determine the average number of cells that were in the S-phase at the developmental stage used. The distribution of silver grains over each nucleus was determined. A sample of 6,300 cells derived from 12 brain ganglia was studied (Table 1). These included both diploid and polytene cells. Most of the nuclei (83.6%) did not show any label in either euchromatin or heterochromatin. Among the labeled nuclei three different types of label distribution could be distinguished. 54.6% showed label over the euchromatin and over the chromocenter, 14.9% showed label only over the euchromatin and 30.5% showed label only over the chromocentral heterochromatin (see Figure 4). Therefore, it can be concluded that the two kinds of chromatin replicate asynchronously (see also BARIGOZZI *et al.* 1966).

Cytophotometric measurements: The absorbancy integrals of 79 interphase nuclei, 68 prometaphase and metaphase nuclei and 237 "large" nuclei were measured. Most nuclei of the last group showed polytene chromosomes, but in the smaller nuclei of this group there was no cytological evidence for a polytenic structure of the chromosomes although their DNA content was higher than that of a 4C nucleus (see Figure 2 e).

The values presented in this investigation are derived from nuclei of both sexes.

TABLE 1

Number of replicating cells and the pattern of thymidine label distribution over the nuclei in the brain ganglion of D. hydei

| Preparation of two brain ganglia	Number of cells without label	Number of cells with label over euchromatin and heterochromatin	Number of cells with label over euchromatin only	Number of cells with label over heterochrom. only	Total number of cells labeled	Total number of cells studied
A	1006 (85.2)	90 (7.6)	31 (2.6)	54 (4.6)	175 (14.8)	1181
B	764 (82.2)	71 (7.7)	39 (4.2)	55 (5.9)	165 (17.8)	929
C	698 (81.7)	82 (9.6)	27 (3.2)	47 (5.5)	156 (18.3)	854
D	1236 (83.9)	141 (9.6)	29 (2.0)	67 (4.5)	237 (16.1)	1473
E	1081 (82.6)	128 (9.8)	22 (1.7)	77 (5.9)	227 (17.4)	1308
F	480 (86.5)	53 (9.5)	7 (1.3)	15 (2.7)	75 (13.5)	555
Total	5265 (83.6% ± 1.6)	565 (8.9% ± 0.9)	155 (2.5% ± 0.9)	315 (5.0% ± 1.1)	1035 (16.4% ± 1.6)	6300

The percentage of each group of cells is in parentheses,
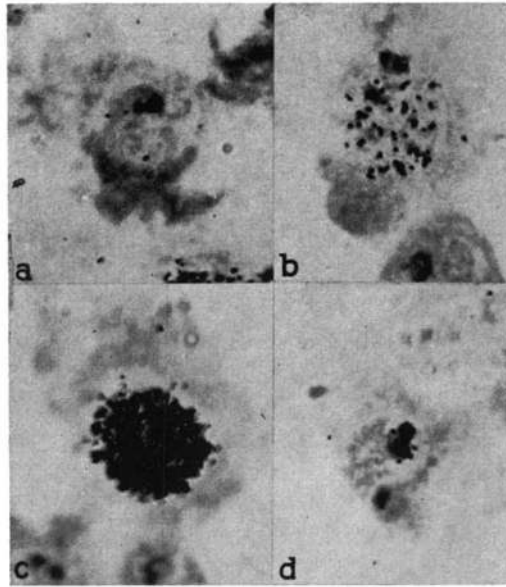
FIGURE 4.—Different patterns of thymidine-H³ incorporation in nuclei of the brain ganglion of *D. hydei.* ×3000.

Separate measurements of diploid nuclei of males and females did not show any significant difference in their DNA content.

All relative values for total nuclear DNA measured on different nuclei are presented in Figure 5. It is apparent that these values show a broad variation. Only two classes are readily distinguishable in this graph, one which centers around an average value of 16.2, and one which centers around an average value of 31.2. Clearly these correspond to the basic 2C and 4C classes of mitotic cells. Among the larger nuclei no distinct classes could be found.

With the spectrophotometer used, it is possible to obtain in addition to the absorbancy integrals of the nucleus as a whole, partial absorbancy integrals from individual structures within the nucleus, e.g. chromocenters. Calculations of this kind were restricted to nuclei containing only one compact chromocenter. The chromocenter values calculated in this way from the spectrophotometer graphs showed a clear cut distribution into geometrical classes. Four classes could be distinguished with average values of 5.4, 10.2, 19.2 and 38.5 respectively (Figure 6). The highest class observed in our material (38.5) was present in only two of the nuclei representing a 16C chromocenter value. The high number of observations of the first class values (5.4) is due to the large number of interphase nuclei measured. However, it is more difficult to explain the high number of values in the second class (10.2). This class of chromocenter values should belong to nuclei with a 4C DNA content. The number of chromocenter values that could be calculated from such nuclei is very low (7). Most of these nuclei were in late prometaphase or metaphase stages and did not show clear chromocenters. Therefore, these chromocenter values must necessarily belong to nuclei

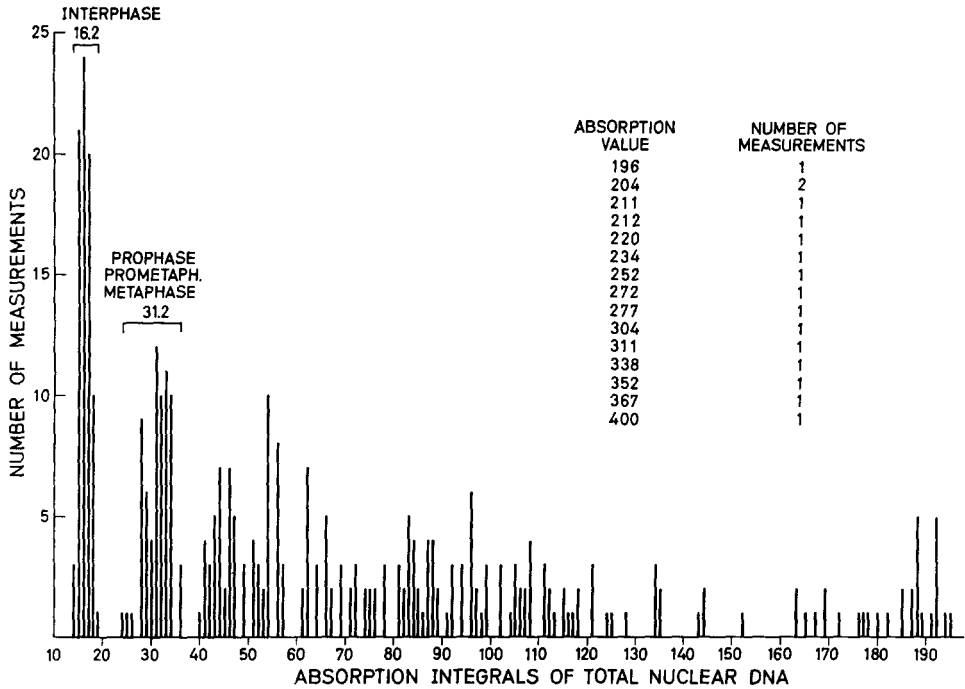| ABSORPTION VALUE	NUMBER OF MEASUREMENTS
196	1
204	2
211	1
212	1
220	1
234	1
252	1
272	1
277	1
304	1
311	1
338	1
352	1
367	1
400	1

FIGURE 5.—Graph of the total absorbancy values of nuclear DNA. All observations on nuclei with an absorbancy greater than 195 are recorded in the table. From this graph only two clearcut classes could be discerned. The interphase nuclei constituted one class with an average value of 16.2 and the prometaphase and metaphase nuclei constituted the second class with an average of 31.2.
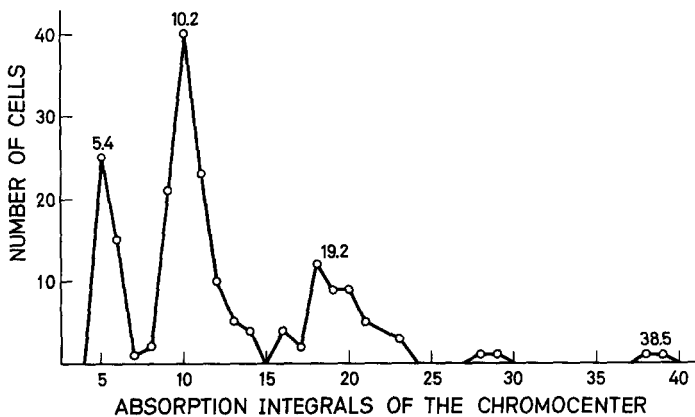

FIGURE 6.—Distribution of the absorbancy integrals of the chromocenters of 194 brain ganglion nuclei. Four classes can be distinguished, with average values of 5.4; 10.2; 19.2 and 38.5 respectively. The first class is derived mainly from interphase nuclei. The second class can not be derived from prophase nuclei only (see Figure 7) and is therefore necessarily constituted by nuclei which possess a DNA content that is greater than the tetraploid amount of DNA.

with a DNA content that is different from the "tetraploid" one. The data showed
further that the chromocentral heterochromatin makes up about one third of the
DNA of interphase nuclei (54/162).

A comparison of the chromocenter values with the total values of the nuclei
showed that most of the larger nuclei were deficient for chromocentral DNA (see
also Figure 8). Many nuclei with a total DNA content higher than 4C possess a
chromocenter with a diploid value. It was also observed that nuclei with a high
total value can possess any of the different chromocenter class values.

The absence of a geometrical class distribution among the larger nuclei as
demonstrated in Figure 4 might be the result of different combinations of euchro-
matin and heterochromatin classes. The euchromatin values of these nuclei can
be estimated by subtraction of the chromocenter value from the total value in
each nucleus. The euchromatin values of these nuclei did indeed show a distri-
bution into geometrical classes (Figure 7). The average values of the euchromatin
classes were, 10.2, 21.5, 39.8, 81.2, and 173.5. The low number of values in the
"tetraploid" class is due to the low number of measurements on prophase nuclei
which contained a clear chromocenter. The random choice of the nuclei measured
involves measurements on nuclei that were in a replication phase. The number
of nuclei showing a value beyond the limits of the classes indicated in Figure 6
is 24%. This percentage is in good accordance with that obtained from autoradio-
graphical experiments (see Table 1).

The highest euchromatin values found were 333 and 342. These values pre-
sumably euchromatin class 64C, which is not considered in Figure 7 on account
of the low number of measurements available. From an inspection of the data
given in Figure 8, it follows that the classes of euchromatin higher than 4C may
be combined with each of the classes of heterochromatin. For instance, the values
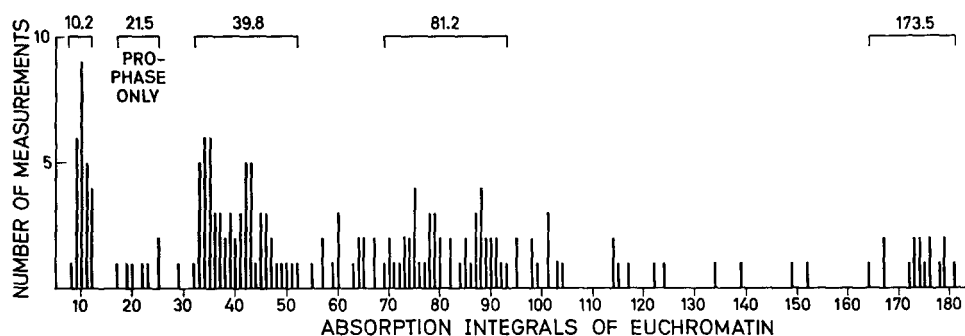333 and 342 were associated with a chromocenter value of 5 and 10 respectively.

FIGURE 7.—Graph of the absorbancy integrals of the euchromatin obtained by subtraction of
the absorbancy values of the chromocenter from the absorbancy value of the whole nucleus. The
number of data presented is less than in Figure 5. Nuclei recorded in Figure 5 which contained
more than one distinct chromocenter and nuclei in which the chromocenter value could not be
determined exactly were excluded from this graph. In addition to the euchromatin values pre-
sented in the graph, nine nuclei with absorbancy integrals higher than 182 were found, the
value 201, was found twice, and the values 219, 262, 268, 281, 306, 333, and 342 one time each.
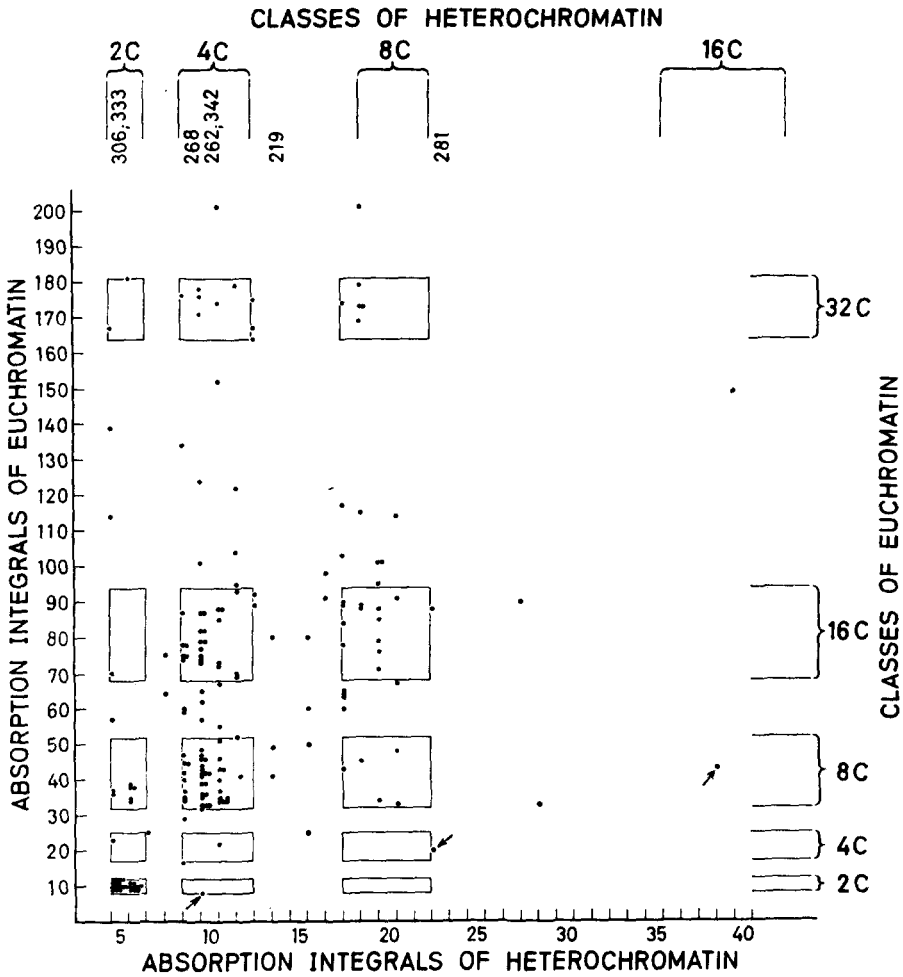
FIGURE 8.—Diagram of the combinations of euchromatin and heterochromatin values in 194 nuclei. The various combinations of euchromatin and heterochromatin classes are indicated by quadrangles. It is evident that, though chromocenter values higher than 2C are more abundant in nuclei with euchromatin values higher than 2C, especially nuclei with euchromatin values falling into the 8C, 16C and 32C, classes are not preferably associated with similar heterochromatin classes. Since the second class of euchromatin (21.5) represents the 4C value (metaphases and prometaphases) only few measurements on the chromocenter could be made. Arrows indicate nuclei which show a higher class of heterochromatin than euchromatin.

It was evident, however, that the high classes of heterochromatin are more abundant in nuclei with higher classes of euchromatin than in those with a lower class value. Since all nuclei with integrated absorbancy values higher than 80 (Figure 2f) show a cable-like organization of the euchromatin, which points to a polytenic structure of the chromosomes, this figure also illustrates the discrepancy of euchromatin and heterochromatin values in polytene nuclei. These nuclei,

generally showed a much lower heterochromatin value than expected from their euchromatin value.

In a few nuclei (3) indicated by arrows in Figure 8, the heterochromatin made up more than one third of the total nuclear DNA. It is likely that these nuclei represent cases in which the heterochromatin has completed one replication step more than the euchromatin.

<div align="center">DISCUSSION</div>

Our data clearly demonstrate that the proportion of heterochromatin and euchromatin in polytene nuclei is different from that in diploid nuclei. By separation of the relative part of the nuclear DNA present in the chromocenter from the total DNA of the nucleus, a clear geometrical distribution of the euchromatin values was established. In appraising the distribution of these euchromatin values (Figure 7), methodological difficulties must be regarded. These difficulties are a common feature in cytophotometry when sharply defined structures with high absorption values (chromocenters) and large surfaces with very weak absorption values (large polytene nuclei) are compared. The calculation of absorption values for the chromocenter is contaminated with inaccuracies which arise always when a value originates from addition of several individual small values (absorbancies of different scanning lines) (KEYL 1965). However, these inaccuracies proved to be of little influence, as both the euchromatin and heterochromatin values showed an evident pattern of geometrical classes.

The broad scatter of values within the three highest classes of euchromatin in Figure 7 reflects the unfavorable situation of measuring large surfaces with low absorbancy.

Our data also provide evidence that the polytene nuclei can show a substantial lack of heterochromatin, as already indicated by HEITZ (1934). In addition to the possibilities that could explain this lack of heterochromatin, proposed by RUDKIN (1963), other possibilities might exist. Heterochromatin may replicate at the same rate as euchromatin, but part of it might be eliminated during polytenization. There is no cytological evidence in our material which indicates the elimination of chromatin. Part of the heterochromatin in polytene nuclei might be euchromatinized. This would lead to a decrease in the amount of heterochromatin measured, but would provide a clear pattern of geometric classes of total values for nuclear DNA. Such a pattern was absent in our material (see Figure 5). Since in nuclei with total DNA values lower than 80 no clear polytenic structure of the chromosomes could be recognized, polytene nuclei will be defined as those nuclei which show a cable-like organization of the chromosomes and which have a total absorbancy value higher than 80. Polytene nuclei belonging to one distinct class of euchromatin can contain various classes of heterochromatin. It is likely to assume that a situation as such arises from an independent replication of euchromatin and heterochromatin in the same nucleus. In this process a different rhythm of replication as well as a different number of replication steps of the two types of chromatin might be involved.

By combining the different classes of heterochromatin and euchromatin ob-

TABLE 2

Scheme of possible combinations of euchromatin and heterochromatin classes in nuclei of the brain ganglion of D. hydei

Euchromatin value	Heterochromatin value	Total value calculated	Number of observations of the (calculated) value
2C = 11 (10.2)	2C = 5	16 (diploid)	24
	4C = 10	21	0
4C = 22 (21.5)	2C = 5	27	0
	4C = 10	32 (tetraploid)	10
	8C = 20	42	3
8C = 44 (39.8)	2C = 5	49	3
	4C = 10	54	10
	8C = 20	64 (octoploid)	3
	16C = 40	84	4
16C = 88 (81.2)	2C = 5	93	0
	4C = 10	98	1
	8C = 20	108	4
	16C = 40	128	1
32C = 176 (173.5)	2C = 5	181	0
	4C = 10	186	2
	8C = 20	196	1
	16C = 40	216	0
	32C = 80	256	0

The measured diploid values of euchromatin and heterochromatin were used as a basis for calculations of the total values for nuclear DNA that could be expected. The average values of the euchromatin classes derived from the measurements are recorded in parenthesis.

served (Figure 8; Table 2), we checked whether a specific combination of heterochromatin and euchromatin classes was favored. No particular combination was favored in our material.

In some nuclei the heterochromatin had a higher class value than the euchromatin, indicating that in these nuclei the heterochromatin had completed one replication step more than the euchromatin. The relatively high percentage of nuclei with thymidine label exclusively over their chromocenter in comparison to the percentage of nuclei with thymidine label only over the euchromatin may incorporate these exceptional cases. It cannot be excluded that the difference between the percentages arises by a difference in time necessary for complete replication of each type of chromatin.

From the geometric distribution of the chromocenter values it can be assumed that a clear-cut part of the nuclear heterochromatin, derived mainly from the X and the Y chromosome, behaves as a unit during replication. It is unlikely, however, that the nucleolus organizer region situated on each of the two sex chromosomes is involved in the replication behavior of most of the heterochromatin. In other polytene cells, for instance the salivary gland cells, which attain much higher polytenic stages, this region behaves with respect to its replication be-

havior as euchromatin. It replicates to the same extent and nearly always at the same time as euchromatin (NASH and PLAUT 1965; BERENDES, unpublished). It is assumed that this part of the heterochromatin is either euchromatinized or does not follow the replication of the rest of the chromocentral heterochromatin. Although the replication of this small part of the heterochromatin in polytene brain ganglion cells cannot be demonstrated by autoradiography, the correlated increase in nucleolar size and increase in DNA content of their nuclei at least suggest a similar behavior of the nucleolar organizer in the polytene cells of the brain ganglion.

Thymidine incorporation in heterochromatin and euchromatin as well as the quantitative distribution of the two types of chromatin was studied on a population of cells at a particular stage of development. No discrimination according to cell function, nor to the history of differentiation of the individual cells measured could be made. It might be possible that the various combinations of heterochromatin values in polytene nuclei are correlated with particular functional differences between the individual cells. This question, however, reveals further study.

The relative lack of heterochromatin in most polytene nuclei indicates that a coordinated replication of the two types of chromatin is not necessarily a prerequisite for functional differentiation of polytene cells. This raises the interesting question whether or not a part of the heterochromatin, known to have important functions during spermatogenesis (HESS 1965), is more or less redundant in somatic cells.

SUMMARY

The brain ganglion of Drosophila contains diploid as well as polytene cells. This tissue is therefore particularly suitable for a study of the replication and quantitative distribution of heterochromatin and euchromatin in early polytenic stages. Thymidine-H^3 autoradiography revealed that the heterochromatin and the euchromatin showed an asynchronous, but in most cases overlapping, period of DNA synthesis in the diploid cells. Cytophotometric DNA measurements showed that the total values for nuclear DNA of the nuclei measured could not be arranged into a geometric series of classes originating from the diploid value. When the values for chromocentral heterochromatin and the euchromatin were separated, a good geometric distribution of the values of each of the types of chromatin was established. The polytene nuclei generally showed different classes of heterochromatin, which were sometimes but not always in accordance with the class expected on the basis of the euchromatin value. It is assumed that the process of polytenization involves an independent replication of euchromatin and heterochromatin, and that the number of replication steps completed by each of the types of chromatin can be different.

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