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Differential DNA Synthesis in Heterochromatic and Euchromatic Chromosome Sets of *Planococcus citri*

Gloria Lorick
Department of Biology, University of Rochester, Rochester, N.Y.

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Abstract. In males of the mealy bug *Planococcus citri*, Nur (1966) counted five heterochromatic (H) and about 5, 10, 20, 40, or 80 euchromatic (E) chromosomes in testis sheath nuclei which were undergoing endomitosis. He suggested that the H chromosomes were not replicating and that the nuclei were becoming polyploid as a result of successive cycles of replication of only the E chromosomes. This hypothesis was tested using autoradiography with H^3-thymidine to detect DNA synthesis and microspectrophotometric measurements of the Feulgen reaction in nuclei to detect quantitative changes in DNA. — The integrated absorbance of the whole nucleus and of the isolated clump of heterochromatic chromosomes (H body) in polyploid testis sheath nuclei were measured using the mechanical scanner of the CYDAC system. The absorbance of the H body was similar in all testis sheath nuclei examined and was not significantly different from the absorbance of a haploid set of H chromosomes measured after meiosis. The absorbance of the euchromatic component varied in different sheath nuclei, the values closely corresponding to the terms of the series 2c, 4c, 8c. This series is expected if the DNA in the E chromosomes is exactly doubled at each cycle of replication. — Autoradiographs showed that most labeled sheath nuclei had silver grains localized exclusively over euchromatin. With one exception, the remainder of the labeled nuclei had silver grains over both euchromatin and the H body. The observation that euchromatin was much more heavily labeled than the H body and that labeled H bodies occurred at a low frequency and only in the presence of labeled euchromatin suggests that the H body did not incorporate the label and that the silver grains over the H body were the result of β-particles which originated in proximal euchromatin.

Introduction

Male mealy bugs have one set of euchromatic (E) and one set of heterochromatic (H) chromosomes (Schrader, 1921). Although they begin development as zygotes containing two sets of E chromosomes, one set becomes condensed, or heterochromatic, at the blastula stage (Hughes-Schrader, 1948). The H set is paternal in origin (Brown and Nelson-Rees, 1961), shows little or no genetic activity (Brown and Nelson-Rees, 1961; Berlowitz, 1965; Brown, 1969), and replicates DNA slightly asynchronously with the E set (Baer, 1965). In some somatic tissues, the H

chromosomes may revert to the euchromatic state and presumably to genetic activity (Nur, 1967).

Males do not transmit the H set to their progeny (Hughes-Schrader, 1935). The meiotic sequence is inverted (Schrader, 1921; Chandra, 1962), i.e. at the first division all chromosomes divide equationally and at the second division homologous chromosomes segregate, with E chromosomes moving toward one pole and H chromosomes moving toward the other pole. The meiotic derivatives containing the E chromosomes develop into sperm. Those containing the H chromosomes remain condensed during spermiogenesis and eventually degenerate.

In the species *Planococcus citri* (Risso), Nur (1966) observed that the polyploid nuclei of the cellular sheath surrounding each testis contained a polyploid number of E chromosomes but only a haploid number of H chromosomes. Since the number of E chromosomes formed a geometric series based on the haploid number, he proposed that the E chromosomes underwent several cycles of replication and endomitosis while the H chromosomes remained unreplicated. The concept that some chromosomes may undergo DNA replication while others within the same nucleus do not is relevant to our understanding of the control of DNA synthesis. A somewhat similar process, namely the underreplication of heterochromatin, has also been proposed for salivary gland chromosomes of *Drosophila* (Rudkin, 1965a, b) and the germ-line limited (L) chromosomes in two species of sciarids (Himes and Crouse, 1961; Sauaia and Alves, 1969). The present report describes a test of the hypothesis of nonreplication of the H chromosomes in *P. citri* using autoradiography with H^3-thymidine to detect DNA synthesis and spectrophotometric measurements of the Feulgen reaction in nuclei to detect quantitative changes in the amount of DNA. The observations support the conclusion of Nur, that the E chromosomes can undergo two or more events of DNA replication while the DNA of the H chromosomes remains at the unreplicated level.

Materials and Methods

Cytophotometry

Cultures of the mealy bug, *Planococcus citri* (Risso) (*Homoptera* : *Coccoidea*) were raised in the laboratory on sprouting Irish potatoes as described by Nelson-Rees (1960). Whole male third instar larvae were fixed for one hour in Carnoy-Bradley solution (4 parts chloroform: 3 parts absolute ethanol: 1 part glacial acetic acid) to allow for a quick penetration of the fixative through the waxy cuticle and were transferred to Carnoy fixative (3 parts absolute ethanol: 1 part glacial acetic acid) for 5–11 h. The fixed larvae were stained *in toto* by the Feulgen reaction (Stowell, 1945) following hydrolysis in 1 N HCl at 60° C for 10 min. The stained larvae were placed in 45% acetic acid. Testes and accessory glands were dissected from each bug and squashed on glass slides under coverslips, which were popped off after freezing on dry ice (Conger and Fairchild, 1953). The slides were trans-

ferred through 50% and 35% ethanol to water, rinsed in three changes of SO_2 water, washed in running water, and air dried. The dry preparations were mounted in Cargille oil of refractive index 1.548, selected to match the refractive index of the cytoplasm.

The spectrophotometric measurements were made using the mechanical scanner of the CYDAC system (Mendelsohn *et al.*, 1968; Bostrom and Holcomb, 1963). The mechanical scanner is a dual-beam integrating scanning cytophotometer. The design of the instrument, the scanning operation, and the data processing are discussed by Mayall and Mendelsohn, (1970). The system was used with a 40 × apochromatic oil immersion objective (N.A. = 1.0), an achromatic aplanatic oil immersion condenser adjusted to a N.A. of 0.6, and an interference filter with maximum transmission at 566 mμ.

The measurable field is 18×18 μ, but by means of electronic mask switches the operator may limit the area from which data are collected. The system divides the measurable field into a large number of small elements of area 0.375×0.375 μ. The optical density is measured for every unmasked element and these values are accumulated digitally. A scan sequence takes less than a minute and consists of three successive scans of the object field followed by three successive scans of a clear field of equal size. The values from the clear field are automatically substracted from the total of the object field values and at the end of the sequence the number displayed by the accumulator is the relative integrated absorbance of the object. Each object was measured twice and the mean of the two values was considered to be the relative integrated absorbance of the object. Throughout this report, the terms "integrated absorbance" and "total absorbance", will be used to indicate this value.

Nuclei to be measured were selected in advance and photographed. Later they were relocated with the aid of the photographs and measured. Only nuclei which were completely separated from surrounding nuclei were selected. Some of these had to be eliminated because they were larger than the measurable field. The integrated absorbance of the whole nucleus and the integrated absorbance of the clump of H chromosomes (H body), as shown by the large and small boxed areas in Fig. 1h, were measured. The two values were subtracted to obtain the total absorbance of the euchromatic component.

Before any measurements were made on testis sheath nuclei, spermatocyte nuclei from a second instar larva were examined to see if the Feulgen reaction in *P. citri* were consistent with the results obtained for other organisms and to compare the total absorbance of an E set of chromosomes with the total absorbance of a homologous H set. Such a comparison was in order since other authors (Hale, 1966; Mayall and Mendelsohn, 1967; Mayall, 1968; Garcia, 1969a, b) have observed that condensed chromatin has a lower stain content than diffuse chromatin.

Autoradiography

The testes from third and fourth instar larvae and from adults of *P. citri* were dissected into Drosophila Ringer solution (Beadle and Ephrussi, 1936), pH 6.3–6.7, containing thymidine-methyl-H^3 (sp. act. 3.0 c/mmole, Schwartz Bioresearch). In experiment A they were incubated for 30 min in a drop of solution containing 10 μc H^3-thymidine per milliliter of solution and were fixed immediately thereafter. In experiment B they were incubated for 15 min in a drop of solution containing 5 μc H^3-thymidine per milliliter of solution and were transferred to fresh Drosophila Ringer solution without thymidine for another 15 min before fixation. The testes were fixed in Carnoy solution and squashed on slides in 45% acetic acid. The coverslips were removed and the tissues were stained according to the Feulgen

procedure as described above. Dried preparations were coated with NTB2 liquid nuclear track emulsion (Eastman Kodak Company) which was diluted 1:1 with water and were exposed for 36 to 48 h. Autoradiographs were developed in D-19 for 2 min at 18° C and fixed in Kodak acid fixer for 5 min at 18°.

Silver grain counts were used to estimate the relative amounts of incorporation of H^3-thymidine by the H body and euchromatin, respectively. All labeled nuclei were included in the data in order to avoid biasing the results. "Countable" nuclei include all labeled nuclei which were not overlapping another nucleus, which could be identified (by the presence of an H body) as sheath nuclei, and in which individual silver grains in both the H body and euchromatin were resolvable. For these nuclei the number of silver grains over euchromatin and the number over the H body were counted. In some nuclei the density of silver grains, especially over euchromatin, was too high for grain counts. For this group of nuclei the presence or absence of an identifiable H body and the number of grains over it were recorded. Samples of "uncountable" nuclei in which the H body was not visible were photographed and examined again after the silver grains had been bleached with Farmer's reducer.

Grains overlying the H body or touching its border were recorded as grains over the H body. Notation was made as to the number of grains which were "central" and the number which were "peripheral", i.e. touching the border from inside or outside. All other grains lying within the Feulgen positive area were recorded as grains over euchromatin. The background level was determined from grain counts over areas of the slide containing only spermatids and H residues.

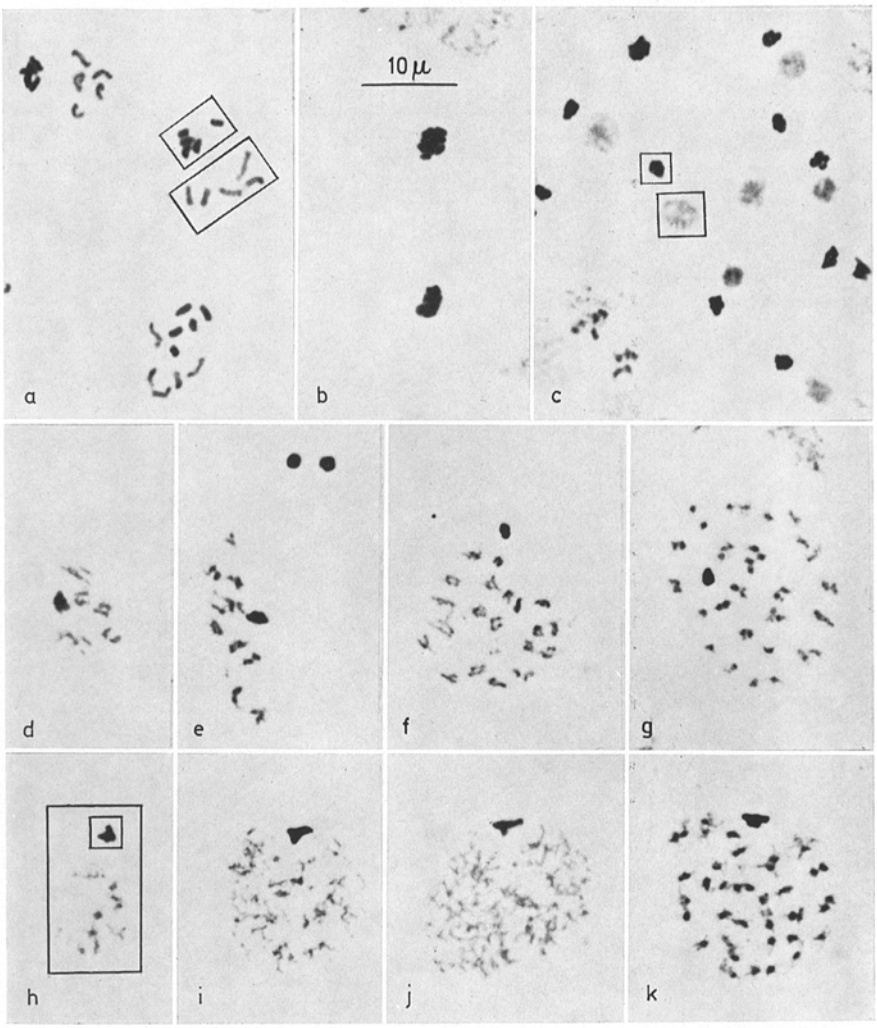
Results

Cytophotometry

1. Meiotic Nuclei

The Feulgen reaction in homologous H and E sets of chromosomes may be examined at prophase I and after telophase II of meiosis. In male mealy bugs homologous chromosomes do not pair at prophase I and the H and E sets can be distinguished by differences in condensation (Fig. 1a). Each set is expected to have replicated and therefore to contain the diploid (2c) amount of DNA. All chromosomes appear equally condensed in metaphase I spermatocytes (Fig. 1b) and the total absorbance of the two sets is expected to correspond to the tetraploid (4c)

Fig. 1a–k. Photographs of Feulgen stained nuclei which were measured spectrophotometrically or used for chromosome counts. The boxes indicate areas measured spectrophotometrically. a–c Meiosis in spermatocytes. a Prophase I. The five euchromatic (E) chromosomes are less condensed than the five heterochromatic (H) chromosomes. Nuclei similar to the two upper ones, in which the H and E sets are in separate clusters, were used to measure the integrated absorbance of each set at prophase. b Metaphase I. In both metaphase cells, the H and E chromosomes are fully condensed and the two sets are indistinguishable. c Post-telophase II. Each set of chromosomes has divided equationally and the H and E chromosomes have segregated into different nuclei. The light and dark nuclei each contain a haploid set of E or H chromosomes, respectively. d–g Testis sheath nuclei at endomitosis.



The H body is about the same size in all nuclei, but the number of E chromosomes varies. d Five E chromosomes, each with two chromatids. e Ten E chromosomes, each with two chromatids. f Twenty E chromosomes, each with two chromatids. g A later stage of endomitosis. Some of the chromatids have separated. Altogether, 40 single elements are present. h–k Testis sheath nuclei in which the extinction of Feulgen dye was measured. The total absorbance of the euchromatin approximately corresponds to that expected for the ploidy class shown in the endomitotic nucleus directly above. T = total integrated absorbance of the nucleus. H = integrated absorbance of the H body. The value for the euchromatin (E) is given as a multiple of the H body value, c. h T = 9065; H = 2690; E = 2.37 c. i T = 17248; H = 3340; E = 4.16 c. j T = 27229; H = 2944; E = 8.25 c. k T = 31283; H = 3461; E = 8.04 c

Table 1. *The mean integrated absorbance of the heterochromatic (H) and euchromatic (E) sets of chromosomes at several stages of meiosis in spermatocytes of P. citri. The ratio of the integrated absorbance at each stage to the integrated absorbance of the H set in post-telophase II nuclei ($\bar{x}/\overline{H}$) is also given. Data are from one second instar male*

Spermatocytes	n	H set		E set	
		$\bar{x} \pm s/\sqrt{n}$	$\bar{x}/\overline{H}$	$\bar{x} \pm s/\sqrt{n}$	$\bar{x}/\overline{H}$
Post-telophase II	14	2979 ± 45	1.00	3109 ± 56	1.04
Prophase I	11	5930 ± 126	1.99	6303 ± 105	2.12
Metaphase I		$\bar{x} \pm s/\sqrt{n}$		$\bar{x}/\overline{H}$	
(H and E)	11	12597 ± 172		4.23	

n = number of nuclei; $\bar{x}$ = mean integrated absorbance; $s/\sqrt{n}$ = standard error of the mean

amount of DNA. At the second meiotic division the H and E chromosomes segregate into different nuclei. Shortly after telophase II the euchromatic derivatives begin to decondense and can be distinguished from the heterochromatic derivatives (H residues), which remain condensed (Fig. 1 c). Each post-telophase II nucleus is expected to contain the haploid (1 c) amount of DNA. Meiosis has been completed by third instar, when the testis sheath nuclei become polyploid. However, the H residues are present and may be used as a standard for the absorbance of a haploid set of H chromosomes.

The results of measurements on spermatocyte nuclei from one *Planococcus citri* male are given in Table 1. The integrated absorbance of each H or E set of chromosomes at prophase I is twice the value observed after telophase II. Also, as would be expected, the total absorbance of the chromosomes in metaphase I spermatocytes is about four times the total absorbance of the post-telophase II nuclei. These measurements indicate that the amount of DNA in each set of chromosomes is doubled prior to meiosis and is halved during the meiotic divisions. Other investigators (Swift, 1953; Vendrely and Vendrely, 1956) have observed that the total absorbance of Feulgen stained spermatocyte nuclei increases to the 4 c value during the interphase preceding meiosis and that as a result of the two meiotic divisions each of the four products has the 1 c value. Therefore the results of the Feulgen reaction in *P. citri* are entirely consistent with the spectrophotometric results reported for other organisms.

Possible differences in the amount of dye bound by heterochromatin and euchromatin may be examined by comparing the total absorbance of the H set of chromosomes with the total absorbance of a homologous E set. Fig. 2 is a graph of the integrated absorbance of the H set *vs.* the

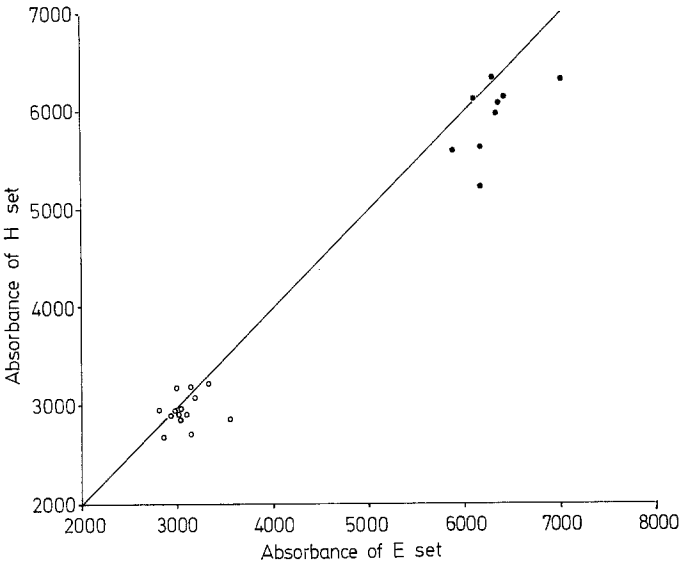


Fig. 2. The integrated absorbance of the heterochromatic (H) set of chromosomes (vertical axis) and the euchromatic (E) set of chromosomes (horizontal axis) in individual spermatocytes of *P. citri* at post-telophase II (o) and prophase I (•). The integrated absorbance of each set at prophase I is twice the integrated absorbance of each set after telophase II. The measured values fall near the line with equation $y = x$. Data are from one male

integrated absorbance of the E set in post-telophase II and prophase I nuclei. The straight line indicates the points at which the values for the two sets are equal. Nine out of fourteen measurements on post-telophase nuclei and seven out of nine measurements on prophase nuclei lie below the line in the direction of higher values for the E set. The ratio of the mean value for the E set to the mean value for the H set is 1.06 at prophase and 1.04 after telophase (Table 1). Thus the integrated absorbance of euchromatin is 6% higher than the integrated absorbance of heterochromatin at prophase and 4% higher after telophase. A t-test for paired observations showed that the values for each set within the same nucleus are significantly different in prophase ($p<0.01$) but not in post-telophase nuclei ($P > 0.05$).

The H chromosomes are already condensed at early prophase and become only slightly more condensed as prophase progresses, whereas the E set changes from diffuse chromatin to condensed chromosomes during prophase. If the total absorbance decreases as the chromosomes become more condensed, then the value observed for metaphase chromosomes should be lower than the value observed for prophase chromosomes. The

sum of the values for the two sets at prophase I is 12233 and the value for the metaphase I complement is 12597. In this case there is no indication of a decrease in total absorbance associated with an increase in condensation. It is not clear why the total absorbance of the H set of chromosomes is slightly lower than the total absorbance of the E set, while the total absorbance of metaphase chromosomes is not lower than that of prophase chromosomes. At any rate, the differences in total absorbance between the euchromatic and heterochromatic sets of chromosomes in *P. citri* are small enough so that values for euchromatin and heterochromatin may be compared to obtain estimates of the relative extent of DNA replication.

2. Testis Sheath Nuclei

The primary spermatocytes of male mealy bugs are arranged into groups, or cysts, of 16 cells each. A cyst is surrounded by a layer of cyst wall cells. The entire testis is surrounded by a cellular sheath, one cell thick. In spermatocyte and sheath cell nuclei the heterochromatin is clumped into a single mass (the H body), usually near the periphery of the nucleus. The nuclei of cyst wall cells lack heterochromatin. This is one of the tissues in *P. citri* in which the paternal set of chromosomes has undergone reversal from the heterochromatic to the euchromatic state (Nur, 1967).

Prior to meiosis, which occurs late in the second instar, the sheath cells are somewhat obscured by the larger and more numerous spermatocytes. At the beginning of the third larval instar, the testis sheath cells are easily recognized from the euchromatic spermatids and the H residues of meiosis. Many large sheath cells are present, some of which are in stages of endomitotic division. During endomitosis chromosomes can be counted. Nuclei with 5, 10, 20, 40, or 80 euchromatic chromosomes and an H body are seen (Fig. 1 d–g; Nur, 1966). The size of the H body appears to be the same in all nuclei regardless of the number of E chromosomes and occasionally the H body is resolved into five chromosomes.

The total absorbance of the H body and the total absorbance of the euchromatin in individual testis sheath nuclei from six animals are shown graphically in Fig. 3. The animals were prepared at different times and values from different animals are indicated by different symbols. Although there is variation between animals, within each animal the total absorbance of the H body is similar in all nuclei. In contrast, the total absorbance of the euchromatin varies over a sixfold range. The mean value for the H body and the standard error of the mean are given in Table 2. In each animal the variance between measurements on the H

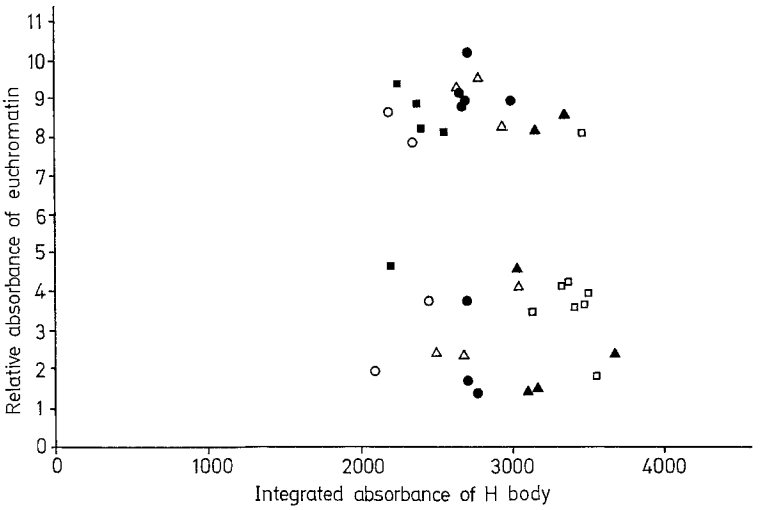


Fig. 3. Relative integrated absorbance of euchromatin (vertical axis) *vs.* integrated absorbance of the H body (horizontal axis) in individual Feulgen stained nuclei from testis sheath cells of *P. citri*. The integrated absorbance of the euchromatin is expressed as a multiple of the H body value in the same nucleus. The distribution along the horizontal axis shows that there is considerable variation in measurements between animals but only slight variation within an individual. Male 1 = ○, Male 2 = △, Male 3 = ■, Male 4 = ●, Male 5 = ▲, Male 6 = □

body in different nuclei was not significantly greater than the variance between replicate measurements ($P<0.05$, F distribution). It is concluded that the variation within animals is attributable to random measuring error and that the total absorbance of the H body is the same in all sheath nuclei.

Table 2. *The mean integrated absorbance of the H body in testis sheath nuclei and the mean integrated absorbance of the H residues of meiosis in several third instar males of P. citri*

Male	H body		H residues	
	$\bar{x} \pm s/\sqrt{n}$	n	$\bar{x} \pm s/\sqrt{n}$	n
1	2263 ± 79	4	—	—
2	2765 ± 83	6	—	—
3	2347 ± 63	5	—	—
4	2733 ± 37	8	2724 ± 32	12
5	3257 ± 100	6	3228 ± 33	12
6	3406 ± 48	8	3424 ± 35	12

$\bar{x}$=mean integrated absorbance; $s/\sqrt{n}$=standard error of the mean; n= number of nuclei.

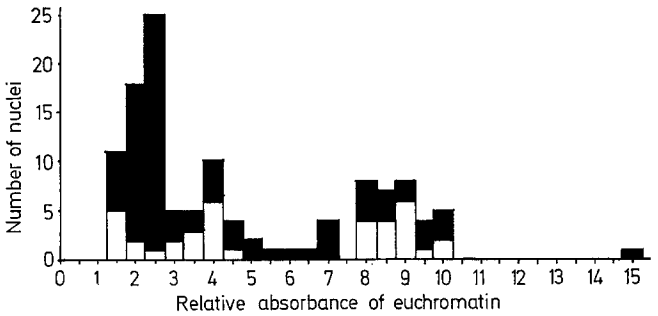


Fig. 4. A histogram showing the distribution of testis sheath nuclei with respect to the relative integrated absorbance of the euchromatin in individual nuclei from six different males. The shaded areas represent 83 nuclei in which only the whole nucleus was measured and the unshaded areas represent the 37 nuclei in Fig. 3, in which both the whole nucleus and the H body were measured. The mean integrated absorbance of the H body in each male was calculated from the latter values. The relative integrated absorbance of euchromatin in each nucleus is expressed as a multiple of the mean value for the H body

In three of the animals the total absorbance of the H body was compared with the total absorbance of the H residues, which contain the 1c amount of DNA. The value for the H body was similar to the value for the H residues (Table 2). It is concluded, therefore, that the H body also contains the 1c amount of DNA.

Variation in measurements between animals are observed for the H body and the H residues, both of which have consistent values within animals. Since the six animals used for these measurements were prepared at different times, differences between animals might be explained as a result of variation in hydrolysis time and in the SO_2 content of different dye solutions (Swift, 1955; Deitch, 1966).

In contrast to the H body, the total absorbance of euchromatin varied widely. The graph in Fig. 3 shows that the ratios of euchromatin to H body roughly approximate the coefficients of the geometric series 2c, 4c, 8c, ..., which would be formed by successive doublings of the DNA in euchromatic chromosomes. In many nuclei the H body was not separated from the euchromatin and so could not be measured separately from the whole nucleus. The histogram in Fig. 4 shows the distribution of nuclei with respect to the amount of euchromatin, expressed as a multiple of the mean total absorbance of the H body for each animal. The data include the 37 nuclei (unshaded areas) shown in Fig. 3 as well as 83 nuclei (shaded areas) in which separate measurements on the H body were not possible. As in Fig. 3 there is a tendency for the total absorbance of the euchromatin to be clustered about multiples of the 2^n series.

The less well-defined classes in the shaded areas suggest that some of these nuclei were undergoing DNA synthesis at the time of fixation. Many nuclei in which the H body was separated from the euchromatin appeared to be in endomitosis. This observation might explain the lower frequency of intermediate values among nuclei represented by the unshaded areas.

In comparing the spectrophotometric and the autoradiographic results it was relevant to know whether there was a tendency for the absorbance of the H body to increase as the ploidy of the nucleus increased. Therefore, for each sheath nucleus, the ratio of the integrated absorbance of the whole nucleus to the integrated absorbance of the H body was calculated and the nucleus was assigned to the nearest ploidy class in the $(2^n + 1)$ series. In the sample of 37 nuclei the coefficient of correlation between the integrated absorbance of the H body and the ploidy of the nucleus was —0.248. This negative correlation is not statistically different from a zero correlation. Since there is no tendency for the total absorbance of the H body to be greater in higher ploidy nuclei, spectrophotometric measurements provide no evidence for an increase in the amount of DNA in the H body during the course of endoduplication of the euchromatin.

Autoradiography

Polyploid nuclei in the testis sheath are first recognized in third instar larvae. When the testes, each surrounded by a sheath, were incubated in a solution containing H^3-thymidine, some sheath nuclei incorporated the label (Fig. 5a–c), but the spermatids, the H residues of meiosis, and the cyst wall cell nuclei did not. The fact that some, but not all, of the sheath nuclei incorporated label and that the amount of label incorporated varied in different nuclei indicates that DNA synthesis is not synchronous in all sheath nuclei. This observation is consistent with the visual observation that the nuclei within a sheath are not at the same stage of interphase or endomitosis or at the same level of polyploidy.

In order to determine whether DNA synthesis in sheath nuclei terminates with maturity, autoradiographs of testis sheath tissue from third and fourth instar larvae and adult males were studied. Labeled nuclei were observed in all nine third instar larvae examined and in two out of fourteen fourth instar larvae. Only one labeled nucleus in one individual was observed among the seven adults examined. Therefore DNA synthesis has virtually ceased by the adult stage.

Third instar larvae were used to examine the relative amounts of incorporation by the euchromatin and the H body. Silver grains were counted in autoradiographs from two experiments. It was mentioned previously that the periods of incubation in H^3-thymidine solution were

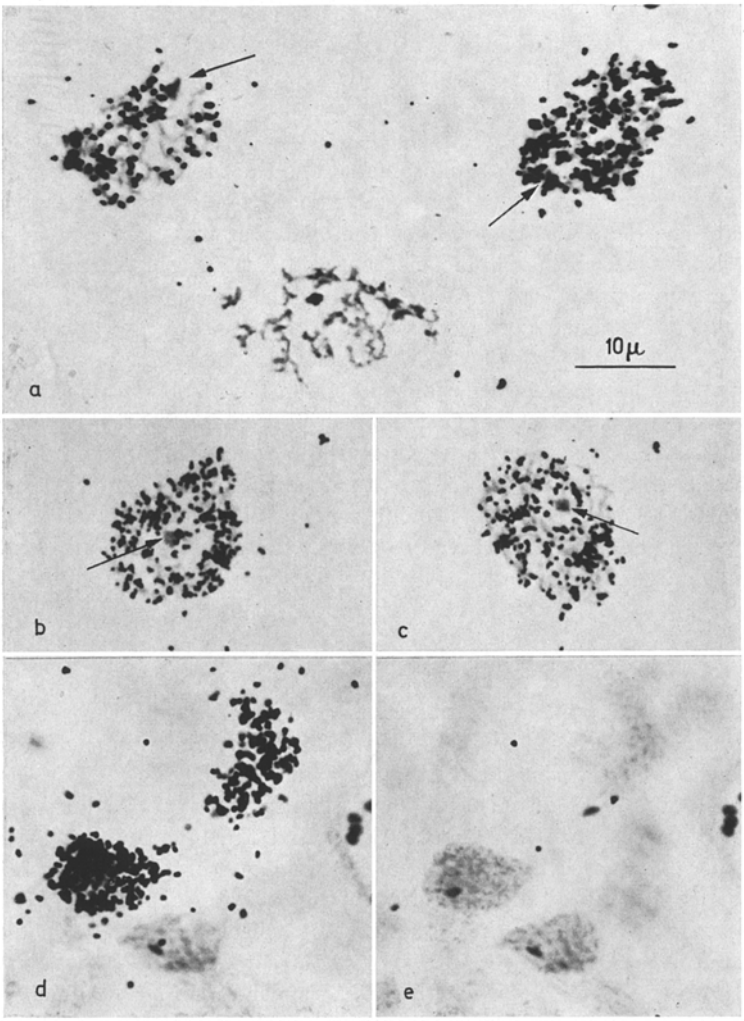
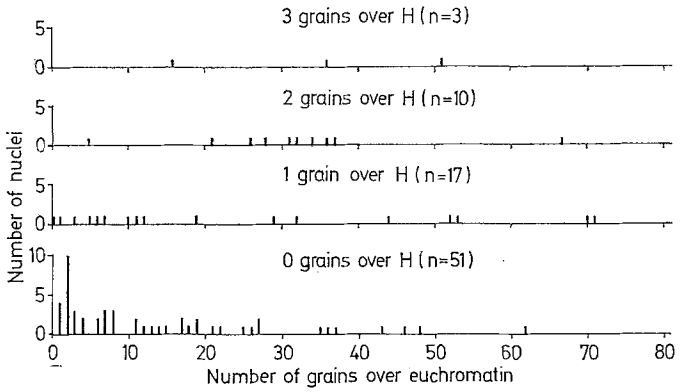
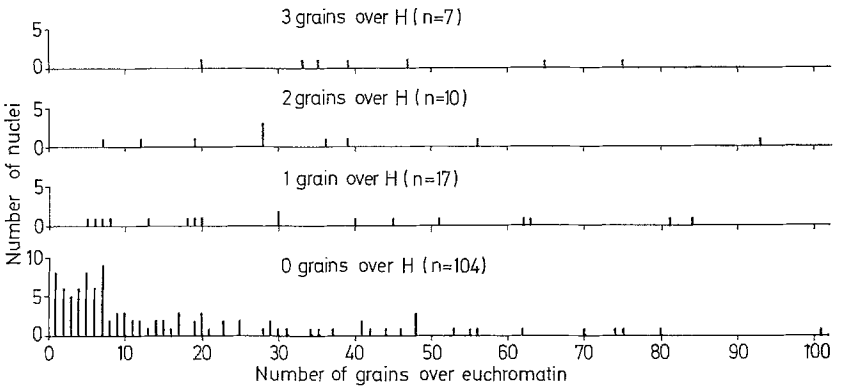


Fig. 5a–e. Photographs of autoradiographs of testis sheath nuclei from *P. citri* exposed to H^3-thymidine. The arrows point to the H body. a Two nuclei have incorporated label and one nucleus is unlabeled. A single silver grain is seen at the periphery of the H body in the nucleus at the left. Several grains are localized at the periphery of the H body in the nucleus at the right. b Both euchromatin and the H body are labeled. The grains are localized at the periphery of the H body. c Label is localized exclusively over euchromatin. d Two densely labeled nuclei in which the H body could not be identified with certainty. e Same nuclei as in d after the silver grains had been bleached. The H body was partially obscured by silver grains in the upper nucleus and completely obscured in the lower nucleus



a



b

Fig. 6a and b. A series of frequency diagrams showing the distribution of silver grains over euchromatin in testis sheath nuclei which incorporated H^3-thymidine. The nuclei are grouped according to the number of grains over the H body (H). a Data are from experiment A. b Data are from experiment B. There were no nuclei with 4 grains over the H body. One nucleus had 5 grains over the H body and 25 grains over euchromatin

different in the two experiments. Within an experiment all of the animals were incubated for approximately the same length of time but not simultaneously. Since the distribution of nuclei with respect to the number of silver grains was similar in animals from the same experiment, the individual results have been combined. The data for experiment A include results from six animals and the data for experiment B include results from fourteen animals.

The grain counts over euchromatin and those over the H body in individual nuclei are given in a series of frequency diagrams in Fig. 6. The counts have not been adjusted for background grains since actual counts show that only one grain or less per nucleus could be attributed to background activation. The data show that the amount of label incorporated by different nuclei within a short time period varies widely and that the variability is greater in euchromatin than in the H body. Although most of the nuclei in which counts are possible have fewer than 20 grains per nucleus, the grain counts for euchromatin range from one to 101. The counts for the H body range from one to five. Most of the grains were localized at the "periphery" of the H body. Nuclei in autoradiographs were not measured spectrophotometrically and therefore no correlation between the amount of DNA in a nucleus and the number of silver grains can be made.

Sixty-three percent of the nuclei in experiment A and 75% of the nuclei in experiment B had silver grains localized exclusively over euchromatin. A single nucleus in experiment A had one silver grain over the H body and was unlabeled over the euchromatin. The remaining nuclei had grains localized over both the H body and the euchromatin (Fig. 5).

Correlation coefficients were used to examine the relationship between the number of silver grains over the H body and the number over euchromatin in individual nuclei. The coefficients calculated from the data of experiments A and B were 0.304 and 0.314, respectively. They are positive and significantly different from zero ($P<0.005$). Thus there is a tendency for a higher grain count in the H body to be associated with a higher grain count in euchromatin. However, this result would be expected if the silver grains over the H body were due entirely to scatter from euchromatin and not to actual incorporation into the H body.

All labeled nuclei were examined and recorded in order not to bias the results. In addition to the nuclei just considered, others in the same animals were so densely labeled that grain counting over the euchromatin was impossible. Here the frequency of nuclei with a labeled H body was increased over that observed for the less densely labeled group; however, the actual number of silver grains over the H body was low. For experiment B the numbers ranged from 1 to 6 with a mean of 2.6 ± 0.2 grains per H body as compared with a range of 1 to 5 and a mean of 1.8 ± 0.2 for the less densely labeled nuclei. These results are consistent with an increase in scatter which would be expected as the adjacent euchromatin becomes more densely labeled.

The data obtained from autoradiographs of testis sheath tissues exposed to H^3-thymidine for periods of 15 or 30 min may be summarized

as follows. Some, but not all, of the testis sheath nuclei in third and fourth instar larvae incorporated the label. Labeled nuclei had silver grains localized either exclusively over euchromatin or over both euchromatin and the H body. Nuclei with silver grains localized exclusively over the H body were not observed. The number of silver grains observed over euchromatin in different sheath nuclei varied over a wide range of values. The H body was either unlabeled or had only a few grains over it, generally localized at the "periphery". There was a small but statistically significant positive correlation between the number of silver grains over the H body and the number over euchromatin in the same nucleus. The latter observation supports the explanation that the label over the H body is due to scatter from the euchromatic areas of the nucleus. It is concluded that there is no evidence for specific measurable incorporation of H^3-thymidine into the H set of chromosomes.

Discussion

Cytochemical Variables

1. Feulgen Reaction in Relation to Eu- and Heterochromatin

Atkin and Richards (1956) reported that human leukocytes in a Feulgen-stained vaginal smear had a 10% lower dye content than diploid cervical epithelial cells. Their observation was confirmed by Hale (1963), who found that immature cells of the bone marrow had the same dye content as diploid kidney and liver cells but mature bone marrow cells bound 10% less dye. He observed further that in phytohemagglutinin cultures when lymphocytes are transformed to blast cells, the nuclei enlarge and the apparent dye content increases before DNA synthesis begins (Hale and Cooper, 1965). Hale suggested that the decrease in dye binding was the result of the "compact manner in which the nucleoprotein is packed in mature leukocytes" (Hale, 1966). Similarly there is a decrease in dye binding capacity concomitant with an increase in chromatin compaction during spermiogenesis in bull (Gledhill, Gledhill, Rigler and Ringertz, 1966) and rat (Garcia, 1969b). Using nuclear area as an index of compaction for interphase chromatin in human leukocytes and chromosome length as an index of compaction for metaphase chromosomes, Mayall (1968) and Mayall and Mendelsohn (1967) observed a decrease in the apparent stain content corresponding to a decrease in nuclear area and chromosome length. Sandritter, Jobst, Rakow, and Bosselman (1965) propose that during hydrolysis the aldehyde groups of heterochromatin are freed more slowly than those of euchromatin. In such a case, nuclei with the same amount of DNA but with different proportions of heterochromatin and euchromatin would bind different amounts of dye after similar hydrolysis times.

Measurements on the heterochromatic and euchromatic sets of chromosomes in a male mealy bug indicate that the heterochromatic set has a slightly lower total absorbance than the euchromatic set and agrees with observations on human leukocytes that compacted chromatin tends to have a lower dye content than diffuse chromatin. On the other hand, the euchromatic chromosomes become condensed during prophase but the total absorbance of the chromatin does not decrease between early prophase and metaphase. This observation is not consistent with the proposed correlation between compaction of chromatin and decreased dye binding and suggests the possibility that the condensed state of heterochromatin is different from the condensed state of chromosomes at metaphase.

2. Autoradiography

The relationship between the amount of label incorporated into a sample and the number of silver grains observed in an autoradiograph is influenced by the concentration of halide crystals in the emulsion, the thickness of the emulsion, the energy of the radiation, the distance of the radiation source from the emulsion, and the density of the material between the source and the emulsion. In the present case, many of these variables are not known. It is clear that a quantitative relationship between the number of grains in the autoradiograph and the number of emitted β-particles cannot be established. Furthermore, the factor of self-absorption might be expected to be greater for heterochromatin than euchromatin because the mass per unit area is greater in heterochromatin (Maurer and Primbsch, 1964). Consequently, grain counts over euchromatin and heterochromatin are not strictly comparable. Nonetheless, disintegrations from the upper surface of both euchromatin and heterochromatin would reach the emulsion. Therefore if label is incorporated into the H body, it is unlikely that all of it would be obscured as a result of self-absorption.

Nonreplication of the H Body

Spectrophotometric measurements indicate that the total absorbance of the H body remains at the 1c level while the total absorbance of the euchromatin varies in different testis sheath nuclei. The fact that the amount of DNA in the H set of chromosomes remains at the haploid level and the number of E chromosomes varies as 2^n multiples of the haploid chromosome number demonstrates that polyploidy is achieved by differential replication of one set of chromosomes, the euchromatic set. These observations eliminate the possibility that the heterochromatic chromosomes are replicating and half of the resulting daughter chromosomes subsequently undergo reversal to the euchromatic state. In such a

case the H body should have absorbance values greater than 1c during the period of DNA synthesis and prior to reversal, and there would be 5, 15, 35, or 75 euchromatic chromosomes after 0, 1, 2, or 3 cycles of replication and endomitosis instead of the observed 5, 10, 20 or 40 E chromosomes.

Although the absorbance measurements indicate that the amount of DNA in the H body does not increase, in autoradiographs a few silver grains may be localized over the H body. The number of grains cannot be accounted for by background radiation. Therefore they must arise either from a small amount of incorporation of H^3-thymidine by the H body or as a result of scatter of β-particles from radioactive label incorporated into adjacent euchromatin. The possibilities that H^3-thymidine is incorporated as a result of DNA turnover or that there is a very small amount of net synthesis of DNA not distinguishable by the microspectrophotometric technique cannot be eliminated. However, the explanation of scatter as the source of silver grains localized over the H body seems most reasonable in view of the absence of sheath nuclei with only the H body labeled and the fact that no selection was made for nuclei in which the H body was clearly separated from the euchromatin.

Replication Failure in Heterochromatin

Nonreplication of part of the chromosomal DNA often, but not always is associated with polyploidy or polyteny and involves chromosomes or regions of chromosomes which are heterochromatic. The mass of chromocentric heterochromatin in salivary gland nuclei of *Drosophila melanogaster* appears to be smaller than is expected on the basis of the level of polyteny of the chromosomes (Rudkin, 1965a, b). Spectrophotometric measurements of Feulgen stained nuclei indicated that (1) the total absorbance of polytene salivary gland nuclei fell below multiples of the absorbance measured in presumably diploid ganglion nuclei and (2) the percent of dye bound by chromocentric heterochromatin decreased with an increase in polyteny. Rudkin suggested that the deviations from the expected values could be accounted for by non-replication of the chromocentric heterochromatin during polytenization.

Spectrophotometric measurements on polyploid brain ganglion nuclei in *Drosophila hydei* indicated that DNA in euchromatin and heterochromatin undergoes stepwise replication but not necessarily in a coordinated manner (Berendes and Keyl, 1967). Usually heterochromatin progressed through fewer replication steps than euchromatin. However, in some nuclei the heterochromatin had progressed through the same or an even greater number of steps than euchromatin. Using a short

exposure to H^3-thymidine, the authors observed the typical asynchrony of DNA synthesis in hetero- and euchromatin.

Himes and Crouse (1961) observed that the "L" chromosomes of *Sciara coprophila*, which are present only in germline nuclei and are heterochromatic, appear not to replicate during endoduplication in the nurse cells. A related observation in another sciarid, *Bradysia hygida*, has been reported by Sauaia and Alves (1969). In *B. hygida* the mass of heterochromatin composed of the "L" chromosomes does not appear to increase to the same extent as the euchromatic chromosomes, but the heterochromatin does incorporate H^3-thymidine, suggesting that DNA synthesis in the "L" chromosomes is not completely repressed during endoduplication.

Therefore partial or complete repression of DNA synthesis in specific heterochromatic regions of chromosomes is not unique to mealy bugs. However, the observation on polyploid nuclei in *P. citri* is a unique example of control in DNA synthesis in that the entire set of heterochromatic chromosomes remains unreplicated while the euchromatic chromosomes undergo repeated cycles of replication.

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Dr. Gloria Lorick
Department of Biology
University of Chicago
Chicago, Ill. 60637
U.S.A.