HETEROCHROMATIN IN HUMAN MALE LEUKOCYTES

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ABSTRACT

Tritiated thymidine was added to peripheral blood cultures containing phytohemagglutinin so that DNA synthesis in interphase nuclei of white blood cells in the human male could be studied. After 57 hours in culture, a large heterochromatic body with a central position is seen in unlabeled Feulgen-stained nuclei. In labeled nuclei in which DNA synthesis was taking place in both the eu- and heterochromatin at the time the thymidine became available, the heterochromatin shows a higher number of silver grains per unit area, accompanied by a stronger Feulgen reaction, an indication of its higher DNA content. The time of DNA synthesis in the heterochromatin blocks is different from that in the surrounding euchromatin. The large heterochromatic block is composed of chromosome segments gathered together around the nucleolus but it is not part of this organelle. In preparations stained with azure A and acid fuchsin for demonstrating both the nucleolus and the chromosomes, six distinctly heteropycnotic chromosome segments can be seen associated with the nucleolus. Cells of all size categories were found to incorporate tritiated thymidine. The distinct appearance of autosomal heterochromatin in white blood cells may be the result of the new physiological conditions to which the cells are subjected in the medium containing phytohemagglutinin.

INTRODUCTION

The heterochromatin so far described in human somatic cells is confined to the sex chromosomes and to the human female. Studies on various human tissues have revealed a small dark body in the resting nuclei of females that is not present in the nuclei of males or occurs in less than 1 per cent of the nuclei in males. This difference in nuclei permits one to determine the sex of individuals by inspection of their cells and to detect some intersexual conditions. This small dark body, which has normally a peripheral position in the nucleus, has been attributed to the heterochromaticity of one of the two X chromosomes of the female (Ohno and Makino, 1961).

Autosomal heterochromatin does not seem to have been described in man. This is probably due to the fact that aceto-orcein has been the stain most widely used in studying human chromosomes. Since orcein is not a specific stain for DNA, the large dark regions found in interphase nuclei could not be identified accurately.

In the study of DNA synthesis in human chromosomes with tritiated thymidine, chromosomes are usually stained with the Feulgen reaction since it withstands best the autoradiographic procedure (Lima-de-Faria, Reitalu, and Bergman, 1961). Interphase nuclei of human leukocytes stained by this procedure reveal the presence of a large heterochromatic body positioned centrally in the nucleus.

MATERIALS AND METHODS

Blood from a healthy male with forty-six chromosomes was used in these experiments. This individual had a normal karyotype with one X and one Y chromosome, and no visible rearrangements in the autosomes. No heterochromatin was present in the nuclei of Feulgen-stained smears of the oral mucosa.

1. *Autoradiography*

The technique used has been described in detail by Lima-de-Faria, Reitalu, and Bergman (1961). Only the main steps will be mentioned here. Twenty ml of venous blood were taken with a syringe wet with heparin and after suitable treatment were centrifuged and cultured. Phytohemagglutinin was present in the culture medium. Tritiated thymidine was added to the medium in a concentration of 3 μc/ml with a specific activity of 1.9 curie/mM. The labeled thymidine was removed by centrifuging the culture and washing the sediment thoroughly with new culture medium free of tritiated thymidine. The culture was then allowed to proceed as before. Colchicine and hypotonic treatment were used. The colchicine concentration was 4 drops of a 0.001 per cent solution per 1.5 ml of culture medium. After the hypotonic treatment the cells were fixed in acetic-alcohol (1:3) for 10 minutes. The fixative was decanted and 2 drops of 45 per cent acetic acid were added. The cells were squashed between slide and coverslip and the latter removed by the dry ice technique (for references to this technique see Hungerford and Di Berardino, 1958). The slides were then passed through the alcohol series and finally immersed in water. They were then stained with fuchsin–sulfurous acid after which Kodak stripping film AR-10 was applied. The film was exposed for 2 days.

2. *Nucleolar Staining*

Besides the cultures used for autoradiography, to which H^3-thymidine was added, and which were stained with the Feulgen reaction, another series of cultures was grown in a similar way, but the cells were stained instead with a nucleolar stain in order to identify the nucleolus in leukocytes. The staining technique was the same as that employed by Reitalu (1957). The nucleolar stain used was acid fuchsin made by dissolving 0.5 per cent of the dye in 90 per cent alcohol containing 0.5 per cent picric acid. Azure A was used in these slides as a stain for chromosomes.

3. *Combined May-Grünwald-Giemsa's Stain*

A few drops of a control culture not treated with H^3-thymidine were taken after 57 hours of incubation and the cells smeared on a slide. These slides were subsequently stained with the May-Grünwald-Giemsa stain by Professor G. Glimstedt, Institute of Histology, University of Lund, to identify the types of cells present in the culture.

Some of the preparations stained with the Feulgen reaction and used in the autoradiographic studies were subsequently stained through the film with the May-Grünwald-Giemsa stain by Dr. A. Norden of the Lund University Hospital. These two histologists agreed on the identification of the smallest cell types found in the cultures as lymphocytes, and Professor Glimstedt identified the large dividing cells in prophase as being most probably neutrophilic leukocytes or juvenile forms.

RESULTS

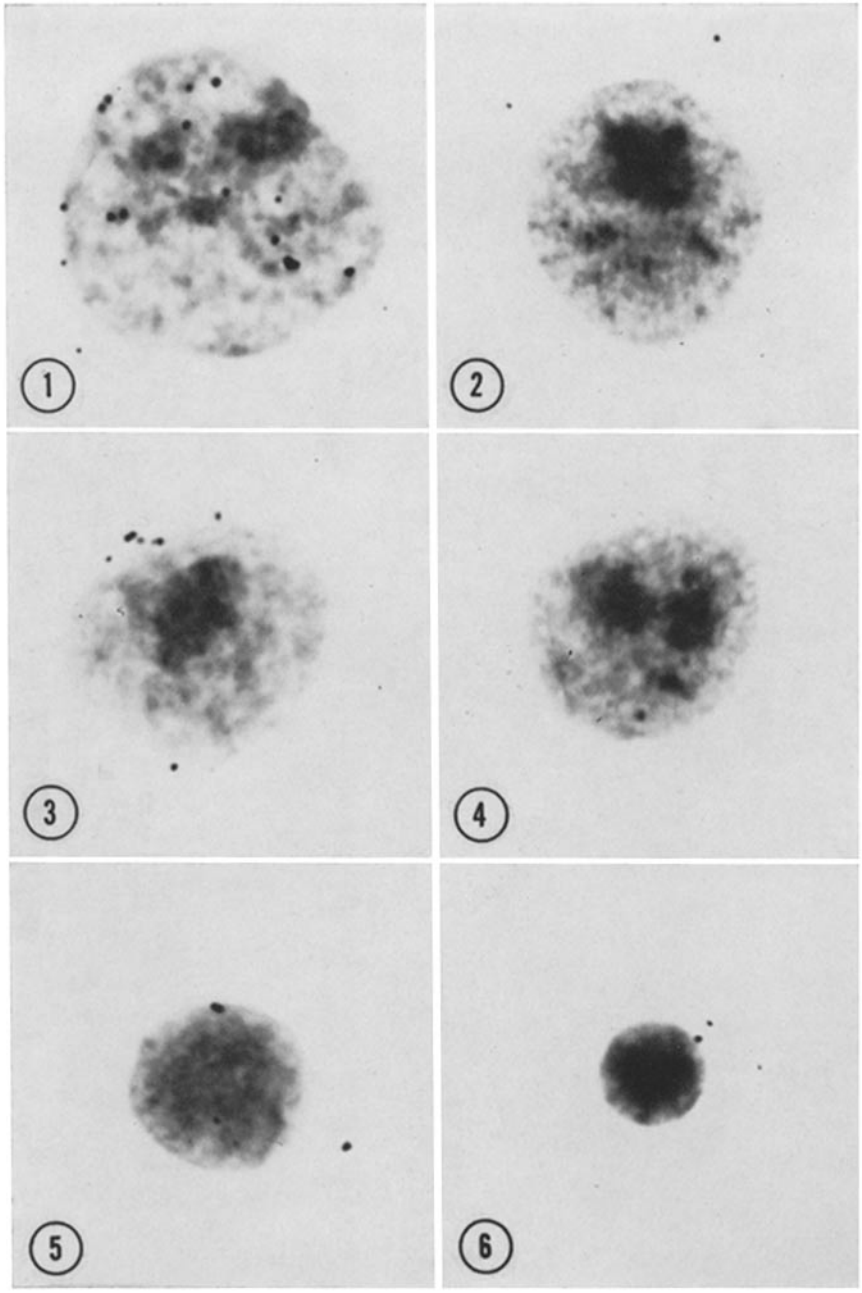
The preparations in which DNA synthesis was studied came from a blood culture that received the following treatment. After 57 hours of incubation at 37°C, tritiated thymidine was added to the medium and it remained in contact with the white blood cells for 3 hours and 30 minutes. The H^3-thymidine was then removed and the culture was allowed to proceed for 16 hours. Colchicine treatment followed for 2 hours, after which hypotonic treatment was applied and then the cells were fixed.

In the preparations that were stained with the Feulgen reaction and covered with transparent autoradiographic film, the nuclei show a distinct and large heterochromatic region that is intensely Feulgen-positive. In unlabeled nuclei, this region is seen to be well delimited, especially in the large- and medium-sized cell types. It is composed of one large block as seen in Figs. 2 and 3, or of two (Fig. 4) or three blocks (Fig. 1). In the smaller cell types (Figs. 5 and 6) it is less well defined or not detectable. The heterochromatic blocks have usually a central position in the nucleus.

In the nuclei which were synthesizing DNA at the time the H^3-thymidine was added to the culture, and hence became labeled, it can be seen that the heterochromatic regions contain four to five times more silver grains per unit area than the surrounding euchromatin (Figs. 7 to 11).

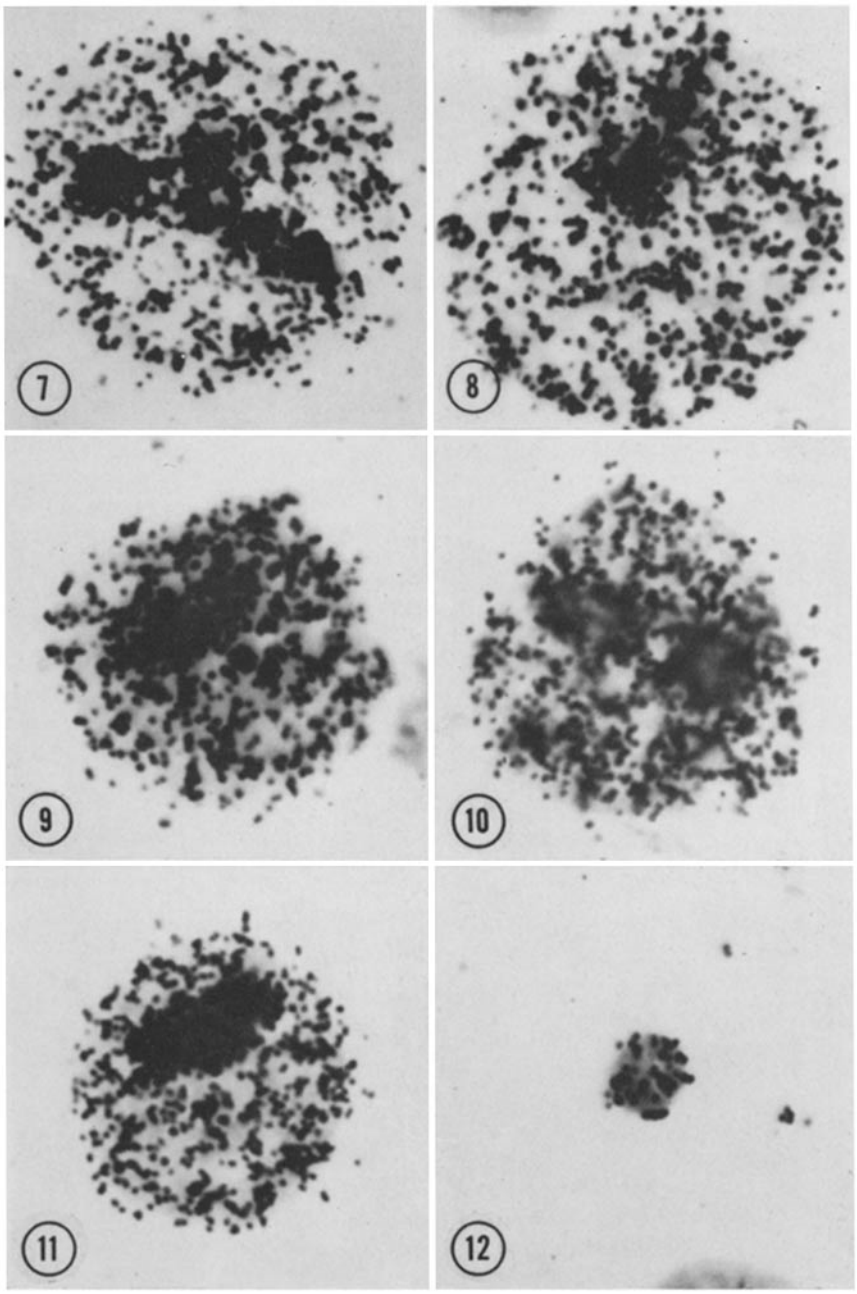
The nuclei of all sizes, from the smallest (Fig. 12) to the largest (Fig. 7), are found to be synthesizing DNA. This is taken to indicate that all cell types present after 57 hours of culture either show an increase in their chromatid number or subsequently undergo mitosis.

There is a period in DNA synthesis when the euchromatin alone replicates. This is seen in Figs. 13, 15, and 17, where the central, darkly stained region is unlabeled. Within the euchromatic regions there is also an asynchrony of replication. Not all chromosomes replicate simultaneously, as seen in Figs. 14, 16, and 18. In Fig. 14, especially, it can be seen that the silver grains are disposed in rows following the chromosome threads.



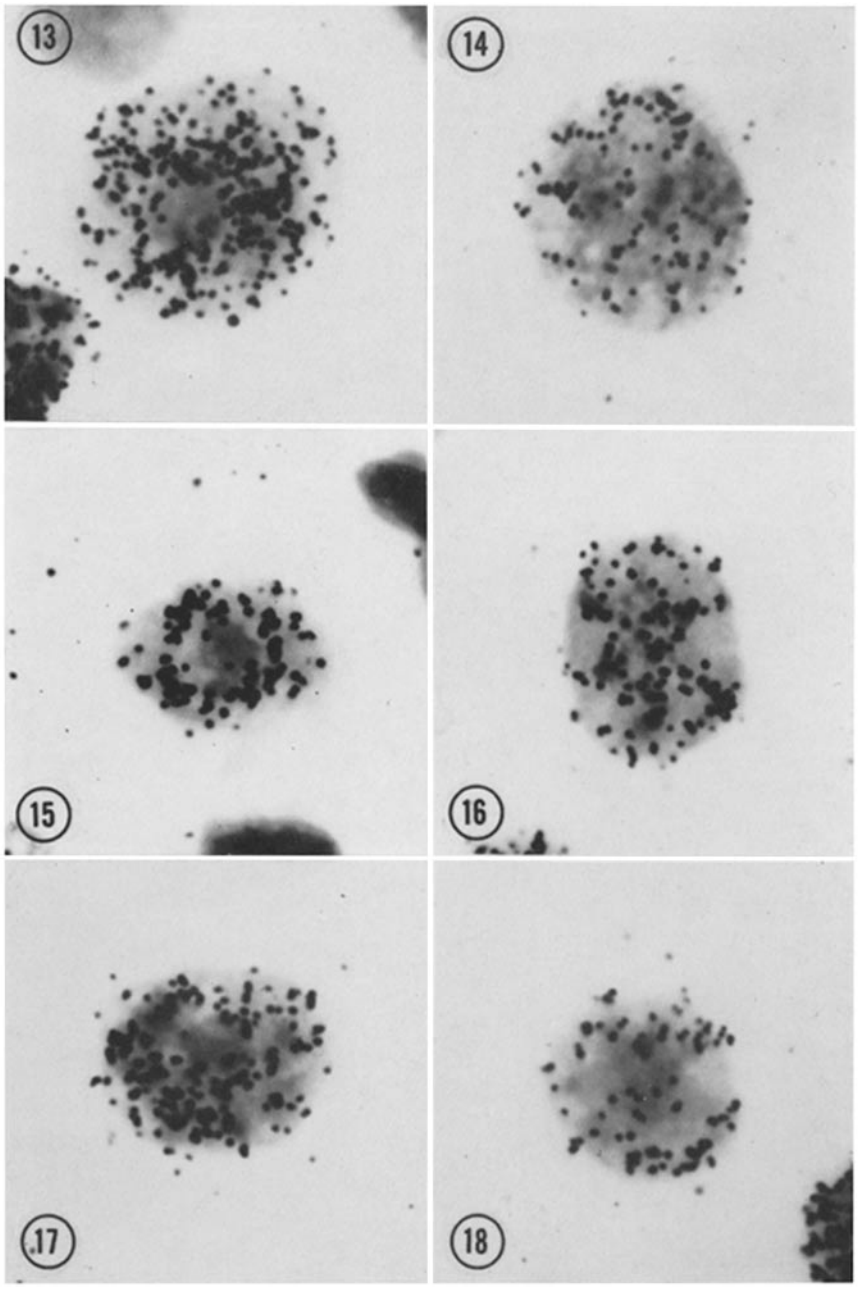
FIGURES 1 TO 6

Unlabeled (or only slightly labeled) white blood cell nuclei in the human male showing one large heterochromatic Feulgen-positive body (Figs. 2 and 3), two such bodies (Fig. 4), or three such bodies (Fig. 1). In the smaller size types the boundaries of the heterochromatic block are not so well defined (Figs. 5 and 6). Feulgen staining. Autoradiographic stripping film. × 3000.



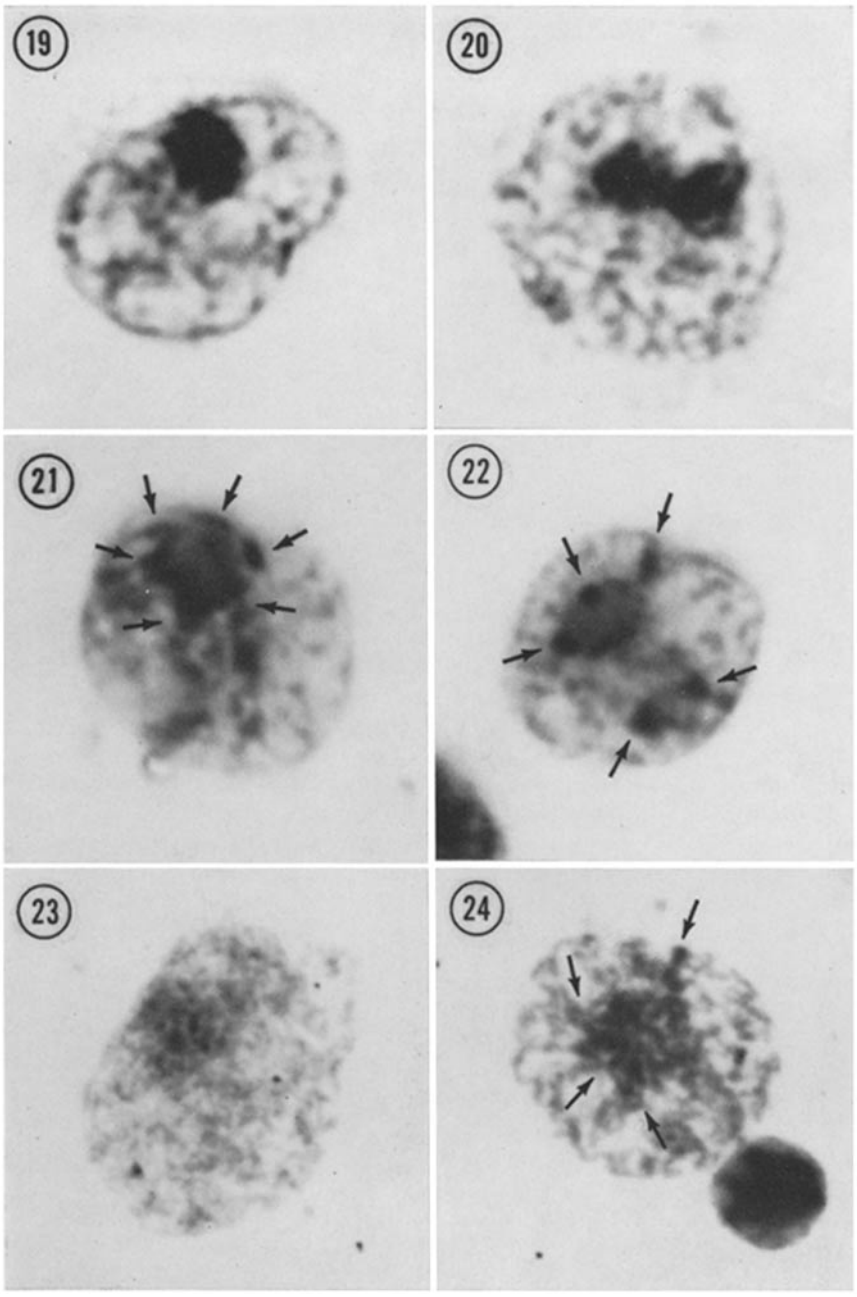
FIGURES 7 TO 12

White blood cell nuclei labeled with tritiated thymidine. The large heterochromatic regions of the nucleus contain a much higher number of silver grains per unit area than do the euchromatic regions of the nucleus. Feulgen staining. Autoradiographic stripping film. × 3000



FIGURES 13 TO 18

DNA synthesis in the euchromatin not accompanied by synthesis in the heterochromatin, which is unlabeled (Figs. 13, 15, 17). In Figs. 14, 16, and 18, only certain chromosomes are labeled, revealing an asynchrony of DNA synthesis within the complement. Feulgen reaction. Autoradiographic stripping film. × 3000.



FIGURES 19 TO 24

Figs. 19 to 22. Nuclei stained with azure A and acid fuchsin. One nucleolus is seen in Fig. 19 and two in Fig. 20. In Figs. 21 and 22 are seen the positively heteropycnotic regions of the chromosomes associated with the nucleoli (arrows). In Figs. 23 and 24 the nuclei are stained with the Feulgen reaction and covered with stripping film. Two early prophases show the chromosome segments involved in the formation of the heterochromatic block (arrows). × 3000.

That the heterochromatic regions are DNA-rich regions of the chromosomes is indicated by the intense Feulgen reaction and by the large number of silver grains. These regions are not part of the nucleolus, which is Feulgen-negative and does not incorporate H^3-thymidine.

In order to determine whether these heterochromatic regions were in any way associated with the nucleolus, a series of preparations was stained with azure A and acid fuchsin in order to determine the size and position of the nucleolus relative to that of the chromosomes (Figs. 19 to 22). The nucleoli appear to be about the same size as the heterochromatic block. One or two nucleoli are present in one nucleus (Figs. 19 and 20). In Fig. 21, it can be seen that six chromosome segments are associated with the nucleolus and that they are more deeply stained at the region of association. In Fig. 22, the nucleolus at the left is surrounded by three chromosome segments which have a deeply stained region at the zone of contact with the nucleolus, and the nucleolus at the right displays clearly two such chromosome segments. There are five to six chromosome segments with positively heteropycnotic regions associated with nucleoli, and it is the close association of these regions about the nucleolar material that leads to the formation of the heterochromatic blocks seen in Feulgen preparations. At early prophase in such preparations the chromosomes involved in the formation of the heterochromatic block are less entangled with one another, and the manner in which they associate to form the block can be partly traced (Figs. 23 and 24).

DISCUSSION

In cultured human leukocytes, it is known that five pairs of autosomes possess secondary constrictions accompanied by satellites and that these chromosomes appear to be associated with each other at metaphase during mitosis (Ferguson-Smith and Handmaker, 1961; Ohno *et al.*, 1961). These autosome pairs are Nos. 13, 14, 15, 21, and 22. In prophase studies of peripheral blood cultures, Ohno *et al.* (1961) found that six chromosomes were associated with the nucleolus. In our preparations we found six chromosome segments associated with this body but we cannot ascertain that they actually represent six chromosomes. The chromosome segments that constitute the bulk of the heterochromatic block are associated with the nucleolus, but this does not mean that they necessarily participate in the formation of this organelle. The phenomenon may simply represent a non-homologous heterochromatin association, such as that observed in many plant and animal species.

It is striking that this conspicuous heterochromatin block has not been described before. Its appearance may be attributable to the fact that the conditions of culturing change the phenotype of the chromosomes toward stronger heteropycnosis. Cells in other tissues taken directly from the body of the human male, such as oral mucosal cells, do not seem to reveal any large heterochromatic body when stained with Feulgen or Cresylecht violet (Battaglia, 1959; Ferguson-Smith, 1961). Occasionally, in Feulgen-stained squamous epithelial cells of the male one or two bodies that have been mistaken for sex chromatin have been described (Ferguson-Smith, 1961). The oral mucosal cells of the male individual used in our study did not show any heterochromatin in Feulgen-stained smears. In different tissues of an organism in which different physiological environments prevail, a given chromosome segment may exhibit at a corresponding stage quite different degrees of heteropycnosis. This is particularly clear in *Ornithogalum virens*, a plant with a haploid number of three chromosomes. Each chromosome can be recognized in most tissues of this plant. However, a strongly heterochromatic region which appears as a large knob at the prophase of meiosis is hardly present at the corresponding stage in the pollen grains (Lima-de-Faria, Sarvella, and Morris, 1959).

The addition of phytohemagglutinin to human blood cultures creates new physiological conditions that stimulate cell division. This new biochemical environment may lead to a different chromosome phenotype which is expressed by a strong heteropycnosis of the chromosomes that are gathered around the nucleolus.

Since the blood cells change morphologically as they develop in the phytohemagglutinin culture, it is of importance to know which of the original white blood cell types undergo mitosis. The labeling with tritiated thymidine is particularly helpful in this connection, because from our present knowledge of DNA chemistry we can assume that a nucleus which is labeled with H^3-thymidine is synthesizing its DNA in preparation either for cell division or for chromosome replication leading to endomitosis. Since the auto-

radiographic studies reveal that cells of all size categories found after 57 hours in culture incorporate tritiated thymidine, and since we have not found evidence of extensive polyploidy, it follows that all morphological categories of cells present at this stage can divide. At the same time, the May-Grünwald-Giemsa stain indicates that the smallest cell types are lymphocytes and that some of the largest cells may be neutrophilic leukocytes or juvenile forms. This result is not necessarily at variance with the work of MacKinney *et al.* (1962), because the drastic changes in cell phenotype make an accurate identification of the cell types very difficult.

The behavior of the heterochromatin in human male blood cells is similar to that of the heterochromatin in nuclei of grasshopper spermatocytes and rye leaf cells. In the nuclei of these cells, as in human leukocyte nuclei, it was found that the time of DNA synthesis in the heterochromatin is different from that in the euchromatin, and it was noted that there is an intermediate period during which the synthesis of the two types of chromosome material overlaps (Lima-de-Faria, 1959 *a* and *b*). The relation between asynchrony of replication within chromosomes and heterochromatin has been discussed by Lima-de-Faria (1962). In the spermatocytes of the grasshopper, *Melanoplus*, the absorbancy recorded in spectrophotometric measurements of Feulgen-stained cells was found to agree with the number of silver grains present in the same nuclear area. A higher number of silver grains and a stronger Feulgen staining per unit area are both the result of a greater DNA content (Lima-de-Faria, 1959 *b*).

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