Chromosomes of the Rat

II. DNA replication sequence of bone marrow chromosomes *in vivo*

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Autoradiography with tritiated thymidine has allowed us an insight of several cytological problems. By using this technique it has been possible to determine both *in vivo* and *in vitro* the cell generation time in several species (Cameron and Clefmann 1964, Prescott and Bender 1963, Quastler and Sherman 1959, Taylor 1960). Many reports have also communicated the pattern of chromosome replication in plants, insects and vertebrates (Bianchi and Bianchi 1965, Evans 1964, German 1962, 1964, Hsu 1964, Lima de Faria 1959, Lima de Faria *et al.* 1961, 1964, Schmid 1963). Moreover, autoradiography with tritium has furnished an exact method to test and analyse the semiconservative mode of chromosome replication (Prescott and Bender 1963, Taylor 1963, Taylor *et al.* 1957).

As outlined above, many questions have been answered by means of autoradiography with tritium. However, perhaps still more important, are the questions that autoradiography with tritium has left open to an answer.

In the present paper we shall study the duration of the cell cycle and the pattern of chromosome replication in the bone marrow cells of the rat *in vivo*. Results obtained will be used in the future as reference for studies about the behaviour of rat chromosomes in different situations.

Material and methods

Ten female and ten male albino rats weighing 100 to 200 gr. were studied. The animals belonged to a non-inbred strain maintained at the Institute of Physiology of Buenos Aires for more than 20 years.

Thymidine- H^3 (Sp. Act. 1.9C/mole.) was intraperitoneally injected in the amount of 2.5 μ C by gr. of body weight. Animals were killed by cervical dislocation at 25, 20, 15, 12, 11, 10, 5, 3, 2 and 1 hours after the injection. One cc of 0.04% solution of colchicine was intraperitoneally injected 3 hours before the killing of the rats. In cases in which radioactive material was administered 2- and 1 hours before sacrificing the animals, colchicine was injected immediately after.

Cytological preparation from bone marrow cells were prepared and stained as described elsewhere (Bianchi and Molina 1966).

Autoradiographic procedures—By means of pilot slides the time intervals at which a high or a low percentage of labeled metaphases appeared were checked.

At moments in which a high amount of metaphases became radioactives preparations were studied unmounted and well spread metaphases photographed. Once the film or emulsion was applied exposed and developed, autoradiograph was studied again and labeled metaphases re-photographed.

At moments in which a small percentage of metaphases became labeled, the double-photograph technique outlined above is time consuming. In these cases it is necessary to photograph a considerable amount of metaphases to obtain a suitable amount of labeled ones. To avoid useless work the following procedure was employed: a) preparations were

mounted exposed and developed; b) only well spread labeled metaphases were photographed; c) silver grains and gelatin of the film were removed; d) the same metaphases without labeling were re-photographed.

The method for removing silver grains and gelatin has been previously detailed (Bianchi *et al.* 1964). However its main steps are the followings: a) transfer slides to distilled water for 10 minutes to rehydrate the film; b) remove silver grains with 7.5% solution of $K_3Fe(CN)_6$ for 3 minutes; c) transfer to a 20% solution of $Na_2S_2O_3 \cdot 5H_2O$ for 3-5 minutes; d) rinse in water, three changes one minute each; e) immerse slides in 0.1% trypsin (1/250) solution in phosphatium buffer, for 10 minutes at 37°C; f) wash in tap water for 5 minutes, rinse in distilled water; g) dry in air, re-stain if necessary.

Kodak AR-10 stripping film or undiluted Ilford L-4 emulsion were applied onto the cytological preparations. In each experiment half of the slides were mounted with AR-10 film and the other half with Ilford L-4 emulsion. Exposure time varied between 75 to 90 days. Autoradiographs were developed 5 minutes in D-19 Kodak developer, fixed for 10 minutes in Kodak fixer and washed three times in distilled water 10 minutes each.

In each one of the 20 rats studied, 25 to 50 photographs were taken with a total of 610 metaphase plates photographed. Among these photographs 140 were selected for karyotype construction. Table 1 indicates the photographs taken and the karyotypes studied in each thymidine- H^3 treatment.

Table 1. Number of metaphases and karyotypes studied in each thymidine- H^3 treatment

Time-interval (in hours) be- tween the thy- midine- H^3 in- jection and the cellular fixa- tion	1	2	3	5	10	11	12	15	20	25	Total
Number of metaphases photographed	50	50	70	86	84	80	80	40	40	40	610
Number of karyotypes studied	—	10	30	40	30	30	—	—	—	—	140

Identification of chromosomes—Combination of chromosome morphology and tritium picture was avoided in the identification of chromosomes. Therefore unlabeled chromosomes were first arranged and autoradiographs were afterwards placed underneath. In this way the pattern of chromosome labeling introduced no bias into the identification procedure.

Chromosomes were sorted out according with their length, arm ratio and location of secondary constrictions. Homologue pairs were classified into five groups: group A formed by pairs 1-3; group B formed by pairs XY-4-10; group C formed by pairs 11-13; group D formed by pairs 14-17; group E formed by pairs 18-20. Chromosome morphology and reliability in the criteria of identification have been detailed elsewhere (Bianchi and Molina 1966).

Results

Duration of the treatments with thymidine- H^3 . When tritium is added to a cell population *in vitro* the time lapse during which the isotope is available to the cells can be determined with accuracy. On the other hand, when thymidine- H^3 is injected to animals it is very difficult to know how much time a given tissue has used the tritium. In our experiments as no scintil-

lation counts of body fluids were done, no direct information about the turnover of non-DNA tritium can be afforded. However, a previous report of Quastler and Sherman (1959) indicates that after 5 minutes of the intra-peritoneal injection labeling per cell in the intestinal epithelium of the mouse reaches half its final value and saturation is reached between 10 to 20 minutes.

Although the uptake of tritium is influenced by different factors according to the tissue analysed (Steel and Lamerton 1965), the report of Quastler and Sherman can be probably made extensive to the bone marrow cells. Hence thymidine- H^3 treatments performed in our experiments must be considered as equivalent to a 10-20 minute-pulse treatment.

Cell generation time and percentage of labeled nuclei. The number of totally and partially labeled metaphases and the percentage of labeled interphase nuclei were analysed in each experiment (Fig. 1).

Fig. 1. Treatments with thymidine- H^3 *in vivo*. Percentage of unlabeled, partially and totally labeled metaphases. The figure also points out the references taken to determine the duration of the cell generation time, the S- and the G_2 periods (see text).

Fig. 1 points out that in the animals sacrificed 1 hour after the tritium injection no labeled metaphase was found. At the 2 hour-interval treatment a 10% of metaphases showed radioactivity; increasing this figure progressively up to 100% at the 5 hour-interval treatment. Later percentages fell to a 50% and 30% at the 10- and 11 hour-interval treatments respectively. In all these treatments both sister chromatids in each chromosome appeared labeled indicating that cells had divided once after the thymidine- H^3 injection (Fig. 1).

At 12, 15 and 20 hour-interval treatments the number of labeled metaphases was 10%, 82% and 100% respectively. Afterwards percentages

decreased to 85% at the 25 hour-interval treatment. In the 12-25 hour-interval treatments only one chromatid of each labeled chromosome was labeled indicating that cell population had reached second division since being labeled (Fig. 1).

Thus far the following information can be obtained (Fig. 1): a) the interval between the end of DNA synthesis and metaphase varies between 2 and 3 hours for a 90% of the cells (G_2 period); b) a measure of the average generation time is the interval between the two maximal peaks of labeling or about 15 hours (Taylor 1960); c) if metaphases labeled in both chromatids are only considered, the time interval between the 50% of the ascending and descending slope of percentages (7 hours interval) can be assessed as the average duration of the S period (Quastler and Sherman 1959, Siskin 1964); d) by subtracting to the cell generation time the S and G_2 periods the duration of the G_1 period is obtained (5 hours); e) the coexistence of totally and partially labeled metaphases at each thymidine- H^3 treatment, reveals that bone marrow cells synthesize DNA asynchronously.

It is actually held that when a growing cell population is labeled and harvested before any cell had reached the mitosis the percentage of labeled nuclei represent the percentage of the total cell cycle taken up by the S period (Siskin 1964, Taylor 1960).

As demonstrated before, in the bone marrow of the rat the S phase occupies about 45% of the total cell cycle. However the percentage of labeled nuclei in the 1 hour-interval treatment is only 32% (Fig. 1). Furthermore this percentage progressively increases in the 1 to 25 hour-interval treatments up to 73% (Fig. 1). To explain why interphase nuclei become labeled in a lower percentage than the expected one and to explain the progressive increase of percentages in the 1 to 25 hour-interval treatments, we have to bear in mind that bone marrow is formed by dividing (myeloid series) and non-dividing cells (mature cells). A population like that is only partially growing. Therefore the percentage of labeled nuclei is lower than the expected because among the unlabeled cells we are scoring not only dividing cells at G_1 and G_2 stages, but also mature cells which synthesize DNA no longer. When the tritium labeling is performed farther enough from the moment of harvesting some cells will divide once. Hence, according with the semi-conservative fashion of duplication, one labeled cell will give rise to two daughter labeled cells. On the other hand, percentage of unlabeled nuclei do not increase in the same way because mature labeled cells do not divide anymore. Consequently, the farther the moment of labeling is to the moment of harvesting so much larger will be the number of cells which divide and therefore so much larger will the relative number of labeled nuclei be.

Pattern of chromosome replication. Considering the problem of chromosome duplication it is possible to analyse which chromosomes are labeled

in each one of the pulse treatments performed. However it is evident that the picture obtained will be somewhat blurred. At any thymidine- H^3 treatment there are cells in different stages of the S period. Hence what is obtained with the above system are overlapping patterns of replication from an asynchronous cell population.

Figs. 2-5. 2-3, thymidine- H^3 treatment 11 hours before harvesting. Metaphase plate of a male cell (pre- and post-autoradiography) at the S_1 stage. Note the labeling scattered almost all over the complement. Arrow points out the Y chromosome unlabeled. $1000\times$. 4-5, thymidine- H^3 treatment 10 hours before harvesting. Metaphase plate of a female cell (pre- and post-autoradiography) at the S_2 stage. Note the labeling over the entire length of every chromosome. Arrow points out a chromosome tentatively identified as one of the X chromosomes; note its heavier labeling in respect to the other elements of the set (see text). $1000\times$.

In solving the problem, all the karyotypes were analysed without taking into account the moment of labeling. By using this criteria it was possible to separate the karyotypes into four groups according to their pattern of labeling (Groups S_1 , S_2 , S_3 , S_4). Afterwards, when the moment of labeling

was considered for each karyotype it appeared clearly enough that each one of the groups was representative of a different stage of the S period.

Group S₁—All the metaphases included in this group were partially labeled. Silver grains were scattered over the chromosomes and no special intrachromosomal patterns of labeling were observed (Figs. 2, 3). In the few cases in which silver grains became packed enough to form clumps they were

Figs. 6-9. 6-7, thymidine- H^3 treatment 5 hours before harvesting. Metaphase plate of a male cell (pre- and post-autoradiography) at the S_3 stage. Note the patches of radioactivity localized over small regions of the chromosomes. Arrow points out the Y chromosome heavily labeled. $1000\times$. 8-9, thymidine- H^3 treatment 3 hours before harvesting. Metaphase plate of a male cell (pre- and post-autoradiography) at the S_4 stage. Arrow points out the Y chromosome labeled. Note the slight labeling over the rest of the set. $1000\times$.

located over the telomeric regions of chromosomes Nos. 1 and 2 (Figs. 11, 12, 13, 14, 15).

When less than one-half of the complement was labeled, density of grains over the chromosomes was low. As the number of labeled chromo-

somes increased, density of silver grains over them also increased. In this way the pattern of labeling we shall describe in group S_2 was progressively reached.

With the exception of pair No. 2 all the other pairs showed homologue asynchrony.

Metaphases within this group were only observed in the 11- and 10 hour-interval treatments. Hence they were considered as representatives of the initial stage of the S period (S_1 stage).

Fig. 10. Treatment with thymidine- H^3 15 hours before harvesting. Metaphase plate of a male cell (post-autoradiography). Note only one chromatid labeled indicating that the cell was engaged in the second division since being labeled. Arrows point out two sister chromatids exchanges. 1500 $\times$.

Group S_2 —At the moment in which all the chromosomes of a metaphase became labeled the density of silver grains over them increased considerably. Consequently, metaphases included within this group exhibited radioactivity over the entire length of every chromosome (Figs. 4, 5, 11, 12, 13, 14, 15, 16).

Metaphases with the described pattern of labeling were found in 10- and 5 hour-interval treatments. They were considered representatives of the intermediate S period in account of: a) these metaphases were found farther

inside the S period than those of the previously described group; b) the progressive increase of radioactivity from metaphases at the S₁ stage up to metaphases at the present stage (Figs. 4, 5).

An outstanding fact of the S₂ stage was the finding of metaphases with a chromosome of the group XY-4-10 showing about twice more grains, than that observed in the other elements of the set. This chromosome was always one of the largest of the group and it was only seen in cells from female individuals. Consequently it was tentatively identified as one of the two X chromosomes in the female (Figs. 4, 5, 11, 12, 13, 14, 15).

Group S₃ —

Metaphases included within the present group were encountered among the cells treated 5- and 3 hours before harvesting. Thus they were interpreted as belonging to cells engaged in a late stage of the S phase (S₃ stage) at the moment of tritium uptake.

Metaphases at this stage were labeled in more than half of the complement. These metaphases showed chromosomes or chromosome regions which have already finished their duplication. Consequently, patches of radioactivity were found over small chromosome areas (Figs. 6, 7).

Pair No. 1 showed bands of radioactivity located in the junction of the proximal third with the two distal thirds of the long arm. Over chromosomes No. 2 silver grains were located along the halfway-point of the arm. One chromosome No. 3 showed radioactivity over the short arm while the other exhibited a patch of silver grains in the midway of the long arm. (Figs 11, 12, 13).

Group XY-4-10 did not reveal a consistent pattern of labeling. However in several cases silver grains were located over the kinetochore regions letting the lower chromosome half free (Fig. 16). A heavy labeling over the Y chromosome was almost constantly seen in this group (Figs. 6, 7).

Pairs No. 11, 12 and 13 showed localized spots of radioactivity over their short arms (Fig 15). A preferential labeling over one arm of chromosomes 14-20 was very often seen.

Fig. 11. Pattern of labeling of pair No. 1 throughout the S period (autoradiographs placed underneath). S₁: silver grains scattered over the chromosomes but showing a certain tendency to locate on the telomeric regions. S₂: labeling over the entire length of both chromosomes. S₃: labeling in the junction of the proximal third with the two distal thirds of the long arm. S₄: homologue asynchrony.

Figs. 12-14. 12, pattern of labeling of pair No. 2 throughout the S period (autoradiographs placed underneath). S₁: predominant labeling over the telomeric regions. S₂: labeling over the entire length of both chromosomes. S₃: labeling in halfway mark of the arm. S₄: homologue asynchrony. 13, pattern of labeling of pair No. 3 throughout the S period (autoradiographs placed underneath). S₁: labeling scattered over the chromosomes. S₂: labeling almost over the entire length of both chromosomes. S₃: labeling over the

The pattern of labeling over the pairs No. 1, 2, 3, 11, 12 and 13 and the pattern of labeling over the Y chromosome were detected in almost all the metaphases included into this stage. The remaining pairs of the set, labeled in a characteristic way, were less frequently found.

Asynchrony of duplication between homologues could be detected over several pairs (Fig. 16).

Group S₄—Metaphases in this group exhibited a similar pattern of labeling than that of the preceding group. The main differences between the two groups were that the present one showed: a) labeling in less than one-half of the complement; b) a more striking asynchrony between homologues (Figs. 11, 12, 13, 14, 15, 16); c) very often the Y chromosome labeled and the remaining chromosomes of group XY-4-10 unlabeled or slightly labeled (Figs. 8, 9, 14).

Metaphases with the above pattern were found in the 3- and 2 hour-interval treatments. Thus they were considered as representatives of the final S period (S₄ stage).

Chronology of chromosome duplication. The classification of the karyotypes in four groups corresponding to four different stages of the S period allowed to determine the time-course of chromosome duplication.

According to their pattern of labeling metaphases were ranked from the initiation up to the end of the S period. Thus, chronology of chromosome duplication could be determined by determining the percentage of labeling of each chromosome in each one of the four groups. In the case of some easily identifiable pairs (Pairs No. 1, 2, 3, 11, 12 and 13) the time-course of replication could be followed with accuracy. In the other chromosomes,

Fig. 15. Pattern of labeling of pairs 11, 12 and 13 throughout the S period (autoradiographs placed underneath). S₁: labeling scattered over the chromosomes. S₂: labeling over the entire length of every chromosome. S₃: labeling localized over the short arm of all the chromosomes. S₄: homologue asynchrony.

short arm of one chromosome and on the midpoint of the long arm in the other. S₄: homologue asynchrony. 14, pattern of labeling of group XY-4-10 throughout the S period (autoradiographs placed underneath). First pair in each row are the presumptive sex chromosomes. S₁: labeling scattered over the chromosomes. Note the Y chromosome unlabeled and the rest of the chromosomes in the group labeled. S₂: labeling over the entire length of every chromosome. Note the heaviest labeling over a presumptive X chromosome (first chromosome in the row). S₃: labeling over the kinetochore regions or in the upper half of most chromosomes. S₄: Y chromosome labeled and the rest of the chromosomes in the group unlabeled or slightly labeled.

due to difficulties in their identification, sequence of replication is less precise.

Fig. 18 shows the sequence of chromosome replication and the percentage of homologue asynchrony at the four stages of the S phase. At the S_1 stage the first chromosomes to replicate were the X chromosomes and the pairs 6, 9 and 10. Immediately after duplicated chromosomes No. 1, 5, 7, 11, 12, 13, 17, 19 and 20. Later, pairs No. 2, 3, 4, 8, 14, 15, and 16 began DNA synthesis. Afterwards replicated pair No. 18 and finally the Y chromosome was the last to start DNA synthesis.

With the exception of pair No. 2 homologue asynchrony at the initial S period was met over all pairs.

Fig. 16. Chronology of chromosome replication and number of karyotypes analysed. S_1 : initial stage of the S period. S_2 : intermediate stage of the S period. S_3 : late stage of the S period. S_4 : final stage of the S period. Black full lines represent the pairs of chromosomes and their percentage of labeling at each stage of the S period. Black dotted lines represent the percentage of homologue asynchrony. Note the Y is the last chromosome to begin replication. Also note the absence of a late replicating X chromosome.

At the S_2 stage all the metaphases exhibited radioactivity over the entire length of each chromosome and no homologue asynchrony could be detected.

At the late and final stages of the S period (S_3 and S_4 stages) several chromosomes finished replication and homologue asynchrony could be observed again. The first pair to finish duplication was the No. 13. Later, pairs No. 18, 19 and 20 concluded replication. At the halfway mark of the S_4 stage pairs 8, 16 and one X chromosome in the female completed DNA synthesis. Afterwards, pairs 1, 4, 5, 6, 7, 9, 10, 14, 15, 16 the remaining X chromosome in the female and the X chromosome in the male finished their replication. Chromosomes 11, 12, 3, 2 and the Y chromosome were the last to replicate finishing the DNA synthesis in the above numerical order.

No late replicating X chromosome was found in any metaphase of female individuals.

Discussion

Experiments dealing with the cell generation time and with the pattern of chromosome duplication have been performed on several species (Benirschke *et al.* 1964, Bianchi and Bianchi 1965, Fraccaro *et al.* 1965, Galton and Holt 1964, 1965, Gartler and Burt 1964, German 1962, 1964, Kikuchi and Sandberg 1964, Hsu 1964, Moorhead and Defendi 1963, Mukherjee and Sinha 1964, Schmid 1963, Taylor 1960, *et al.* 1957). However differences in the methods employed make the results homologueably difficult. Most authors have focused their investigation on the pattern of chromosome labeling at the final stage of the S phase or only on the behaviour of the sex chromosomes (Fraccaro *et al.* 1965, Galton and Holt 1964, 1965, German 1964, Gilbert *et al.* 1962, Gartler and Burt 1964, Grumbach *et al.* 1963, Morishima *et al.* 1962, Schmid 1963). Others have studied the duration of the S period or the cell generation time without analysing in full the pattern of chromosome replication (Taylor 1960, Kikuchi and Sandberg 1964). Studies facing the behaviour of the chromosomes throughout the S period have been only reported for normal human leukocytes and Chinese hamster fibroblast maintained *in vitro* (Bianchi and Bianchi 1965, Hsu 1965). Similar studies in the *in vivo* state have not been undertaken up to date and only partially aspects of the problem have been reported by Srinivasan (1964) in the lens of the rabbit.

Although the information obtained was variable according to the species studied and the design of the experiment, some patterns of chromosome duplication were found out every time they were sought. Consequently attempts at generalization can be made for some facts such as the semi-conservative mode of chromosome replication and the different kinds of asynchronous DNA synthesis.

Under the following headings we shall try to compare our results in the rat *in vivo* with the results obtained by other authors in others species both *in vivo* and *in vitro*.

Replication of DNA. It is actually held that DNA replicates in a semi-conservative fashion (for a revision see Taylor 1963). In account of this it is accepted that each chromosome is at least formed by two complementary chains of DNA, which at the moment of replication become separated and act as a template for the synthesis of two new strands. Hence, after mitosis, each daughter cell receives an old DNA strand and a new synthesized one.

If at the moment of replication thymidine- H^3 is taken up by the chromosomes and afterward the cells are left to divide in a tritium free medium, the distribution of labeling can be predicted as follow: a) at the first division after labeling both chromatids of each radioactive chromosome at metaphase

are labeled (Figs. 2, 3, 4, 5, 6, 7, 8, 9) ; b) at the second division after labeling one single chromatid of each radioactive chromosome at metaphase is labeled (Fig. 10).

The above postulates were originally demonstrated by Taylor (1957) in *Vicia faba*. Afterward, similar observations were made in other species of plants, in Chinese hamster and in human beings *in vitro* ; in the rabbit *in vivo* and in the present study in the rat *in vivo* (Hsu 1964, Kikuchi and Sandberg 1964, Prescott and Bender 1963, Srinivasan 1964, Taylor 1963).

Besides to support the semi-conservative mode of chromosome replication in the *in vivo* state, the study of metaphases engaged in the second division after labeling yield information about the sister chromatid exchange. To date such phenomenon has been observed in vicia, Crepis, Bellavalia, Allium, Chinese hamster, human cells and grasshopper (Taylor 1963). In the present experiment distribution of labeling indicating sister chromatid exchange was detected in almost all the metaphases studied in the second division after labeling. Fig. 10 points out two chromosomes in which this exchange is observed. A detailed study of the frequency and location of sister chromatid exchanges will be communicated elsewhere.

Asynchronous DNA synthesis by the chromosomes. Asynchrony of autosome duplication in mammalian was initially proved by Taylor (1960) in Chinese hamster. Ever since asynchrony was found in each species in which it was sought. Thus, it is actually known that asynchrony exists in the case of normal and leukemic human beings and throughout the S period in the Chinese hamster (Bianchi and Bianchi 1965, German 1962, 1964, Kikuchi and Sandberg 1964, Lima de Faria *et al.* 1961, 1964, Hsu 1964).

The present study yields information about the existence of intra- and interchromosomal asynchrony as much at the beginning as at the end of the S period in the rat *in vivo*. Since the factor causing the initiation of DNA synthesis are far from being fully understood, the origin of the asynchronous chromosome replication at the beginning of the S period is not known. Experiments in bacteria (Maaløe 1963) have shown that some type of protein and RNA synthesis is necessary before the cell can initiate DNA synthesis. The need for protein synthesis might indicate that after each replication the enzymes involved are destroyed, having to resynthesize before each new round of replication is started. If we suppose that bacteria and mammalian cells have a similar regulatory mechanism for replication and we assume the necessity of a different concentration of enzyme for each chromosome to start DNA synthesis, a presumptive explanation for the asynchrony at the early S phase is obtained.

It has been demonstrated in bacteria (Kuempel and Pardee 1963) and also in mammalian cells treated with FUDR (Hsu *et al.* 1964) that once the DNA synthesis has began, it follows independently to the nutritional conditions of the cell. Presumptively then, to explain asynchrony at the end

of the S phase we have to consider different factors to those we have assessed to explain asynchrony at the beginning of the S period. Schmid (1963) has demonstrated that asynchronous late replicating regions over human chromosomes coincide with the location of secondary constrictions. In a previous paper the location of secondary constrictions over the rat chromosomes has been described (Bianchi and Molina 1966). The present experiment indicates that late replicating regions over several pairs of the rat (pairs No. 1, 2, 3, 13) are located in the same place just as secondary constrictions. Considering that secondary constrictions represent heterochromatic regions (Saksela and Moorhead 1962, Sasaki and Makino 1963) it can be assumed that asynchronous replications at the end of the S period might be closely related with the heterochromatin indicating, perhaps, a different metabolic activity to synthesize DNA between the hetero- and isochromatin.

In the present report as in the cases of human beings, Chinese hamster and *Vicia faba* (Bianchi and Bianchi 1965, Hsu 1964, Evans 1964) the distribution of silver grains over the chromosomes throughout the S period has been noteworthy. From the data obtained it can be assumed that chromosomes start replication over partial areas. Later they extend DNA synthesis to their total length and at the final S stage only small replicating zones—not those at the starting point—can be detected over the chromosomes.

Replication of sex chromosomes. In 1959 Lima de Faria was able to demonstrate that the X chromosome in prophase of first meiotic division in spermatocytes of *Melanoplus differentialis* synthesize out of phase in respect to the other chromosomes. In the case of mammalia Taylor (1960) was the first to demonstrate that in cells of female Chinese hamster one of the X chromosomes finishes the replication later than the other elements of the set. This late replicating X chromosome was afterward met with in the female cells of normal and leukemic human beings, bovines, Syrian hamster, donkey, mule, horse, donkey-zebra hybrid and canine species (German 1962, 1964, Gilbert *et al.* 1962, Morishima *et al.* 1962, Gartler and Burt 1964, Galton and Holt 1964, Mukherjee and Sinha 1964, Benirschke *et al.* 1964, Fraccaro *et al.* 1965). Moreover, in the case of bovines in which the X chromosomes are easily identifiable, it has been possible to demonstrate that the late replicating X chromosome is also among the chromosomes which start replication the earliest at the beginning of the S period (Gartler and Burt 1964). Experiments of Bianchi and Bianchi (1965) strongly suggest a similar phenomenon for human beings.

Studies performed in the female mule (Mukherjee and Sinha 1964) and in abnormal cases of human beings with one to four extra X chromosomes (Grumbach 1963, Gianelli 1963, Mukherjee *et al.* 1964) have allowed to complete the picture about the late replicating X chromosome. According to the above reports it is actually held that this chromosome is genetically inactive and the cause of the sex chromatin body in interphase nuclei.

X chromosomes in the strain of rats used by us are very difficult to identify. However, taking into account that they were pick out exclusively on morphological grounds, it is possible to assume a correct identification when a given pattern of labeling is repeatedly observed over the presumptive X chromosomes. In accordance with this in all the karyotypes analysed the X chromosomes are among the earliest chromosomes to begin DNA synthesis whereas the pairs easily mistaken with them begin replication later (Fig. 19).

At the end of the S period X chromosomes finish their replication before several other pairs (pairs No. 2, 3, 4, 6, 7, 9, 10, 11, 12 and Y chromosome). Consequently no late replicating X chromosome was observed in the female cells of the rat *in vivo*.

The absence of a late replicating X chromosome in the adult rat agrees with the fact that with the exception of the liver and amnion no other cells of this animal show sexual dimorphism (Bianchi and Bianchi 1963a, 1963b, Moore 1962). A recent report from Galton and Holt (1965) points out the existence of a late replicating X chromosome in the cells of mouse fetuses. If we consider that mouse cells do not exhibit sexual dimorphism and that this species is closely related to the rat, the different behaviour of the X chromosomes of the mouse fetuses in respect to the behaviour of the X chromosomes of the adult rat can be explained in the following ways: a) a real difference between the two species; b) female cells from mouse and rat fetuses present a late replicating X chromosome which after birth progressively shifts the end of its replication to an earlier moment of the S period. At the present moment it is not possible to decide which explanation can account for the different behaviour of the X chromosome in the two species of roentia.

In the case of bovines, Chinese hamster and human beings (Gartler and Burt 1964, Hsu 1964, Bianchi and Bianchi 1965), replication of the Y chromosome has been studied throughout the S period. From the results obtained in these experiments it has been possible to prove that Y chromosome is the last to begin DNA synthesis and one of the last to end it.

Our studies show that the Y chromosome in the rat *in vivo* behaves in a similar way to that described for the above species. This fact would indicate that asynchronous duplication of the Y chromosome is, perhaps, a more general phenomenon than the asynchronous replication of the X chromosomes.

Summary

The duration of the cell cycle and the pattern of chromosome replication throughout the S period are studied in the bone marrow of the rat *in vivo*. From the analysis of 610 metaphases and 140 karyotypes with their corresponding autoradiographs it can be concluded:

1. The cell generation time is about 15 hours ; the G_1 period is about 4 hours ; the S period lasts 7 hours and the G_2 period comprises 2 to 3 hours.
2. The study of the labeling over the chromosomes allows the distinguishing of four different patterns corresponding to the initial, intermediate, late and final stages of the S phase.
3. The results obtained indicate that chromosomes start replication over partial areas. Later they extend DNA synthesis to their total length and at the final S stage only small replicating zones—not those at the starting point—can be detected over the chromosomes.
4. Observation about the chronology of chromosome replication point out that different pairs of chromosomes start and finish DNA synthesis at different moments. Homologue asynchrony was detected as much as at the beginning as at the end of the S phase.
5. The X chromosomes are among those which start replication the earliest ; finishing DNA synthesis before several other pairs. In no case a late replicating X chromosome is observed.
6. The Y chromosome is the last chromosome to start replication and also the last one to finish it.
7. The semi-conservative fashion of chromosome replication and the existence of sister chromatids exchanges were detected by studying metaphases at the second division after labeling.

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