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^{3}H-thymidine labelling pattern of preleptotene chromosome condensation stages in the fœtal sheep ovary

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Summary. A sequence of morphological events from premeiotic interphase to leptotene has been described in the ovaries of 64-day old sheep fœtuses. The nuclear changes throughout the condensation and decondensation stages showed a sequential pattern. After a flash of tritiated thymidine, the labelling pattern of these figures demonstrated that those stages followed premeiotic DNA synthesis, i. e. belonged to meiotic prophase. However, with our methodology, this sequence of morphological events could not be confirmed due to a high asynchronism in the oogenetic processes.

Introduction.

A preleptotene stage of chromosome condensation has been described in numerous plants (for review, see Walters, 1972, 1976) : at the end of the premeiotic interphase, the chromosomes coil and condense, sometimes so that they resemble those found in mitotic prometaphase, then despiralize to the elongate leptotene stage.

The complete sequence of morphological events from premeiotic interphase through preleptotene condensation and decondensation to leptotene has been accurately described in *Lilium* (Walters, 1970, 1972; Bennett and Stern, 1975). A comparable process of preleptotene condensation was observed in fœtal human ovaries (Stahl and Luciani, 1971). At maximum condensation, the diploid number of 46 chromosomes was easily discernible, and each condensed chromosome despiralized to form an elongate leptotene filament.

Using morphological criteria, further observations of this process were reported in rabbit (Devictor-Vuillet *et al.*, 1973), sheep (Mauléon *et al.*, 1976) and mouse (Hartung and Stahl, 1977) oocytes.

The sequential stages in plants were exactly determined by comparing the microsporocytes from buds of different sizes and from different regions of individual anthers. Since this methodology cannot be applied to mammals, we have attempted to ascertain the sequential stages in sheep using autoradiography.

This technique has also been employed to investigate the pattern of premeiotic DNA synthesis and the site and duration of the condensation stage. Some data were recently obtained from *Lilium longiflorum* (Holm, 1977), *Euremurus* (liliaceous plant), and fœtal human oocytes (Therman and Sarto, 1977).

Material and methods.

64-day old female sheep fœtuses were injected in the thigh with 90 μCi (1 μCi/g) of tritiated-methyl thymidine (CEA specific activity 25 Ci/mM) in a 0.1 ml physiological saline solution.

The fœtuses were killed and the ovaries removed 15 and 30 min, and 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30 and 42 hrs after the ^{3}H-thymidine injection.

The ovaries were briefly immersed in a 0.44 p. 100 hypotonic potassium chloride solution, then transferred into fixative (methanol/acetic acid, 3/1 vol), and treated according to the technique described previously by Luciani *et al.* (1974). Following fixation, the ovaries were placed in 45 p. 100 acetic acid. Cellular dissociation was obtained by repeated pipetting. The cellular suspension was spread on precooled slides which were all stained with 1 p. 100 aceto-orcein. Some of them were treated for autoradiographic study. Autoradiographs were prepared by the dipping technique using Ilford K 5 emulsion with a procedure similar to that of Kopriwa and Leblond (1962). They were exposed in the dark room at 4 °C for 10 to 15 days and developed with Kodak D 19*b*.

The oogonial and preleptotene nuclei were identified according to morphological criteria, and the silver grains were counted for each category of nuclei. The background was estimated by the silver grains present over the pachytene stage. The intensity of labelling over each preleptotene stage was expressed as the median number of grains, calculated with an appropriate statistical technique (Lazar and Gerard-Marchant, 1965).

PLATE 1

FIG. 1. — *Precondensation nucleus.* Note the presence of small chromocenters, the network of fine filaments and the heterochromatic chromosome peripherally located (arrow). × 1 600.

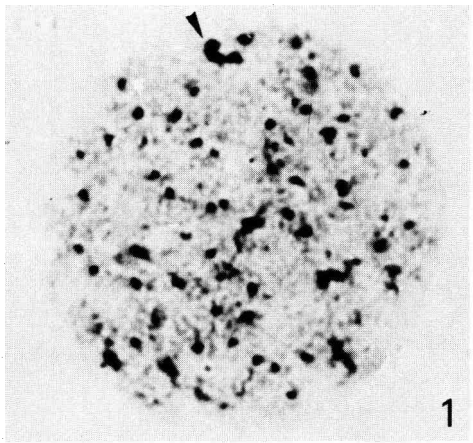
FIG. 2. — *Early-condensation.* Each chromocenter is surrounded by the more individualized filaments. Note the heteropyknotic chromosome (arrow). × 1 600.

FIG. 3. — *Mid-condensation stage* demonstrating the tightening of the filaments about each chromocenter. The heteropyknotic chromosome is still visible at the periphery (arrow). × 1 600.

FIG. 4. — *Slightly more advanced condensation* showing the progressive individualization of the chromosomes. Note the heteropyknotic chromosome (arrow). × 1 600.

FIG. 5. — *Late condensation.* Progression of condensation leads to the formation of 54 chromosomal entities from which radiate fine filaments. × 1 600.

FIG. 6. — *Full condensation.* The chromosomes form about 54 masses from which a few filaments protrude. Note the heteropyknotic chromosome (arrow). × 1 600.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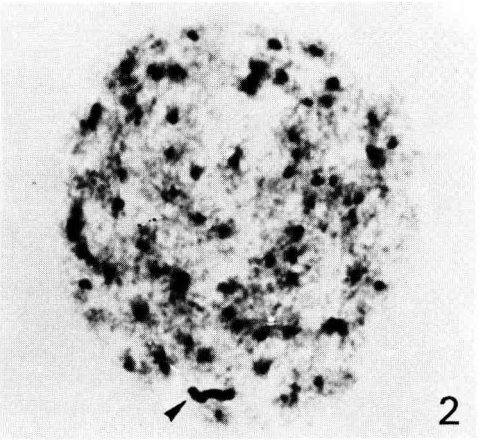
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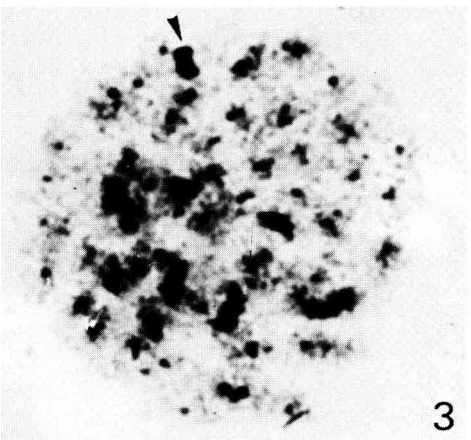
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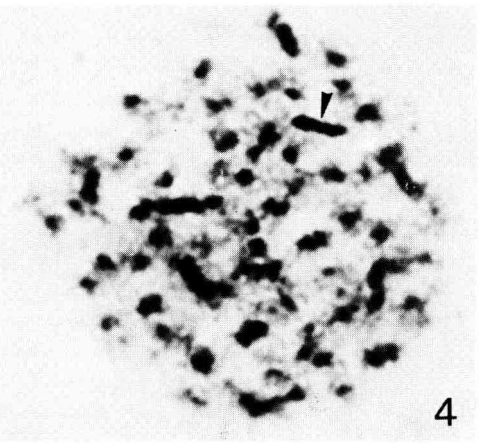
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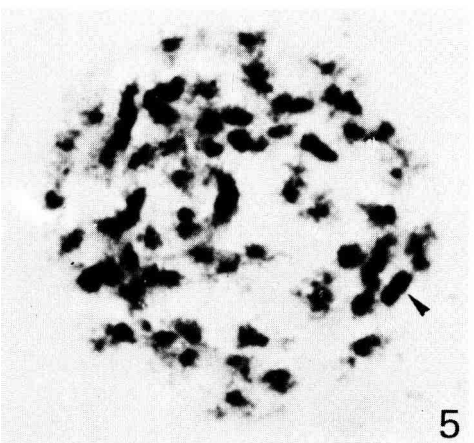
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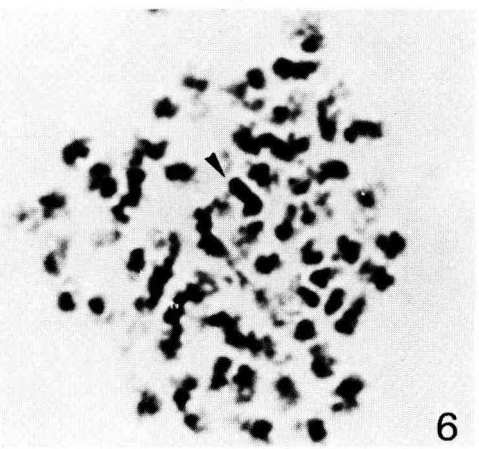
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Some nuclei identified as follows were measured at 280 nm (between 6 and 15 measurements per stage) with a Zeiss UMSP 1 spectrophotometer.

Terminology. — The continuous process of condensation in this study was divided into 4 phases, according to Walters (1970, 1976) : early, mid, late and full condensation. The same divisions were used for decondensation. The terms « condensation », « contraction », and « spiralization » are used without consideration of the intimate process of condensation occurring at the chromosome level.

Morphological description of the stages. — All the stages of the first meiotic prophase could be observed in the sheep embryo at 64 days. Nuclei in the preleptotene condensation stage were also present.

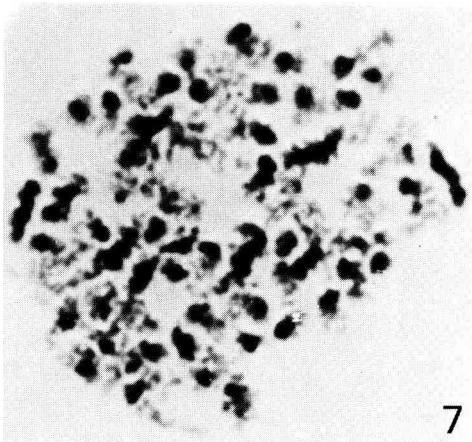
The pictures of the spread nuclei observed on our slides are in good agreement with those described in other mammals. At the precondensation stage, the nucleus was characterized by the presence of numerous, small chromocenters whose discernible number was always close to the diploid number 54 (fig. 1). Specific staining showed that these chromocenters corresponded to centromeric heterochromatin. These darkly stained chromocenters contrasted with the presence of short segments of thin, light-staining chromosomal threads which were barely visible. In addition to these numerous small chromocenters, a heteropyknotic, precocious condensed chromosome, already reported in the mouse female fœtus (Hartung and Stahl, 1977), was usually seen at the periphery. This heteropyknotic chromosome was invisible in the male at the same stage, thus, it probably corresponded to one of the two X chromosomes (fig. 1).

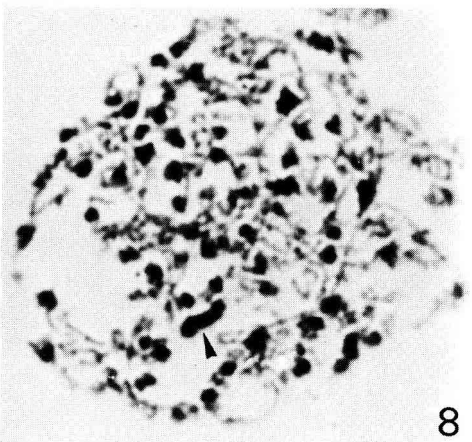
At the onset of condensation, the fine chromosomal threads were more individualized and constituted a filamentous structure surrounding each chromocenter. The heteropyknotic X chromosome was still visible (fig. 2).

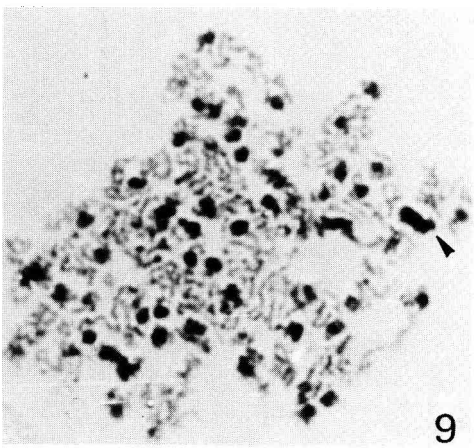
In the following stage (mid-condensation), some chromosomes thickened as a result of filament coiling (fig. 3). Filament condensation increased, and each chromosome was reprensented by a dense, darkly stained mass around which the filaments radiated for a short distance (fig. 4). At a later stage (late condensation), the individualized chromosomes exhibited thicker and thinner regions : short filaments still projected out from the edges, producing an irregular outline (fig. 5). The X chromosome was distinguished by its higher degree of contraction and the absence of protruding filaments (fig. 6). The onset of decondensation was characterized by the reappearance and elongation of the filaments surrounding each chromosome. The stages of condensation and decondensation could always be distinguished : chromosomes in despiralization appeared thicker and more precisely defined than those in spiralization (fig. 7).

As decondensation progressed (mid-decondensation), the volume of each chromosome decreased, and loops were seen along the length of the defined filaments (fig. 8).

At the end of the decondensation stage, the nucleus contained chromosomes of filamentous appearance (fig. 9), with the exception of an heteropyknotic mass usually located at one extremity of the filaments. The position of this mass conformed to that of the centromere in sheep chromosomes (fig. 9). As with condensation, the various chromosomes underwent unequal decondensation : the heteropyknotic X chromosome decondensed later than the other chromosomes (figs. 8 and 9).







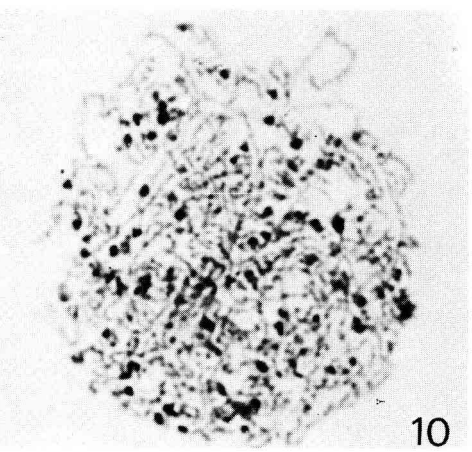


PLATE 2

FIG. 7. — *Early-decondensation.* Reappearance and elongation of the filaments originating from each mass. × 1 600.

FIG. 8. — *Mid-decondensation.* The filaments thicken and form loops along their length. Note the presence of a heteropyknotic chromosome (arrow). × 1 600.

FIG. 9. — *Late decondensation.* The filaments show a heteropyknotic mass usually located at one extremity. The heteropyknotic chromosome can still be seen (arrow). × 1 600.

FIG. 10. — *Early leptotene.* Chromosomes appear as typical individualized leptotene filaments. × 1 600.

In very early leptotene, the nuclear chromosomes were long, decondensed filaments. Nevertheless, the region corresponding to the centrometric heterochromatin was less advanced in this respect (fig. 10), and the heterochromatin is no longer visible at mid-leptotene stage.

Pattern of labelled stages after a tritiated thymidine flash. — The percentage of labelled oogonia regularly increased from 15 min to 1 hr after the injection, reached 45 p. 100, remained almost constant from 1 to 10 post-injection hrs, and then decreased. This

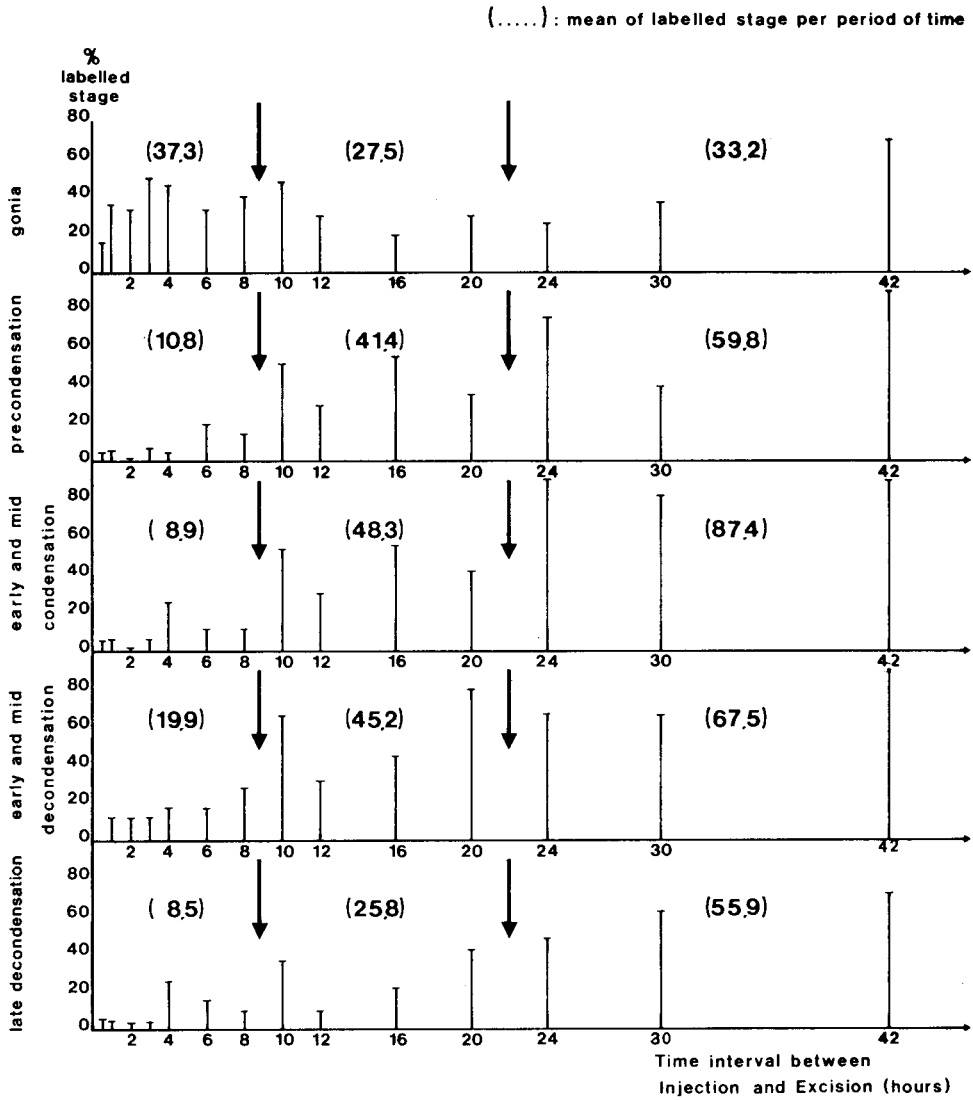


FIG. 11. — *Percentages of labelled stages after different time intervals between tritriated thymidine injection and excision.* The arrows divide the period of observation in three parts referred to as presumed successive peaks of oogonial division.

decrement of about 10 p. 100 was due to labelling dilution. Labelling intensity was calculated by the median number of silver grains present over the nuclear area : 50 to 60 grains were counted from 1 to 8 hrs after the injection. This number was 25 to 30 from 10 to 20 hrs, and then decreased once again to 12 to 18 grains after 24 post-injection hrs. These data indicated that between 8 and 10 hrs after the injection, there was mitotic division involving at least 50 p. 100 of the oogonia labelled with ^{3}H-thymidine during the flash labelling. Another mitotic division was completed after 20 to 24 hrs by an identical percentage of oogonia. The evolution of the oogonial labelling pattern was similar to that observed in any other cell population after a pulse (see review in Aherne *et al.*, 1977).

The labelling pattern of the preleptotene stages was longer than that of the oogonial cell cycle.

Within 10 hrs after the injection about 10 p. 100 of the nuclei, morphologically identified as either condensed or decondensed preleptotene stage, were labelled. Between 10 and 20 hrs after the injection, the percentage of labelled cells of all the stages increased identically from 41 to 48 p. 100, and tended to increase more after 24 hrs post-injection (55 to 87 p. 100). The percentage of labelled nuclei, morphologically identified as preleptotene stage, increased when the period between the injection and the ovariectomy was lengthened. Oogonial nuclei showed the opposite labelling pattern (fig. 11).

The preleptotene stage labelling pattern was also characterized by uniform labelling intensity, except at 6 post-injection hours for nuclei in precondensation and early and mid-decondensation : these preleptotene nuclei, originating from oogonia which immediately incorporated ^{3}H-thymidine during their period of premeiotic DNA synthesis, exhibited a higher median number of silver grains than the nuclei reaching this stage later. Nevertheless, this number of grains never reached that counted over oogo-

TABLE 1

Intensity of labelling (median number of grains). *Preleptotene stages*

Time interval between injection and excision (hrs)	Precondensation		Early and mid condensation		Early and mid decondensation	
1					9.7 (6)	6.5-14.5
2					8.6 (13)	6.2-11.9
3						
4						
6	96.8 (27)	38.7-242.0	20.4 (18)	7.9-53.1	23.0 (31)	13.5-39.2
8	14.5 (19)	9.7-21.7	11.0 (12)	5.1-23.4	16.1 (23)	11.0-23.5
10	8.0 (22)	5.2-12.3	19.0 (28)	15.0-24.0	15.5 (21)	11.6-20.9
12	9.7 (21)	7.3-12.9	4.9 (22)	3.1-7.6	11.8 (26)	10.0-14.0
16	21.3 (42)	16.8-27.1	18.6 (70)	15.7-22.1	17.2 (45)	14.2-20.9
20	19.6 (14)	14.3-26.9	16.6 (8)	8.3-33.3	20.3 (8)	10.4-39.7
24	16.6 (45)	14.2-19.3	17.3 (37)	15.1-19.8	9.8 (61)	8.6-11.2
30	20.6 (26)	16.4-26.0	23.2 (37)	18.1-29.8	11.4 (14)	8.0-16.2

( ) Number of labelled nuclei counted. Confidence interval.

nial nuclei. This lower intensity could result from the long duration of the premeiotic DNA synthesis, as reported by Hochereau-de Reviers (1971) in the male ; compared to a normal mitotic cycle, the tritiated thymidine was only available during a short period of the S-phase.

According to this labelling behavior, the nuclei identified as in preleptotene stage have to be considered as in prophase. The oogonial metaphase was also labelled 6 hrs after the injection of tritiated thymidine ; but the absence of labelling dilution during the 10 to 42 hr period when the first leptotene stage appeared to be labelled (table 1), and the uniformity of the maximum labelling pattern up to pachytene stage (maximum number of grains over the nuclear area : 45-50), demonstrated that the nuclei were in a true preleptotene stage. The UV-DNA microspectrophotometric values confirmed that these stages were prophase, i. e. they had 4n DNA content. In arbitrary units these values were in the early and mid-condensation, and early and mid-decondensation, stages : 18-22 vs 9.9-11.0 values over interphase nuclei (2n) of somatic cells. The precondensation stage seemed to be close to the premeiotic DNA-S phase, having a larger range of values (14 to 22) than the following stages.

The characteristics of these parameters (percentage of labelled stages and of their labelling intensity) did not allow us to either confirm or invalidate the filiation of these stages based on morphological criteria.

The advance of a labelling front is a classical method for studying the sequential filiation between various cellular types and the precise duration of the different stages of the meiotic prophase (Bennett, 1977). Due to the high degree of meiotic asynchronism in the fœtal sheep ovary, the technique was not accurate in our study, and we were unable to demonstrate the existence of that filiation : 6 hrs after the injection of tritiated thymidine, the first condensation stage was labelled as well as the first decondensation stage, and some decondensation stages were even labelled before the sixth hour. Either the duration of each stage was very short (an hypothesis not supported by the observations of *Lilium* ; Bennett and Stern, 1975), or the sequence of development from precondensation and early to full condensation was not obligatory. The latter hypothesis was previously suggested during a preliminary study of preleptotene nuclear labelling (Mauléon *et al*, 1976). The proportion of full condensation stages was always very low, and thus they could not be quantitatively studied. The velopment of the labelling pattern of this stage was identical to that reported above for the other stages of condensation. The scarcity of this full condensation stage emphasized that there might be a by-pass from precondensation or early condensation to decondensation.

Observations of the labelling front advance showed that the preleptotene, including the early leptotene stages, lasted a long time (20 to 40 hrs). We think that the short duration of the premeiotic interphases (15 min in *Euremurus* and 3 hrs in humans) reported by Therman and Sarto (1977) results from a confusion at the morphological level between leptotene stage, decondensation stage and oogonial mitotic prophase. A similar mistake was made by Callebaut (1967) studying the chicken embryo : the histological aspect of the nuclei, classified by that author as types 2 and 3 and labelled 1 hr after the pulse of ^{3}H-thymidine, corresponded to preleptotene condensation stage. In the same way, the nuclei identified as leptotene and labelled 10 hrs after the pulse were probably preleptotene decondensation stage. This emphasizes that it is difficult to

identify early meiotic nuclei with histological techniques. The same misinterpretation was made in the sheep embryo from the histological pictures of nuclei labelled 2 hrs after the injection of tritiated thymidine (Mauléon, 1974) : the precociously labelled nuclei were probably preleptotene condensation stage.

In conclusion, the analysis of the data obtained from the development of the labelling pattern clearly demonstrates that during condensation and decondensation, the nuclei are in preleptotene stage, preceded by a period of premeiotic DNA synthesis occurring before and during the early precondensation phase. However, we failed to demonstrate the sequential filiation of the stages hypothetically established from morphological criteria because such a filiation could not be obtained from a highly asynchronic population of germinal cells.

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Résumé. La séquence des événements morphologiques qui s'étend de l'interphase préméiotique au leptotène a été décrite dans l'ovaire de fœtus de brebis de 64 jours. Les changements nucléaires montrent une évolution à travers des passages par des stades de condensation et de décondensation. Le déroulement dans le temps du marquage de ces figures après une injection de thymidine tritiée démontre que ces stades morphologiques suivent bien une synthèse d'ADN préméiotique, c'est-à-dire sont bien au stade de prophase méiotique. Cependant, la validité de la filiation des événements morphologiques n'a pu être confirmée avec cette méthodologie d'étude par suite de l'asynchronisme très marqué entre cellules subissant les processus oogénétiques.

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