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MEIOTIC STAGES IN THE FEMALE CHINESE HAMSTER (*CRICETULUS GRISEUS* MILNE-EDW.)

A THESIS

Presented to

The Faculty of the Graduate Division

by

Brenda Maxine Waldon

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CHINESE HAMSTER (*CRICETULUS GRISEUS* MILNE-EDW.)

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SUMMARY

Meiotic prophase, observed in 22 immature female hamsters, was present at birth for 96 hours. One hundred and twenty hours after birth, all oocytes were in the dictyate stage which persisted until a stimulus initiated the resumption of meiosis.

Superovulation was induced in mature female Chinese hamsters by treatment with the gonadotrophins pregnant mares' serum (PMS) followed by human chorionic gonadotrophin (HCG). Oocytes were observed in the dictyate stage 1-16 hours after the injection of HCG. Diakinesis appeared at two hours and also persisted through the sixteenth hour. Metaphase I oocytes were first seen at two hours and last seen at 12 hours. The first anaphase I oocytes were found at four hours and were last observed at 12 hours. Telophase I oocytes appeared at six hours and disappeared after 14 hours. Metaphase II was found from the tenth hour through the sixteenth hour. Ovulation began 12 hours after the injection of HCG.

Anaphase II and telophase II were observed in Chinese hamster oocytes spontaneously activated by placing the oocytes in cell culture media. Fifty-five per cent of the oocytes cultured showed spontaneous activation.

Tjio and Levan's aceto-orcein squash technique provided successful preparations of meiotic prophase and the dictyate stage. Diakinesis, metaphase I, anaphase I, telophase I, and metaphase II

were best identified in fixed, sectioned and stained ovaries. However, a modification of Jagiello's method for preparation of mammalian oocytes proved the most successful of the techniques tested for preparing metaphase I chromosomes. The phase contrast technique was most useful in viewing telophase I, telophase II, and the two, three, and four-cell stages.

CHAPTER I

INTRODUCTION

Many of the reported chromosomal anomalies in man and animals probably arise through non-disjunction or lagging chromosomes during meiosis in the oocyte. Investigations of the origin and primary incidence of such anomalies would be greatly facilitated if meiotic stages in the mammalian oocyte were easily available (Edwards, 1962). The purposes of this investigation are to identify meiotic stages and determine meiotic duration and to establish a technique for observing chromosomes in mammalian oocytes of _Cricetulus griseus_. The striped-back or Chinese hamster, _Cricetulus griseus_ Milne-Edw. was selected for this study because of its excellent cytogenetic properties. It has 11 morphologically distinct pairs of chromosomes; this is the lowest number yet to be observed among Eutherian laboratory mammals (Yerganian, 1958).

In the majority of mammalian species, oocytes undergo the prophase stages of meiosis until diplotene just before or shortly after birth (Edwards, 1962). Brambell (1927) distinguished early prophase stages in the female mouse at 12 days post coitum and synaptic stages at 15 or 16 days post coitum; in four-day old female mice he found that all oocytes were in the dictyate condition. In contradiction to Brambell, Slizynski (1957) observed the initiation of meiotic prophase in female mice 12 hours after birth. He found

dictyate stages 60-72 hours after birth. In female germ cells of man all oocytes in the ovary have completed up to the diplotene stage of meiosis, including DNA synthesis, early in postnatal life (Ohno *et al.*, 1961). After completing diplotene, oocytes enter the long interphase-like dictyotene stage until stimulation of the estrus cycle (Ohno *et al.*, 1961). Thus, to find the early stages of female meiosis, oocytes must be taken from late fetuses or newly-born animals (Edwards, 1962).

Much of the fundamental information on non-disjunction, lagging chromosomes, crossing-over, and associations between normal and structurally altered chromosomes will be provided by studies on meiotic stages after diplotene. After diplotene, oocytes enter the dictyate stage and remain nucleated for the long period from diplotene until shortly before ovulation. In most mammalian species the stages of meiosis between diakinesis and the metaphase of the second maturation division can be recovered from the ovaries just prior to ovulation; but the number of such oocytes is meagre, and the stage of meiosis can vary widely between animals (Edwards, 1962). However, under appropriate endocrinological conditions the dictyate stage in mammalian oocytes can be terminated, and resumption of meiosis can be induced.

The appropriate endocrinological conditions necessary for resumption of meiosis are supplied by the phenomenon of superovulation which in the literature denotes both the production of ovulation in immature animals and ovulation of excessive numbers of oocytes in adult animals. Smith and Engle (1927) first demonstrated

superovulation by anterior pituitary transplants which stimulated follicular growth. Runner (1950) found that ovulation could be induced in immature mice with one to four international units (i.u.) of pregnant mares' serum (PMS) followed 24-36 hours by one i.u. of pituitary extract. The number of oocytes recovered after pretreatment with PMS was three times that found in controls. In 1958 superovulation was induced in immature rats by injection of PMS followed by injection of human chorionic gonadotrophin (HCG) (Zarrow et al., 1958). Multiple ovulations also were induced in calves by the same method (Jainudeen, 1966).

After establishment of PMS and HCG as hormones causing superovulation, their different roles were considered. Rowlands (1944) stated that PMS, although effective in producing follicular growth, had only limited capacity to cause ovulation. Hunter (1966) confirmed Rowlands' observation concerning PMS when he reported that treatment with PMS merely shortened the follicular phase of the estrus cycle in the pig. In 1952 Maximow and Bloom determined that the follicle stimulating and luteinizing hormones of the hypophysis together cause preovulatory growth and rupture of ovarian follicles. A further distinction was made in 1962 that the degree of ovulation is controlled by both the follicle stimulating hormone, PMS, and luteinizing hormone, HCG; PMS controlling the number of oocytes that can be released from the dictyate stage and HCG determining how many of the mature follicles can be ruptured (Wilson and Zarrow, 1962). Weifenbach (1965) has a different view of the action of FSH and HCG

in superovulation. He believes that the increase of the number of oocytes in mature rats is not effected by stimulation of additional follicles but by prevention of atresia of already stimulated follicles. In contrast, immature rats show true superovulation (up to 80 oocytes per animal), and Weifenbach (1965) contends that this difference is due to the inability of mature rats to stimulate more than one set of follicles either by endogenous or exogenous gonadotrophins.

Several factors have been found to alter the mammalian response to gonadotrophins. The most significant of these are age of animals, method and amount of injections, and time interval between injections.

Fetuses and newborn animals have not been found responsive to pituitary hormones. The gonad of the rat fetus is incapable of ovulatory response (Corey, 1928). Hertz and Hisaw (1934) demonstrated that "infantile" rabbits did not respond to gonadotrophins. They found that the hormones were able to affect only antrum-containing follicles, but they were unable to determine the physiological factors responsible for conditioning follicles so that they will respond to stimulus. Hisaw (1947) proposed that growth processes up to the formation of follicular antra are regulated by a self-contained system of organizers. Gonadotrophins also are incapable of inducing ovulation in the newborn Golden hamster; sensitivity of ovaries to anterior pituitary hormones develops only gradually (Bodemer _et al._, 1959). Initially the follicles become competent to respond to gonadotrophins at approximately 14 days of age in the mouse and 17-18 days of age in

the rat. Sensitivity increases to a maximum within two weeks and decreases rapidly by 50-65 per cent prior to puberty. The decrease in the number of oocytes released with age is due to changes in the sensitivity of the follicles to gonadotrophins (Zarrow and Wilson, 1961). The Golden hamster ovary is first stimulated by gonadotrophins at ten days of age when both weight and hormone production are increased (Ortiz, 1947). Ovulations were inconsistent before 26 days of age, but thereafter ovulations were induced in all treated hamsters. The maximal ovarian reaction occurred at 33-36 days, the approximate time at which the hamster attains sexual maturity (Bodemer et al., 1959). Few experiments using superovulation have been performed with mature animals. However in 1957 Fowler and Edwards reported that they injected mature female mice with PMS and HCG and induced ovulation in 99 per cent of the mice.

In the experiments reviewed, injections were given either subcutaneously, intravenously, or intraperitoneally. Wilson and Zarrow (1962) compared effects of subcutaneous and intravenous injections of hormones in aqueous media and found that intravenous administration increased sensitivity by a factor of approximately ten.

Many variations concerning the quantities of gonadotrophins injected and the interval between injections have been found in the literature. The results given here are those which proved most reliable in producing ovulation. Immature mice injected with two i.u. pregnant mares' serum followed 40 hours later by two i.u. human chorionic gonadotrophin ovulate approximately 12 hours later (Gates

and Beatty, 1954). Wilson and Zarrow (1962) found that the optimum time requirement between PMS and HCG in immature mice is 30-50 hours. Immature rats given 10 i.u. PMS followed 48-72 hours later by 10 i.u. HCG also commence ovulation 12 hours after the injection of HCG (Rowlands, 1944). Wilson and Zarrow (1962) stated that ovulation in the immature rat was complete 17 hours after the injection of HCG. In 1957 Fowler and Edwards produced ovulation in 99 per cent of mature mice tested by the injection of three i.u. PMS followed after 40 hours by three i.u. HCG. Edwards and Gates (1958) confirmed the reliability of three i.u. PMS followed after 40 hours by three i.u. HCG in producing superovulation in mature mice. In 1965 Jagiello used 5-10 i.u. PMS followed after 56 hours by 5-10 i.u. HCG to produce ovulation 12 hours after HCG injection in mice.

The optimum time interval between the injection of HCG and the presence of oocytes in the oviduct is 16-24 hours with the peak reached at 16 hours. The first ova are seen at 12 hours, and at 26 hours oocytes begin to degenerate (Wilson and Zarrow, 1962).

The quantity of gonadotrophins injected also can cause over-stimulation. Hertz and Hisaw (1934) rendered rabbit ovaries refractory to further pituitary stimulation by prolonged treatment. After two to three months none of these animals had recovered their responsiveness to hypophyseal stimulation. In 1944 Rowlands demonstrated that doses of PMS greater than 30 i.u. caused over-stimulation in the rat ovary and prevented discharge of oocytes from follicles. In hamsters, 20-40 i.u. PMS increases significantly the number of cystic

and hemorrhagic follicles; 60-80 i.u. PMS elicits development of numerous hemorrhagic and cystic follicles, thus indicating significant over-stimulation (Bodemer *et al*., 1959). Over-stimulation also reduces the rate of oocyte recovery in adult rabbits. Kennelly and Foote (1965) attributed this partially to the effect of endogenous estrogen production on the rate of oocyte transport and partially to oocytes being trapped in follicles.

After resumption of meiosis is induced by gonadotrophic hormones, stages between diakinesis and metaphase II can be obtained. Edwards and Gates (1958) timed the stages of the maturation divisions, ovulation, and fertilization of eggs of adult mice treated with gonadotrophins. The oocytes were in the dictyate (germinal vesicle)stage until two hours after the injection of HCG. They then completed the prophase of the first maturation division. From approximately four to eight hours all oocytes were in metaphase I. The first maturation division and extrusion of the first polar body were then rapidly completed just before ovulation. Ovulation began 11 hours after the injection of HCG and was virtually complete by 14 hours. The time at which ovulation occurred was very similar in mated and unmated females. The extrusion of the second polar body was not timed (Edwards and Gates, 1958). Mandl (1962) made an approximate estimate of the duration of each stage of meiotic division up to the second metaphase in the adult rat. Hamilton (1942) and Samuel (1942) illustrate early hamster oocytes, but they do not give the exact age of specimens. Yanagimachi and Chang (1961) found meiotic stages in the Golden hamster from prometaphase I to

anaphase I in oocytes three hours before ovulation. One hour before ovulation they noted the first polar body or telophase I. Graves (1945) compared the normal development of the hamster with the corresponding development of the rat. The formation of the first maturation spindle was seen in both hamster and rat five hours before ovulation. Metaphase stages appeared three hours before ovulation in the hamster and two to three hours before ovulation in the rat. As ovulation commenced, the first polar bodies were formed in oocytes of both animals. Thus, as ovulation occurs, oocytes are discharged into the oviducts at any stage from 'first anaphase' to 'first polar body, second metaphase' (Strauss, 1956).

Anaphase and telophase of the second maturation division are more difficult to obtain, since they occur rapidly after sperm penetration (Edwards, 1962). Huber (1915) stated that in the albino rat the second polar body is given off in the oviduct only after insemination and that formation of the second polar body may begin within five minutes to two hours after sperm are introduced. Kirkham and Burr (1913) observed that formation of the second polar body was started as soon as the sperm head began to penetrate the oocyte cytoplasm of the albino rat. In the preceding case, no resting phase was observed between the first and second maturation divisions. In 1932 Pincus and Enzmann questioned earlier data which indicated that formation of the second polar body is due to the action of a proteolytic enzyme carried by the sperm, but their only conclusion was that the second polar

body was given off 45 minutes or longer after sperm penetration into the rabbit oocyte. Lewis and Wright (1935) stated that the second polar body was not formed in mouse eggs unless they were fertilized. In the Golden hamster, two hours after copulation two polar bodies were seen, one small and flattened and located either in the zona pellucida or between the zona pellucida and a second internal membrane; the other was obviously free between the vitellus and zona pellucida (Venable, 1946). In 1956 Austin stated that hamster oocytes appear unique among known mammalian oocytes in that nearly 80 per cent undergo spontaneous activation, extruding the second polar body within 24 hours after ovulation. In the Golden hamster, which has not been mated, two to six hours after ovulation all oocytes were at metaphase II. At 12 hours after ovulation, 94 per cent of 66 ova were at metaphase II; and six per cent were at anaphase II. Eighteen hours after ovulation, approximately 69 per cent of 65 oocytes were spontaneously activated: six per cent showed anaphase II or telophase II; 45 per cent showed the second polar body; and 18 per cent formed two pronuclei. No stages were observed past pronuclei formation. At 52 hours after ovulation, deterioration of ooplasm was obvious and some oocytes had more or less completely disintegrated (Yanagimachi and Chang, 1961).

While spontaneous activation of mammalian oocytes is a rarely observed phenomenon, a technique has been developed to stimulate mammalian oocytes to continue their maturation _in vitro_. All that is necessary is to liberate the oocytes into a simple culture medium by

puncturing the follicles; many oocytes then immediately resume their maturation (Edwards, 1962). According to the definition of artificial parthenogenesis (Arey, 1965) this stimulation of the oocyte is actually a means of artificial parthenogenesis. Pincus (1930) was one of the first to use this technique in the rabbit. Out of 213 oocytes liberated and cultured, 63.8 per cent "divided". He demonstrated that regular parthenogenetic division appeared after oocytes had been in culture eight to nine hours and that the first segmentation of unfertilized ova dividing regularly into two cells occurred from 6-14 hours after being cultured or as late as 26 hours. Pincus (1930) also noted that all ova in culture stopped segmenting and degenerated after some time. Practically no development occurred after ova had been in culture 36 hours.

In 1935 Pincus and Enzmann made further studies on artificial parthenogenesis and postulated possible causes. Implications were that the associated follicle cells serve either to maintain the egg in a nutritional state wherein nuclear maturation is impossible or that they actually supply to the ovum a substance or substances which directly inhibit nuclear maturation.

Pincus and Saunders (1939) stimulated human ovarian oocytes and claimed that approximately 50 per cent displayed meiotic chromosomes or first polar bodies. Pincus (1939) made an intensive study of artificial activation of the rabbit oocyte. He concluded that ductal oocytes of rabbits subjected to hypertonic solution, supranormal temperature, or culture in a moist chamber _in vitro_ undergo development

comparable in every way to normally fertilized rabbit oocytes and that untreated oocytes both *in vivo* and *in vitro* do not exhibit such development. However, artificially activated oocytes did not develop with clock-like regularity of normally fertilized oocytes, and haploid, diploid, or tetraploid parthenogenesis resulted from the treatments employed (Pincus, 1939). Although atretic changes also could inhibit any stage in the developmental process, development was judged truly parthenogenetic, not merely a result of initiation of atresia, for three reasons: prompt formation of polar bodies; completely normal cleavage in some eggs; and young obtained from some eggs (Pincus, 1939).

Edwards (1962) induced large numbers of mouse, rat and hamster oocytes to resume maturation by liberating oocytes into a simple culture medium. When oocytes within their follicles were cultured *in vitro*, no evidence of maturation was found. An inhibitory influence within the follicle rather than a factor in the oocyte evidently causes the persistance of the dictyate stage, and rupture of the follicle releases the oocyte from the inhibitor (Edwards, 1962). Human, baboon, monkey and dog oocytes did not respond as well as those of rabbits and rodents after liberation from their follicles. Neither the addition of gonadotrophins to the culture medium nor the use of widely differing culture media has so far induced the resumption of meiosis in human, monkey, or baboon oocytes (Edwards, 1962).

In order to study most thoroughly all the various meiotic stages in mammalian oocytes, *Cricetulus griseus* Milne-Edw. was chosen because

of its excellent cytogenetic properties and its unique feature of spontaneous activation of oocytes. The following study attempts to determine when each meiotic stage can be found and to define the techniques best suited to observation of each stage.

CHAPTER II

MATERIALS AND METHODS

Source of Material

Meiotic cells were obtained from the ovaries of female Chinese hamsters, *Cricetulus griseus* Milne-Edw. The Chinese hamster, a native of China and Eastern Asia, is a nocturnal rodent closely related to the rat and mouse. It is particularly desirable for cytogenetic studies because it has 11 pairs of morphologically distinct chromosomes (Yerganian, 1958). The Chinese hamsters used in these experiments came from the colony maintained at the Georgia Institute of Technology.

The prophase stages of meiosis were taken from ovaries of immature hamsters; stages from dictyate to telophase II were found in oocytes of mature hamsters. Spontaneous activation of meiosis was studied in ovarian oocytes grown in cell culture.

Preparation of Prophase Stages

Prophase stages of meiosis were taken from ovaries of immature hamsters. At birth and at hourly intervals after birth, hamsters were sacrificed using carbon dioxide anesthesia, followed by cervical dislocation. These new-born female hamsters were dissected to expose the genital organs which include the ovaries, the oviducts, the uterine horns, the cervix and the vagina (Parkes, 1931).

Meiotic cells were prepared from the ovaries as follows: one ovary from each hamster was prepared as aceto-orcein squash preparations (Tjio and Levan, 1954); and the other ovary was fixed and sectioned.

Squash Preparation

The ovary was dissected free and prepared by the aceto-orcein squash preparation of Tjio and Levan (1954). One ovary was placed in a watch glass with a few drops of 1 part 1N HCl and 9 parts standard orcein solution, prepared by dissolving 2 grams orcein in 45 ml boiling glacial acetic acid. The solution was allowed to cool to room temperature, 55 ml distilled water added, and the solution filtered. The watch glass was passed over a flame several times to cause spreading of the chromosomes; care was taken not to boil the solution. The ovary was then transferred to a droplet of standard orcein solution on a clean slide. To insure chemical cleanliness the slides were soaked in a cleaning fluid of 1 part saturated aqueous solution of potassium dichromate and 1 part concentrated sulphuric acid for at least 24 hours (Jones and McClung, 1950). They were then rinsed thoroughly in hot water and transferred to clean, 70 per cent alcohol. Immediately before use, the slides were flamed dry.

A coverslip was placed on the ovary and gentle pressure applied while it was kept in a fixed position under a strip of blotting paper. The slides were sealed with printer's wax and examined with phase contrast optics under 100X oil immersion.

Fixation and Sectioning

Ovaries were fixed in Allen's Fixative, B-15, for one hour at 35°C. They were then transferred to Bouin's fluid and left overnight. Allen's Fixative, B-15, was prepared by adding .75 grams chromic acid and 1 gram urea to Bouin's fluid, previously heated to 37°C (Conn and Darrow, 1946). Allen's Fixative, B-15, was made up immediately before use and was retained for no longer than two hours.

The ovaries were then washed, dehydrated, cleared and embedded in paraffin.* Each ovary was serially sectioned at 7 microns, stained with Weigert's iron hematoxylin, and counterstained with aqueous fast green FCF.

Preparation of Post-prophase Stages of Meiosis

Meiotic cells in stages dictyate, diakinesis, metaphase I, anaphase I, telophase I, metaphase II, anaphase II, and telophase II were obtained from oocytes of mature female hamsters. Two groups of hamsters were given 3 i.u. pregnant mares' serum (PMS)[1] followed after 40 hours by 3 i.u. human chorionic gonadotrophin (HCG)[2]. Four other groups of female hamsters were given 10 i.u. PMS followed after 56 hours by 10 i.u. HCG in order to detect any variation in meiotic time or ovulatory time due to different doses of gonadotrophins. The

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[1] Equinex, Ayerst Laboratories, Inc., New York City.

* See Appendix for details on fixation, sectioning, and staining.

[2] Compliments of APL, Ayerst Laboratories, Inc., New York City.

Five methods were used to prepare meiotic cells from mature hamsters. From one group given 3 i.u. PMS followed after 40 hours by 3 i.u. HCG and from one group given 10 i.u. PMS followed after 56 hours by 10 i.u. HCG, one ovary per hamster was fixed and sectioned. Other methods of meiotic preparation were as follows: aceto-orcein squash preparation (Tjio and Levan, 1954) using whole ovaries; air-drying method for chromosome preparations (Tarkowski, 1966); a modification of Jagiello's squash preparation of mammalian ova (Jagiello, 1965); and a phase-contrast observation technique.

Fixation and Sectioning

The methods of fixation and sectioning were the same as those previously stated for ovaries of immature female Chinese hamsters.

Aceto-orcein Squash Preparation

Whole ovaries were prepared by the aceto-orcein squash method previously described for ovaries of immature hamsters.

Air-drying Method

Female hamsters were injected intraperitoneally with 10 i.u. PMS followed after 56 hours by 10 i.u. HCG. Ovaries were placed in sodium citrate and then treated with hyaluronidase. They were fixed with 3 parts absolute ethyl alcohol and 1 part glacial acetic acid and then air-dried on slides (Tarkowski, 1966).

Modification of Jagiello's Preparation of Mammalian Ova

Superovulation was induced in the female hamster with a priming

dose of PMS followed by an ovulating dose of HCG. Ten i.u. PMS was given intraperitoneally followed after 56 hours by 10 i.u. HCG given intraperitoneally.

Female hamsters were sacrificed at hourly intervals from one hour after the injection of HCG, and the ovaries and oviducts were placed in one per cent sodium citrate in embryological culture dishes. In the case of follicular oocytes, three or four of the larger follicles were dissected from the ovary. Ductal oocytes were secured by breaking the oviduct into short segments and stripping the oocytes out with watchmaker forceps. The oocytes were then placed in one per cent hyaluronidase solution to remove the cumulus cells. Most coronal cells were removed with watchmaker forceps, but it was necessary to pull the oocytes into a micropipette several times to remove remaining coronal cells. The citrate was removed with a sliver of filter paper and a large drop of acetic orcein was placed on the ova. Several minutes later a cover slip was gently dropped on the ova. The slide was blotted with a paper towel, very moderately squashed, and sealed with printer's wax.

Phase Contrast Observation

Superovulation was again used to secure a large number of oocytes. The ovaries and oviducts were placed in one per cent sodium citrate. The oocytes were dissected free and treated with a one per cent hyaluronidase solution to remove cumulus cells. Coronal cells were removed with watchmaker forceps and a micropipette.

A ring of printer's wax was made on each slide. The purposes of

the ring of printer's wax were to prevent destructive squashing of the oocytes and to keep them in a restricted area. The oocytes were placed in this ring of printer's wax which was filled with a hypotonic solution to cause swelling of the cells. A coverslip was placed gently over the oocytes, and the slide was sealed.

Spontaneous Activation of Meiosis

Spontaneous activation of meiosis was studied in ovarian oocytes grown in cell culture. Observing aseptic techniques, the ovaries were dissected free and the follicles pierced with watchmaker forceps to liberate the oocytes. A micropipette was used to transfer the oocytes to 25 ml tissue culture flasks containing cell culture media.*

The oocytes were placed in an incubator maintained at 35°C. Every four hours oocytes were removed and examined by the phase contrast technique defined previously.

Analysis

Stained meiotic cells were viewed with a Leitz Wetzlar phase contrast microscope under 100X oil immersion. Various meiotic stages were recorded and photographed.

Serial sections of ovaries were viewed with bright field optics with 16X and 450X objectives. The number of oocytes in each meiotic stage was noted and recorded for each ovary. Photographs were made

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* See Appendix

using 100X oil immersion objective.

Oocytes grown in cell culture were first viewed with a 50X dissecting scope. More detailed observations were made with phase contrast optics. The number of oocytes activated was recorded and meiotic stages photographed.

CHAPTER III

RESULTS

Meiotic prophase was observed in 22 immature female hamsters. It was found that the nuclei in all oocytes of three newly born female hamsters were in leptotene (Figures 2-5). Numerous leptotene nuclei existed up to 72 hours of age (Figure 1). Zygotene nuclei first appeared at 10 hours, became predominant at 20 hours, and disappeared 72 hours after birth (Figures 6-9). Pachytene nuclei were first seen at 12 hours, and at 48 hours pachytene nuclei were predominant (Figures 10-13). Numerous pachytene stages existed at 72 hours, but at 96 hours only a very few late pachytene stages were found. An occassional early dictyate nuclei appeared from 32 to 48 hours. At 72 hours early dictyate stages were most numerous, but only a few oocytes were accompanied by follicle cells. By 120 hours the number of meiotic cells was greatly reduced. All the oocytes present were in the advanced dictyate stage with accompanying follicle cells (Figures 14-20).

Superovulation was induced in six groups of female Chinese hamsters by treating them with the gonadotrophins PMS and HCG. Table 3 shows the number of oocytes in various meiotic stages at hourly intervals after injection of 3 i.u. HCG preceded 40 hours by 3 i.u. PMS. The resting or dictyate stage was present in ovaries from 1-16 hours after treatment. Diakinesis first appeared at 3 hours and ended at 16 hours. Metaphase I also began at 3 hours and was last seen

at 12 hours. At 4 hours anaphase I appeared; anaphase I disappeared at 12 hours. Telophase I began at 6 hours and ended after the fourteenth hour. Metaphase II lasted from 12-16 hours. Ovulation began at the eleventh hour after injection of HCG.

In another group of Chinese hamsters treated with 10 i.u. PMS followed after 56 hours by 10 i.u. HCG, the dictyate stage was also present from hour 1 through hour 16 (Table 4). Diakinesis first appeared at 2 hours and persisted through the sixteenth hour. Metaphase I appeared at hour 2, disappeared from the eleventh to the thirteenth hour, and reappeared at hours 15 and 16. Anaphase I began at 4 hours and stopped after 11 hours; it reappeared at the sixteenth hour. At hours 7 and 8 and 10-13, telophase I was found. Metaphase II lasted from the tenth hour through the sixteenth hour, excepting hour 14. Ovulation began 12 hours after the injection of HCG.

Table 5 compares the number of developing oocytes in adult hamsters treated with 3 i.u. doses of gonadotrophins and in hamsters treated with 10 i.u. doses of gonadotrophins. The number of oocytes in the dictyate stage varies very little between the two groups. However, the group given the 10 i.u. doses of PMS and HCG consistently has as many or more oocytes in diakinesis, metaphase I, and anaphase I than the group given 3 i.u. injections of PMS and HCG. The group given the larger doses of gonadotrophins also totals more oocytes in telophase I and metaphase II than the other group. The number of oocytes ovulated is greater for the 10 i.u. dose group.

Tjio and Levan's aceto-orcein squash technique (Tjio and Levan, 1954) provided successful preparations of meiotic prophase and the dictyate stage (Figures 2-14, 18 and 20). Diakinesis, metaphase I, anaphase I, telophase I, and metaphase II were best identified in fixed, sectioned, and stained ovaries (Figures 16, 17, 21, 25-32, and 34-36). The phase contrast technique was most useful in viewing telophase I, telophase II, and the 2,3, and 4-cell stages (Figures 33 and 38-45). Tarkowski's air-drying method (Tarkowski, 1966) yielded one adequate preparation out of approximately 96 oocytes tested (Figure 19). The modification of Jagiello's method for preparation of mammalian oocytes proved the most successful of the techniques tested for preparing metaphase I chromosomes (Figures 22-24).

Spontaneous activation, the stimulation of unfertilized oocytes to continue meiosis and commence mitotic divisions, of Chinese hamster oocytes in cell culture was observed. The per cent activated generally increased up to 20 hours and generally decreased through 36 hours (Table 6). At 36 hours many of the oocytes showed signs of degeneration.

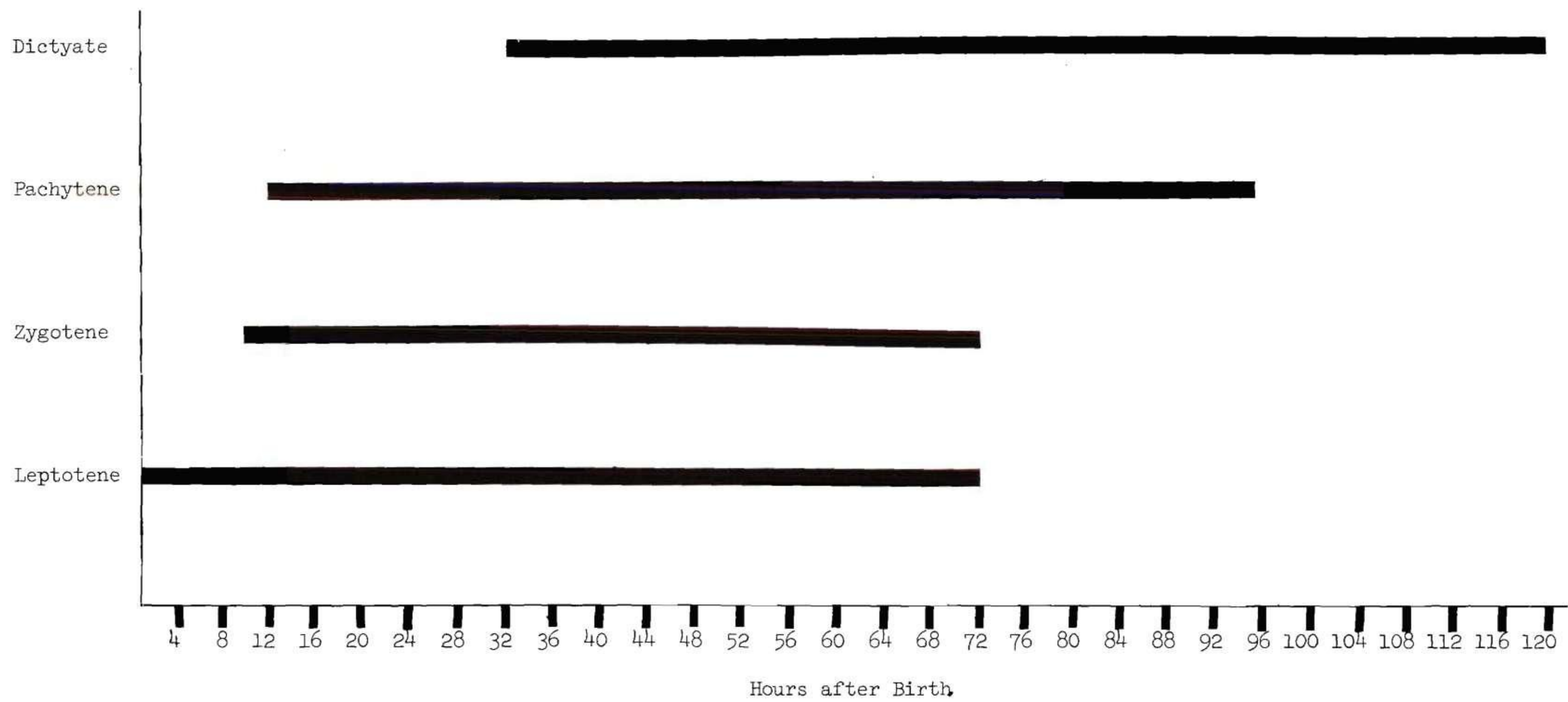

Figure 1. Duration of Prophase Stages of Meiosis in 22 Female Chinese Hamsters.

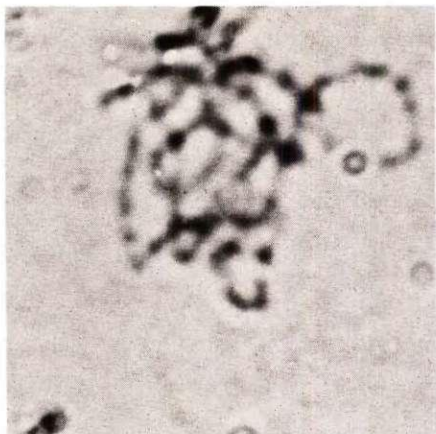

Figure 2. Leptotene. Early Prophase (X10,000).

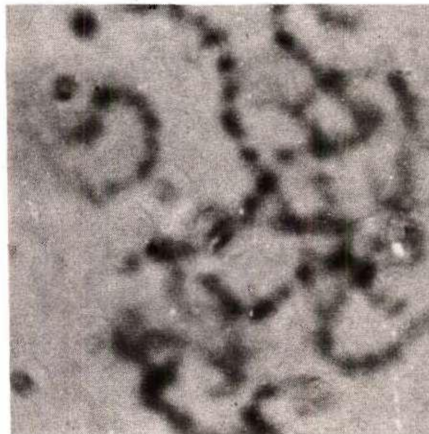

Figure 3. Leptotene. Chromosomes Diploid (X12,000).

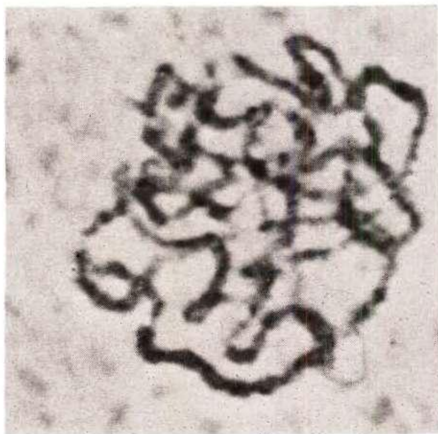

Figure 4. Leptotene. Chromosomes are Thin and Threadlike (X10,000).

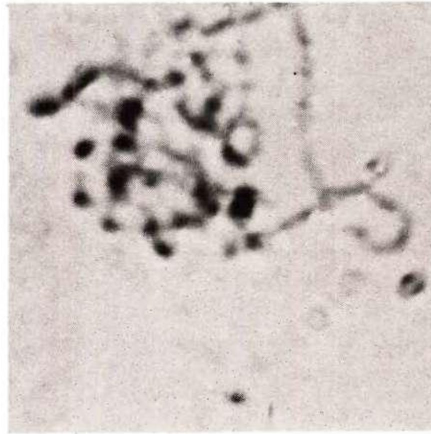

Figure 5. Leptotene. (X10,000).

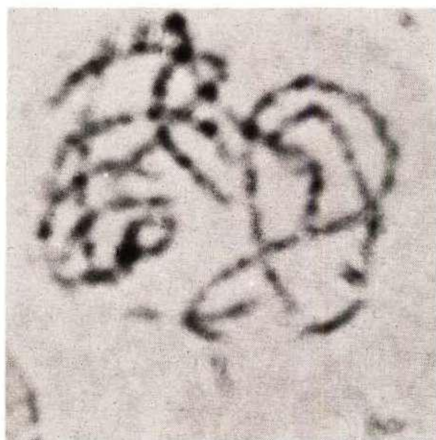

Figure 6. Zygotene. Beginning of Synapse (X10,000).

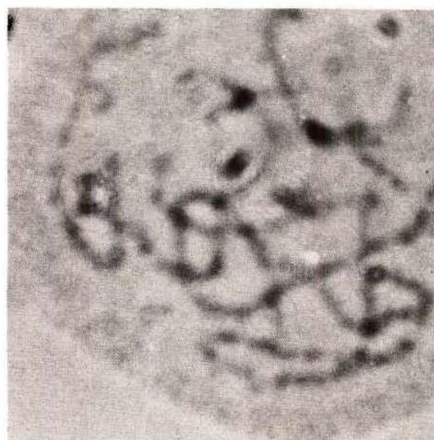

Figure 7. Zygotene. (X12,000).

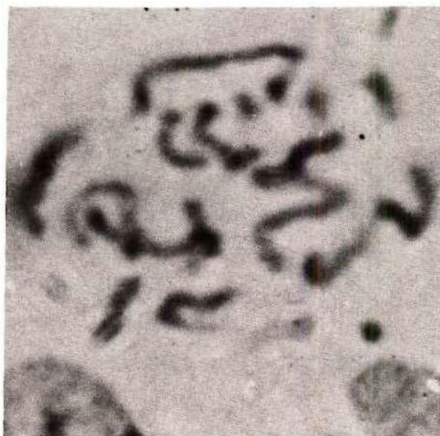

Figure 8. Zygotene. (X10,000).

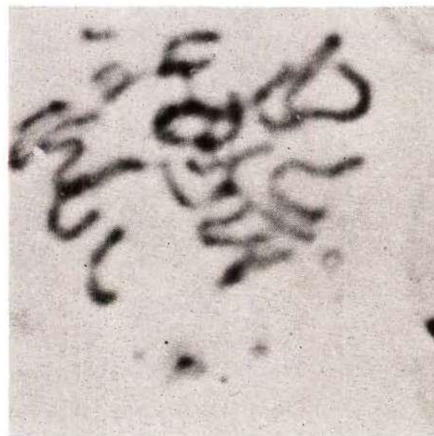

Figure 9. Zygotene. (X10,000).

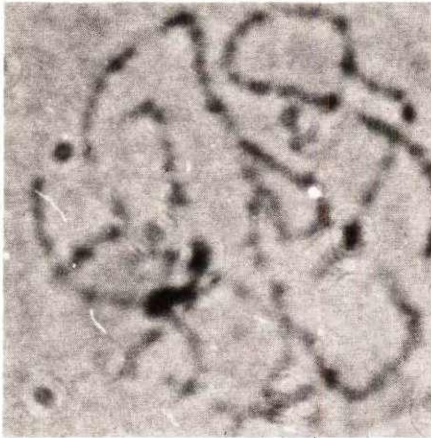

Figure 10. Pachytene. Pairing of Chromosomes is Complete (X10,000).

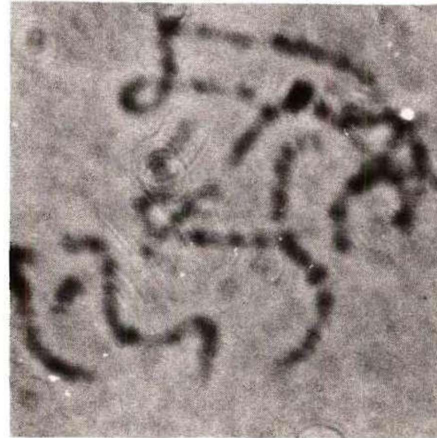

Figure 11. Pachytene. Chromosomes are Thick and Tightly Coiled (X10,000).

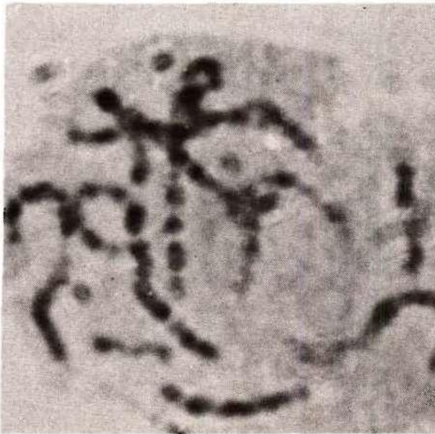

Figure 12. Pachytene. Tetrads Composed of Two Homologous Chromosomes in Close Longitudinal Union (X10,000).

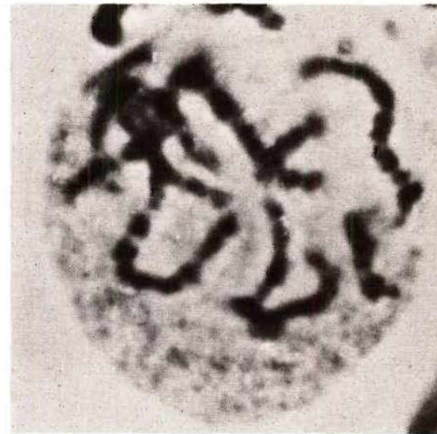

Figure 13. Pachytene. (X10,000).

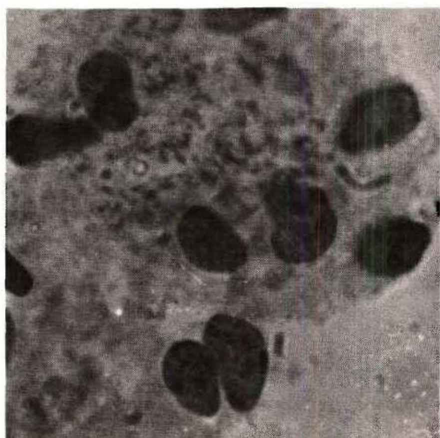

Figure 14. Early Dictyate. (X10,000).

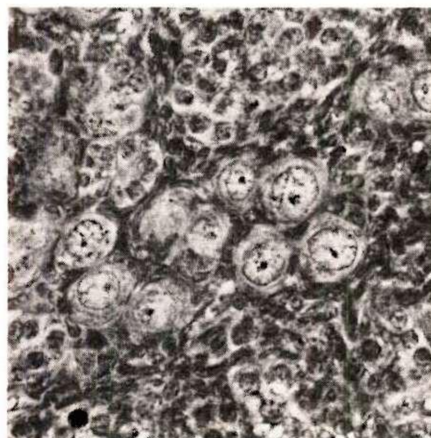

Figure 15. Dictyate. Cross-section of Ovary Showing Egg Nests (X1,600).

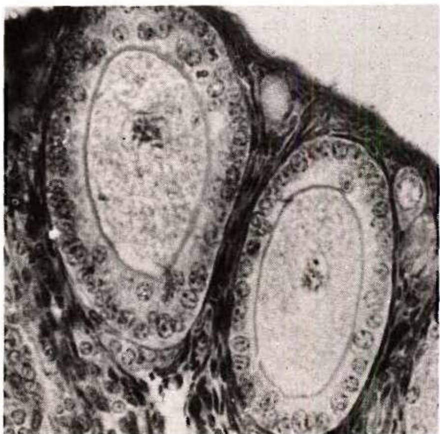

Figure 16. Dictyate. Oocytes Shown in Cross-section Surrounded by One and Two Rows of Follicle Cells (X1,600).

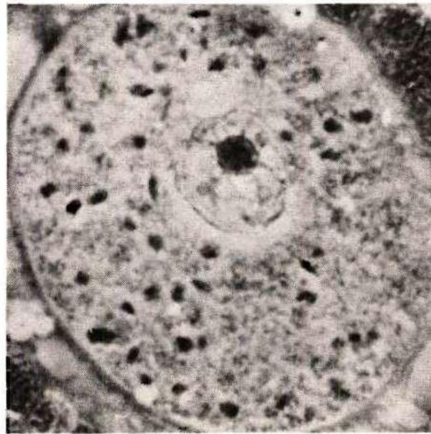

Figure 17. Dictyate. Cross-section of Ovary Showing Oocyte in Maturing Follicle (X4,500).

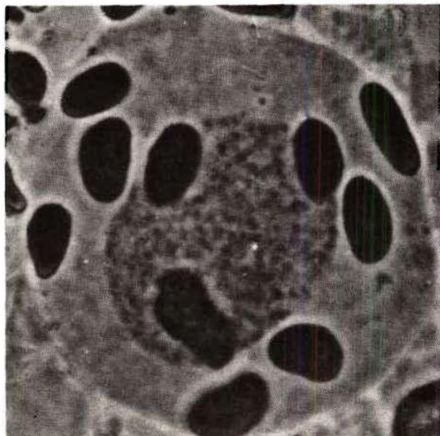

Figure 18. Dictyate. Aceto-orcein Preparation of Whole Ovary Showing Oocyte Surrounded by Follicle Cells (X10,000).

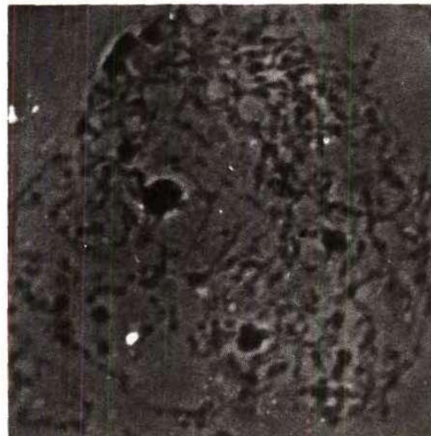

Figure 19. Dictyate. Air-dried Preparation (X10,000).

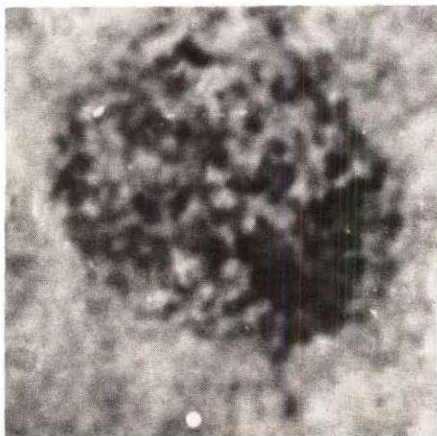

Figure 20. Dictyate. Aceto-orecin Preparation of a Follicle (X10,000).

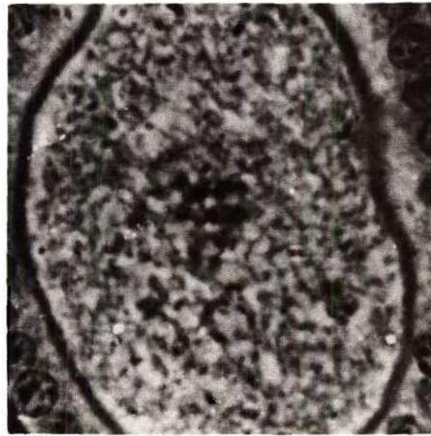

Figure 21. Diakinesis. Chromosomes Short and Tightly Coiled (X4,500).

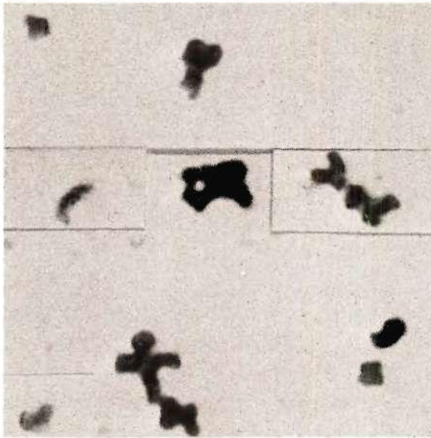

Figure 22. Metaphase I. Each Bivalent Possesses Two Independent and Undivided Centromeres (X5,000).

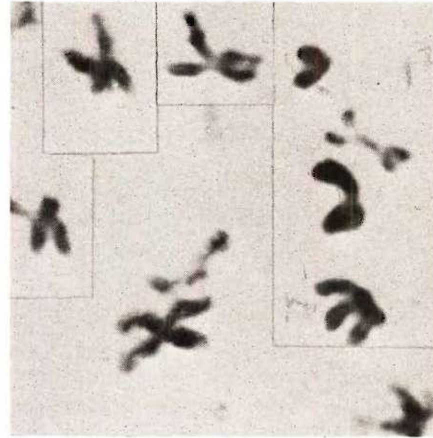

Figure 23. Metaphase I. (X6,000).

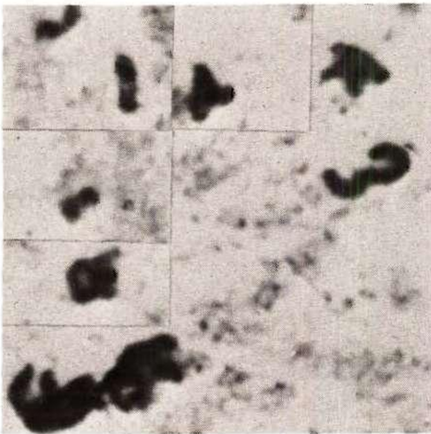

Figure 24. Metaphase I. (X10.000).

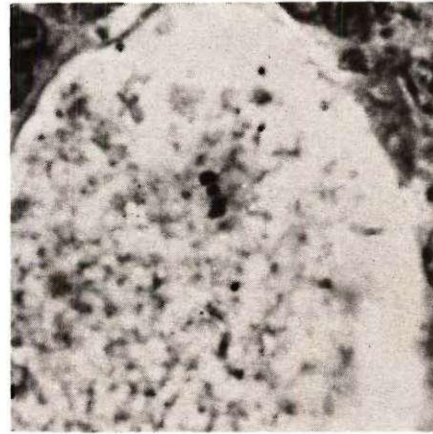

Figure 25. Metaphase I. (X4,500).

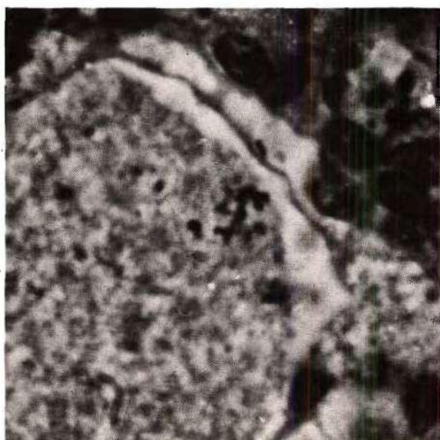

Figure 26. Metaphase I. (X4,500).

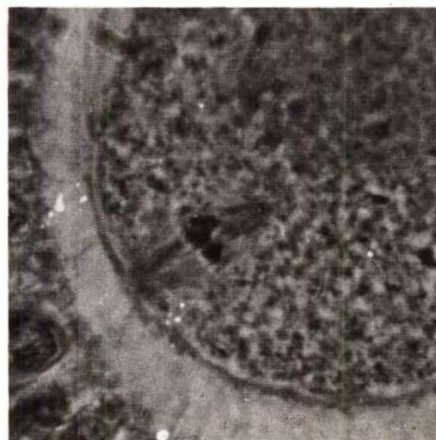

Figure 27. Metaphase I. Different Pairs of Homologous Chromosomes Align Themselves on Equator of Cell in Independent Fashion (X4,500).

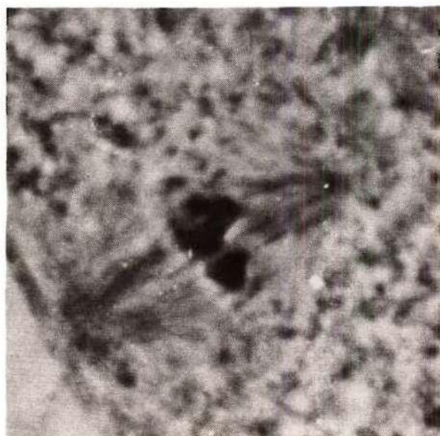

Figure 28. Metaphase I. (X10,000).

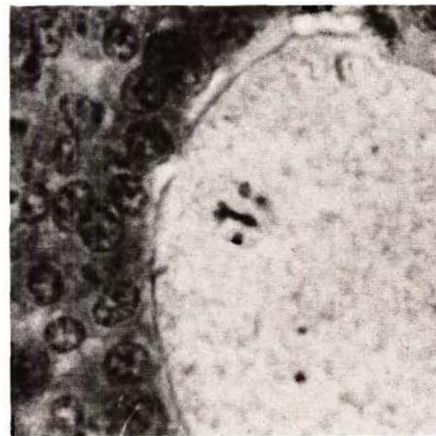

Figure 29. Anaphase I. (X4,500).

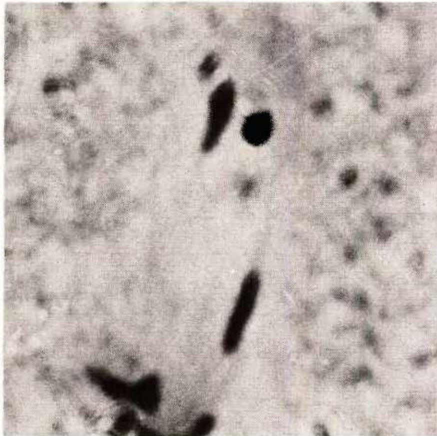

Figure 30. Anaphase I. Daughter Chromatids of Each Homologue Move Toward Their Respective Poles (X4,500).

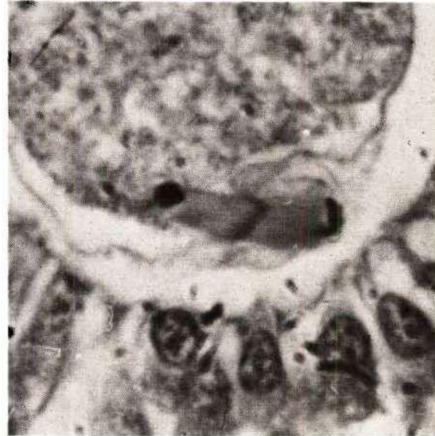

Figure 31. Late Anaphase I. Chromosomes Have Moved to Their Respective Poles (X4,500).

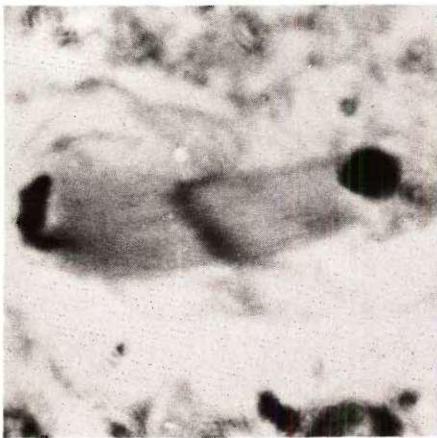

Figure 32. Late Anaphase I. Cell Plate is Forming Between Separated Chromosomes (X10,000).

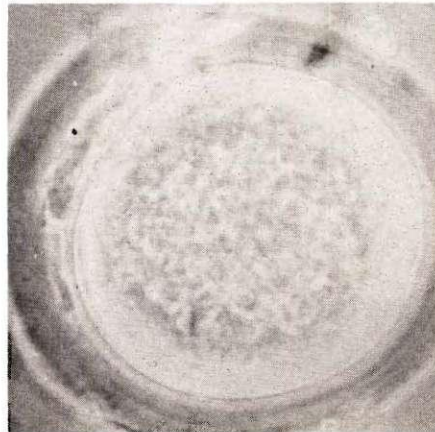

Figure 33. Telophase I. (X10,000).

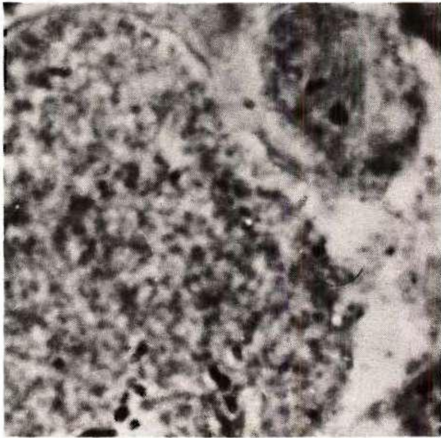

Figure 34. Metaphase II. (X4,500).

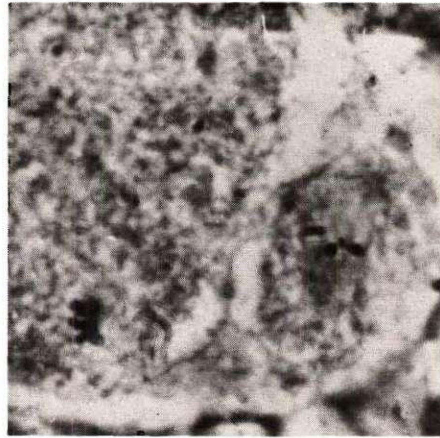

Figure 35. Metaphase II. (X4,500).

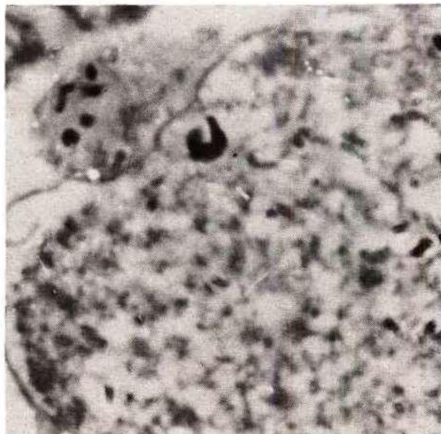

Figure 36. Metaphase II. (X4,500).

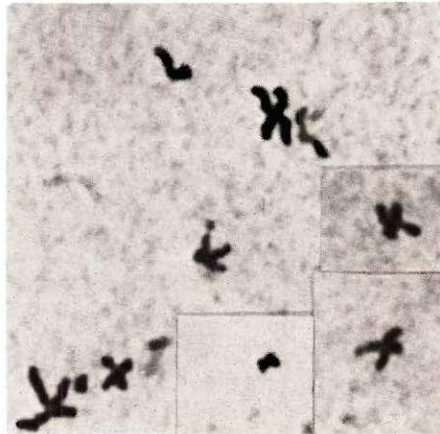

Figure 37. Metaphase II. (X6,000).

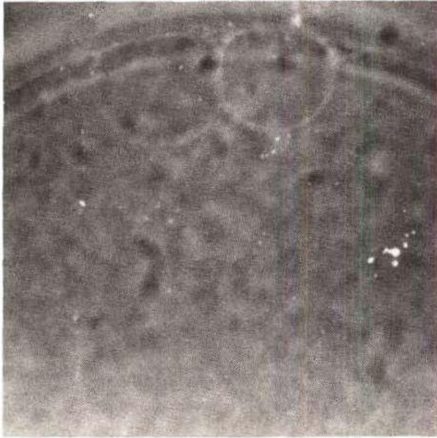

Figure 38. Telophase II. (X10,000).

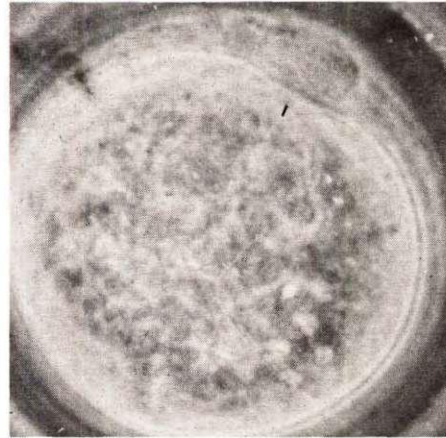

Figure 39. Telophase II. (X10,000).

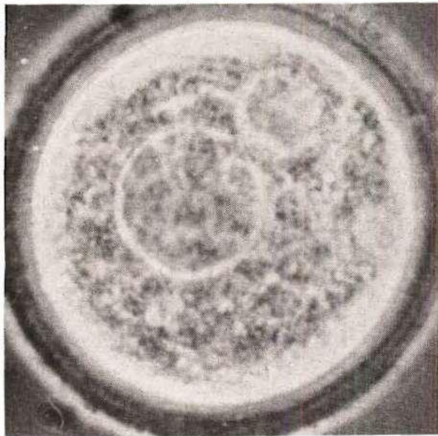

Figure 40. Telophase II. (X10,000).

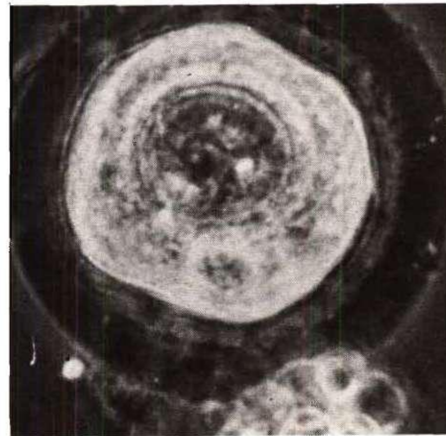

Figure 41. Two-cell Stage. (X10,000).

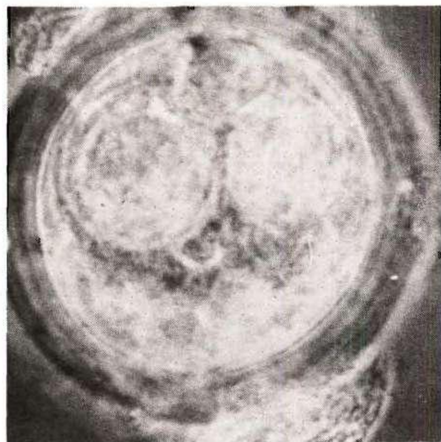

Figure 42. Three-cell Stage. (X10,000).

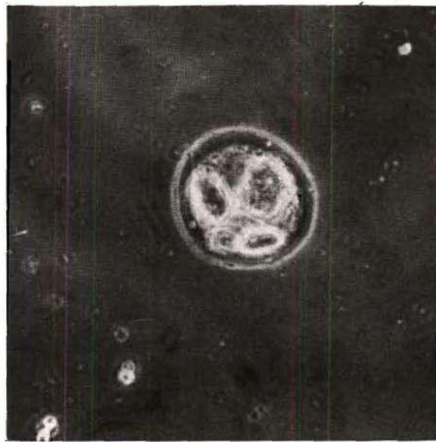

Figure 43. Four-cell Stage. (X1,600).

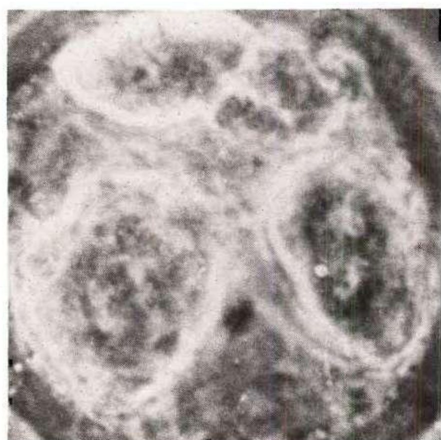

Figure 44. Four-cell Stage. (X10,000).

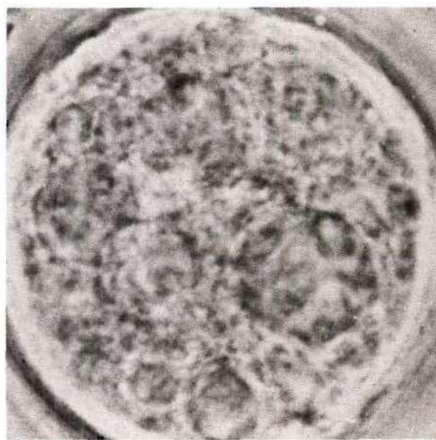

Figure 45. Morula. (X10,000).

Table 2. Duration of Prophase Stages of Meiosis in 22 Female Chinese Hamsters

Number of Hamsters	Hours After Birth	Meiotic Stages
3	0	Leptotene
1	2	Leptotene
1	4	Leptotene
1	6	Leptotene
1	8	Leptotene
1	10	Leptotene, Zygotene
1	12	Leptotene, Zygotene, Pachytene
1	14	Leptotene, Zygotene, Pachytene
1	16	Leptotene, Zygotene, Pachytene
1	18	Leptotene, Zygotene, Pachytene
1	20	Leptotene, Zygotene, Pachytene
1	24	Leptotene, Zygotene, Pachytene
1	28	Leptotene, Zygotene, Pachytene
1	32	Leptotene, Zygotene, Pachytene, Dictyate
1	36	Leptotene, Zygotene, Pachytene, Dictyate
1	44	Leptotene, Zygotene, Pachytene, Dictyate
1	48	Leptotene, Zygotene, Pachytene, Dictyate
1	72	Leptotene, Zygotene, Pachytene, Dictyate
1	96	Pachytene, Dictyate
1	120	Dictyate

Table 3. Meiotic Stages at Hourly Intervals After Injection of 3 i.u. HCG Preceded 40 Hours by 3 i.u. PMS in 22 Adult Females

Hours After Injection of HCG	Number of Oocytes in Dictyate Stage			Number of Oocytes in Diakinesis			Number of Oocytes in Metaphase I			Number of Oocytes in Anaphase I			Number of Oocytes in Telophase I			Number of Oocytes in Metaphase II			Number of Oocytes Ovulated**
	P*	D*	T*	P	D	T	P	D	T	P	D	T	P	D	T	P	D	T	T
1	71	19	90																
2	104	38	142																
3	99	26	125		3	3		2	2										
4	47	6	53		4	4		4	4		1	1							
5	42	6	48		7	7		5	5										
6	8	13	21	31	6	37		6	6		1	1		2	2				
7	6	4	10	25	23	48		5	5					2	2				
8	9	4	13	21	9	30		1	1		3	3							
9	17	9	26	21	7	28					1	1							
10	36	16	52	28	3	31		1	1										
11	9	5	14	8		8		1											
12	24	5	29	19		19		1	1		1	1		3	3		1	1	4
13	18	13	31	21	3	24								1	1		2	2	2
14	42	10	52	46	1	47								1	1		1	1	4
15	54	8	62	25		25											1	1	1.5
16	60	15	75	39	1	40											1	1	

* P represents primary oocytes with 1-2 rows of follicle cells.
D represents developing oocytes with more than 2 rows of follicle cells.
T gives the total number of primary and developing oocytes.

** Each figure given for number of oocytes ovulated is an average for two female hamsters.

Table 4. Meiotic Stages at Hourly Intervals After Injection of 10 i.u. HCG Preceded 56 Hours by 10 i.u. PMS in 22 Adult Females

Hours After Injection of HCG	Number of Oocytes in Dictyate Stage			Number of Oocytes in Diakinesis			Number of Oocytes in Metaphase I			Number of Oocytes in Anaphase I			Number of Oocytes in Telophase I			Number of Oocytes in Metaphase II			Number of Oocytes Ovulated**
	P*	D*	T*	P	D	T	P	D	T	P	D	T	P	D	T	P	D	T	T
1	87	19	106																
2	89	24	113		1	1		2	2										
3	116	30	146		10	10		7	7										
4	78	3	81		4	4		6	6		1	1							
5	34		34	29	26	55		15	15		8	8							
6	8	8	16	37	11	48		7	7		2	2							
7	6	12	18	49	34	83		5	5		11	11		1	1				
8	12	4	16	45	29	74		15	15		10	10		2	2				
9	19	11	30	68	14	82		8	8		5	5							
10	34	11	45	29	2	31		3	3		3	3		14	14		6	6	
11	23	14	37	27		27		4	4		1	1		1	1		3	3	
12	41	12	53	43	2	45								8	8		12	12	1
13	33	9	42	35	2	37								1	1		3	3	1.67
14	35	3	38	33		33											3	3	5.67
15	75	11	86	48		48		4	4								3	3	6.67
16	85	25	110	73		73		6	6		1	1					18	18	5.67

* P represents primary follicles with 1-2 rows of follicle cells.
D represents developing follicles with more than 2 rows of follicle cells.
T gives the total number of primary and developing follicles.

** Each figure given for number of oocytes ovulated is an average for two female hamsters.

Table 5. Comparison of Developing Oocytes in 22 Hamsters Given 3 i.u. Doses of Gonadotrophins and Hamsters Given 10 i.u. Doses of Gonadotrophins

Hours After Injection of HCG	Number of Oocytes in Dictyate Stage		Number of Oocytes in Diakinesis		Number of Oocytes in Metaphase I		Number of Oocytes in Anaphase I		Number of Oocytes in Telophase I		Number of Oocytes in Metaphase II		Number of Oocytes Ovulated**	
	S*	L*	S	L	S	L	S	L	S	L	S	L	S	L
1	19	19												
2	38	24		1		2								
3	26	30	3	10	2	7								
4	6	3	4	4	4	6	1	1						
5	6		7	26	5	15		8						
6	13	8	6	11	6	7	1	2	2					
7	4	12	23	34	5	5		11	2	1				
8	4	4	9	29	1	15	3	10		2				
9	9	11	7	14		8	1	5						
10	16	11	3	2	1	3		3		14		6		
11	5	14			1	4		1		1		3		
12	5	12		2	11		1		3	8	1	12	4	1
13	13	9	3	2					1	1	2	3	2	1.67
14	10	3	1						1		1		4	5.67
15	8	11				4					1	3	1.5	6.67
16	15	25	1			6		1			1	18		5.67

* S represents group given 3 i.u. gonadotrophins.
L represents group given 10 i.u. gonadotrophins.

** Each figure given for number of oocytes ovulated is an average for 2 female hamsters.

Table 6. Spontaneous Activation of Chinese Hamster Oocytes in Cell Culture

No. of Animals	Hours of Culture	No. of Oocytes Recovered	Percent Activated	Metaphase I	Anaphase I	Telephase I	Metaphase II	Anaphase II	Telephase II	2-cell	3-cell	4-cell	Morula
2	4	23	17.4	4									
2	8	25	36.0			1	8						
1	12	28	53.6			4	3	1	4				3
1	16	35	45.7			3	7	2	1				3
1	20	22	100.0			4	4	4	9				1
1	24	20	70.0			1	3		4	1		1	4
1	28	23	73.9			7	2	7			1		
1	32	24	58.3			1			9			1	3
1	36	25	40.0			3	1	1	5				

CHAPTER IV

DISCUSSION

The majority of the meiotic cells in the ovaries of newly born female Chinese hamsters were in the leptotene stage which persisted through 72 hours. Synaptic stages appeared at 10 hours and ended at 72 hours. Pachytene began 12 hours after birth and was completed by 96 hours. At 120 hours all oocytes were in the dictyate stage which apparently existed until there was a stimulus for resumption of meiosis. Evidence for this was the observation that no pre-diakinesis prophase stages of meiosis were observed in the female Chinese hamster after 96 hours. These findings are in agreement with the works of Brambell (1927), Slizynski (1957), Ohno *et al*. (1961), Edwards (1962), and Mandl (1964) who found that mammalian oocytes undergo the prophase stages of meiosis until diakinesis just before or shortly after birth.

In most mammalian species, oocytes are in the dictyate stage until stimulation by a luteinizing hormone shortly before ovulation, but appropriate endocrinological conditions can induce resumption of meiosis (Edwards, 1962). In order to obtain meiotic stages from diakinesis through metaphase II, superovulation techniques were employed to stimulate a large number of dictyate oocytes to continue their maturation division. Several factors affecting mammalian response to gonadotrophins were considered. Pregnant mares' serum (PMS) followed by human chorionic gonadotrophin (HCG) were the gonadotrophins reported

most successful in producing superovulation (Runner, 1950; Zarrow et al., 1958; and Jainudeen, 1966). Other pertinent factors included age of animals, method and amount of injections, and time interval between injections. Mature female Chinese hamsters (8-12 weeks old) were available for experimentation. Therefore two test groups were given injections of gonadotrophins comparable to those given to adult mice by Edwards and Gates (1958). Preliminary evidence indicates that there may be a correlation between body weight and the intensity of ovarian response in Golden hamsters (Bodemer, 1959); therefore, four other test groups were given injections of 10 i.u. PMS followed after 56 hours by 10 i.u. HCG which Jagiello (1965) used to produce superovulation in rats.

In 32 female Chinese hamsters treated with gonadotrophins, the dictyate stage was present in primary and in developing follicles from 1-16 hours after HCG injection; and diakinesis was present after 2-16 hours. These findings agree with Walton and Hammond's (1928) report that ovulation is not the invariable consequence of follicular growth, and that ovulation is independent of actual follicular size but limited to mature follicles. In an animal such as the Golden hamster where a great number of eggs are expelled, all ripe follicles are not expected to rupture, because a small per cent may become atretic just before rupture (Strauss, 1956). Kirkham (1913) also found perfectly normal rat oocytes in the dictyate stage in larger follicles just at, and also subsequent to ovulation. He attributed the presence of the dictyate stage after ovulation to the requirement of more than one

estrus cycle for the development of the oocyte from dictyate to the stage of the first polar body when the oocyte leaves the ovary. In the Chinese hamster, the presence of developing oocytes in dictyate and diakinesis after ovulation supports Kirkham's (1913) report for the rat.

In the female Chinese hamster, metaphase I first appeared 2 hours after HCG injection and was completed by 12 hours. Metaphase I oocytes seen at hours 15 and 16 were atretic. Anaphase I appeared 3 hours after HCG injection and was last recorded at 12 hours. The one anaphase I oocyte seen at 16 hours also was atretic. Telophase I lasted from 6-14 hours after injection of HCG, while metaphase II lasted 10-16 hours. Ovulation began at 12 hours after HCG and reached a peak at 15 hours. These findings compare favorable with the works of Edwards and Gates (1958) and Jagiello (1965) who demonstrated similar results in adult rats and in adult and immature mice. Further experiments using gonadotrophins should determine a gonadotrophic dose which would produce more effective superovulation in the Chinese hamster.

Spontaneous activation was observed in approximately 55 per cent of the oocytes cultured. The presence of Chinese hamster oocytes at telophase I (Figure 33) and metaphase II (Figures 34-37) after eight hours in cell culture media agrees with the earlier finding of Pincus (1930). Telophase II (Figures 38-40) appeared at 12 hours, and a two-cell stage (Figure 41) and a four-cell stage (Figures 43 and 44) appeared after 24 hours of cell culture. In the two-cell and in the

four-cell stage, blastomeres were of unequal size. The presence of a three-cell stage (Figure 42) at 28 hours also indicates asynchronous division. These results agree with Hamilton's (1942) report on the Golden hamster where at first division two blastomeres are produced which are usually of unequal size.

CHAPTER V

CONCLUSIONS

A study of the meiotic stages in the female Chinese hamster (Cricetulus griseus Milne-Edw.) led to the following observations.

1. Oogenesis was not complete in the female Chinese hamster at birth.

2. Oocytes were in leptotene in the newly born female hamster; some oocytes were in leptotene 72 hours after birth.

3. The zygotene stage also appeared in ten-hour old female hamsters and ended at 72 hours.

4. Pachytene was first seen at 12 hours after birth and was last seen at 96 hours.

5. Early dictyate stages first appeared in 32-hour old hamsters; late dictyate stages began at 96 hours and continued until a stimulus initiated the maturation divisions.

6. The gonadotrophins pregnant mares' serum and human chorionic gonadotrophin induced superovulation in the mature female Chinese hamster.

7. The meiotic stages diakinesis, metaphase I, anaphase I, telophase I, and metaphase II were found in Chinese hamster ovaries from 2-16 hours after an injection of HCG preceded by an injection of PMS.

8. Ovulation began 12 hours after injection of HCG.

9. Spontaneous activation of meiosis was observed in ovarian oocytes placed in cell culture media.

10. Anaphase II and telophase II were observed in spontaneously activated oocytes after 12 hours of cell culture.

11. After 24-28 hours of cell culture, two-cell stage, three-cell stage and four-cell stage oocytes were observed.

CHAPTER VI

RECOMMENDATIONS

Based on an insight gained during the course of this investigation, the following recommendations are noted as being of future interest:

1. Detailed study of superovulation in female Chinese hamsters in order to determine the optimum dosage of gonadotrophins and the most favorable conditions of treatment.

2. Further tests on meiotic preparations should be conducted to establish a technique which gives consistently good preparations of Chinese hamster metaphase I chromosomes.

3. Spontaneous activation of oocytes should be further investigated to test the possibility of establishing a cell line from the oocytes.

CHAPTER VII

APPENDIX

FIXATION, SECTIONING, AND STAINING

After fixation in Allen's Fixative, B-15, and Bouin's fluid the ovaries were then washed in distilled water for ten minutes, 50 per cent alcohol for one hour, and frequent changes of 70 per cent ethyl alcohol until the yellow color due to picric acid disappeared.

Dehydration was carried out by hourly changes of the following: two changes of 70 per cent ethyl alcohol; two changes of 95 per cent ethyl alcohol; and two changes of 100 per cent ethyl alcohol.

To clear the ovaries they were placed in hourly changes of 1:1 absolute alcohol and xylol and two changes of xylol.

Next the ovaries were placed in melted paraffin and allowed to remain until the specimens were completely infiltrated with melted paraffin. The steps from dehydration to infiltration were carried out in an autotechnicon®.

The ovaries were next embedded in blocks of solid paraffin. Metal embedding dishes were sprayed with mold-release spray; they were then partially filled with paraffin and placed on a hot plate at 56°C. The ovaries were placed in the paraffin and the metal dishes were immediately placed on blocks of ice to harden the paraffin and to secure the tissue in the center of the blocks before the paraffin had time to crystallize. Tissue-teks® were placed on the metal dishes which were then completely filled with paraffin. After the paraffin hardened the embedding dishes were removed, and the blocks

of paraffin containing the ovaries were placed in a refrigerator overnight before sectioning.

Each ovary was serially sectioned at 7 microns. Sections were laid in order on black paper. From there they were floated on a water bath set at 50°C. The sections were picked up from the water bath onto slides smeared with egg albumin.

Paraffin was removed from the sections by passing them through xylol, 1:1 xylol and 95 per cent ethyl alcohol, and 95 per cent alcohol. The ovary sections were stained 6 minutes in Weigert's iron hematoxylin, washed in tap water, and counterstained for 3 minutes in 1:5000 aqueous fast green FCF. They were then washed in 1 per cent acetic acid, dehydrated with 95 per cent ethyl alcohol and two changes of 100 per cent ethyl alcohol, cleared with one change of 1:1 alcohol-xylol and two changes of xylol, and mounted in permount.

Table 1. Cell Culture Media.

Compound	Quantity in ml	Concentration in mg/lit.
Hanks' Saline Solution	100	
$NaCl$		8000
KCl		400
$CaCl_2 \cdot 2H_2O$		186
$MgSO_4 \cdot 7H_2O$		100
$MgCl_2 \cdot 6H_2O$		
L-Amino Acids in 2N HCl	20	
Arginine		105
Cystine		24
Glutamine		292
Histidine		31
Isoleucine		52
Leucine		52
Lysine		58
Methionine		15
Phenylalanine		32
Threonine		48
Trytophan		10
Tyrosine		36
Valine		46
Phenol Red	5	5
Distilled and Deionized Water	10	
2N NaOH	5	
Phosphate buffer	5	
$Na_2HPO_4 \cdot 7H_2O$		90
KH_2PO_4		60
Glucose		1000
Sodium Bicarbonate		350
Glutamine		290
Penicillium "G" Sodium		50
Dehydrostreptomycin Sulphate		50
Pyruvate		110
Filtered Fetal Calf Serum	200	
Vitamins		
Choline		1
Folic Acid		1
Nicotinamide		1
Pantothenate		1
Pyridoxal		1
Riboflavin		0.1
Thiamine		1
Distilled and Deionized Water	655	

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