NON-DISJUNCTION IN AGING FEMALE MICE

by

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ABSTRACT

Classical studies have shown that reproductive performance declines with maternal age in humans and other mammals. There is an increase of trisomic offspring with maternal age in humans and an increase of trisomic embryos and fetuses with maternal age in mice. It has been suggested that this increase in non-disjunction is due to the decrease in chiasma frequency and increase in univalents observed in the oocytes of old mice. This study was undertaken to determine the effects of maternal age on non-disjunction in the oocytes of CBA mice.

Oocytes from CBA mice varying in age from two to eleven months were cultured to the metaphase II stage of meiosis and the chromosomes were analysed. The oocytes from three maternal age groups were compared with respect to the mean number of oocytes obtained per mouse, the frequency of maturation to metaphase II, and the frequency of numerical chromosome abnormalities. Both the mean number of oocytes obtained per mouse and the frequency of maturation decreased markedly with maternal age. The frequency of chromosome abnormalities in the oocytes increased with

maternal age from the young to the middle aged mice but dropped off in the oldest maternal age group. No hyperploid (n+1) oocytes were observed in the young or old group of mice but 4.9% hyperploidy occurred in the middle age group. It is suggested that the lack of hyperploid oocytes in the old CBA females might be due to a threshold effect in which oocytes which are damaged by the number or type of univalents become atretic and do not progress to metaphase II. The frequency of diploid (2n) oocytes was 1.7% and was not maternal age dependent.

An overall theory of maternal age related non-disjunction is proposed in which the various environmental and genetic factors known to affect non-disjunction are linked to an underlying mechanism of univalent production in oocytes.

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I. INTRODUCTION

Reproductive performance in mammals

Advanced maternal age in mammals is associated with reduced fertility. In humans, the frequency of offspring with chromosome abnormalities, particularly trisomies, increases with maternal age (Penrose 1933; Court Brown et al., 1969; Polani, 1969). Penrose (1933) first discovered that the mean maternal age for mothers of children with Down's syndrome (trisomy 21) was 35 years compared to a mean maternal age of 28 years for the general population. Collman and Stoller (1962) noted that the incidence of Down's syndrome increased dramatically with maternal age: from .043% in mothers between 15 and 19 years to 2.2% in mothers 45 years and older. Lenz, et al. (1966) and Magenis, et al. (1968) have shown that the distribution of maternal age in trisomy D and trisomy E shows a similarity to that for Down's syndrome. There is also evidence for an increased mean maternal age for spontaneous abortions where the abortus has been shown to be an autosomal trisomic, including types of trisomy not yet found in post-natal life (Kerr and Rashad, 1966; Carr, 1967a).

To date, studies in other mammals have not shown an increase in the frequency of chromosomally abnormal offspring with maternal age. However, most mammals spend a considerable portion of their lifespan in a post-reproductive condition and a marked reduction in litter size with increasing maternal age has been documented in several laboratory animals (Talbert, 1968).

Biggers et al. (1962) and Harman and Talbert (1967) have shown that the reduction in litter size in old female mice is not accompanied by a lower ovulation rate. Ingram, Mandl, and Zuckerman (1958) have shown that litter size declines with maternal age in rats but the number of corpora lutea remains constant. Perry (1954) found no reduction in the number of corpora lutea in old pigs that were discarded for low fertility. Similarly, several investigators (Parkening and Soderwall, 1973; Connors et al., 1972; Thorneycroft and Soderwall, 1969) have shown that the ovulation rate is the same in young and old hamsters, although litter size declines with age. But Blaha (1964) found a decrease in the number of corpora lutea in old hamsters. Since the vast majority of studies indicate that the decrease in litter size is not accompanied by a decrease in the number of

oocytes ovulated, many embryos of older female mammals must be dying before birth.

Conners et al. (1972) have shown a highly significant increase in preimplantation and postimplantation death of embryos in old female hamsters. Many of the surviving embryos were retarded in development in the old females. Thorneycroft and Soderwall (1969) established that old female hamsters have a seven-fold increase in preimplantation death and a two-fold increase in resorption of established implantation sites by the eighth day of pregnancy. Embryonic mortality is also higher in old mice: Gosden (1974a) determined that 91% of oocytes from young mice survived to the blastocyst stage whereas only 63% of oocytes from old mice were alive at this stage. Gropp (1973) produced aneuploid embryos by using parental translocation strains and found that no aneuploid fetuses survived to term: monosomic embryos died in preimplantation stages and trisomics survived to become fetuses but died before birth. While some of the prenatal death that occurs in old females may be due to chromosome abnormalities, the decreased survival of embryos and fetuses in old females may also be due to uterine factors.

Gosden (1974a) and Talbert and Krohn (1966) used the technique of ovum transplantation to determine whether prenatal death in old female mice is due to uterine factors or defects in the ova. Blastocysts, recovered from young and old mice, were transferred into the uteri of young mice which had been made pseudopregnant by mating with a vasectomized male. Blastocysts from young and old donors survived equally well in the young recipients. When blastocysts from young and old donors were transferred to old recipients, survival was reduced. Therefore, both studies implicated uterine factors as an important cause of the decline in reproduction. However, the number of blastocysts recovered from old mice was significantly lower and the embryonic loss before the blastocyst stage might be due to chromosomal errors. Blaha (1964) carried out a similar study in hamsters, but in contrast to the results obtained with the mice, 49% of blastocysts recovered from young donors developed into normal term fetuses in young hosts but only 4.5% of blastocysts from old donors developed successfully. This indicates that the blastocysts from old hamsters may have been intrinsically defective. There is no definite explanation for the apparent great difference in the viability of embryos recovered from old

hamsters and old mice. A species variation is possible, but there were also technical differences in timing and handling of the ova which may have influenced the results. Therefore the relative contributions of ovarian and uterine factors toward pre-natal loss in old females is still unclear.

Two recent studies of chromosomal abnormalities in embryos (Gosden, 1973) and fetuses (Yamamoto et al., 1973a) from female mice of various ages demonstrate a significant increase of aneuploidy with maternal age in mice as well as in humans, although in mice autosomal trisomic embryos do not survive to term (Goodlin, 1965). Therefore, mice can be used as an experimental model to elucidate the cause of maternal age related non-disjunction leading to aneuploid embryos.

Theories of maternal age related non-disjunction

Several theories have been proposed to explain the effect that maternal age could have on non-disjunction. These theories can be divided into those that depend on the aging of various maternal systems and those that depend on the aging of the ovum due to the prolonged dictyotene stage of meiosis in mammals. Theories of non-disjunction due to aging of maternal systems are: intrafollicular and tubal aging of eggs, pH

disturbances in the female reproductive tract, and autoimmune disease. Theories of non-disjunction due to aging of the ovum during the dictyotene stage are: satellite association of chromosomes, X irradiation, and production of univalents in oocytes.

A) Theories of non-disjunction due to aging of maternal systems

1. Intrafollicular aging of ova

In 1922, Witschi first suggested that delayed ovulation could lead to intrafollicular aging of the ovum and subsequent abnormalities in the embryo. In the African clawed toad, Xenopus, if the sexes are separated, the female retains her eggs and will ovulate only after an injection of gonadotropic hormones. In a series of experiments, Witschi (1969) determined that Xenopus eggs aged in this manner had a greatly increased incidence of mortality and embryonic abnormality. Witschi hypothesized that delayed ovulation in humans could lead to chromosomal abnormalities as well as malformations. Delayed ovulation could be caused by hormonal irregularities which are most often seen at the extremes of maternal age——in very young women whose cycles are just getting established and in

older women approaching the menopause.

There have been some studies in humans which lend support to this theory. In 1955, Hendricks determined that, according to the 1953 Ohio records, women less that 15 years old or greater than 35 years old had a higher incidence of offspring with congenital malformations (2%) than women between the ages of 15 and 35 (1%). Carr (1971a) determined that there was an increase of chromosomal abnormalities in spontaneous abortions from young and old women. Women younger than 17 years had 40% heteroploid abortions; women older than 40 had 33% heteroploid abortions. The general frequency for heteroploid abortions in women of all ages was only 22%. However, these were both retrospective studies and the data was not analysed statistically. Furthermore, the increase of abnormalities in the young and old females was not necessarily due to hormone irregularities or delayed ovulation; they could have been caused by other factors. Hertig (1967) related the frequency of abnormal human embryos to the time of conception. He determined that women who ovulate regularly have a 92% chance of producing normal offspring if conception occurs on day 14 of the menstrual cycle. If

conception occurs on day 15 or later, the possibility of a normal conceptus drops to 42%. Therefore he felt that a delay in ovulation could cause abnormalities. This study was also retrospective and depended on estimates of the timing of ovulation which is difficult to determine exactly.

Only one experimental mammal, the rat, has been used to study this hypothesis. The reproductive cycle in female rats is normally 4 days; however, old rats have a 6 day cycle (Fugo and Butcher, 1970). Therefore ovulation may be delayed in these old rats. In a series of experiments Butcher and Fugo (1966, 1967, 1969a) used nembutal to cause a two-day delay in ovulation in young rats. The rats treated with nembutal had a decreased fertilization and implantation rate, an increased frequency of abnormal embryos, and a three-fold increase in chromosomally abnormal embryos. The majority of the chromosomally abnormal embryos were mosaics but there was also a small increase in the number of aneuploids. However, one cannot be certain whether the increase was due to the delay in ovulation or some other effect of the nembutal. No studies of chromosome abnormalities in untreated old rats have been reported to date.

2. Tubal aging of ova

German (1968) proposed that the increase of trisomy with maternal age could be explained by tubal aging of the egg due to delayed fertilization. He reasoned that older women had less frequent intercourse and this caused a delay in fertilization. The human studies by Hendricks (1955) and Carr (1971a) demonstrating an increase of abnormal offspring and embryos in young and old mothers could be interpreted as supporting a delayed fertilization hypothesis rather than delayed ovulation. Very young women, as well as older women, could have infrequent intercourse. Hertig's (1967) results could also be explained by delayed fertilization. He found that embryonic abnormality increased if conception occurred on day 15 or later in the cycle and this could be due to a delay in the sperm reaching the egg rather than to delayed ovulation. However, all three studies were retrospective and the increase of abnormalities could still be caused by something other than delayed ovulation or fertilization.

Several people have criticized German's hypothesis on statistical grounds. Matsunaga and Maruyama (1969) believe

that the age dependency for the frequency of intercourse is not sufficient to account for the great increase in Down's syndrome with maternal age.

There had been studies on the effect of delayed fertilization in many experimental animals——guinea pigs (Blandau and Young, 1939); rats (Braden, 1959); rabbits (Shaver and Carr, 1969); mice (Vickers, 1969); hamsters (Yamamoto and Ingalls, 1972); pigs (Hunter, 1967). In all these studies, there was an increase in embryonic abnormalities and death, but the commonest effect of the delayed fertilization was triploidy. After delaying fertilization for seven hours in mice, Vickers (1969) got a slight increase in trisomy and a nine-fold increase in triploidy. Shaver and Carr (1969) found that triploidy increased from 1.4% to 13% after delaying fertilization for 6-10 hours in rabbits.

This dramatic increase in the frequency of triploid embryos may be due to a defect in the spindle fibre structure in aging eggs. Szollosi (1971) has determined that the spindle in aging eggs often rotates and migrates to the centre of the ovum. This would inhibit the second meiotic division and a diploid egg would result. The triploid embryo could also be caused by failure of the aging oocyte

to prevent more than one sperm entering the egg.

Therefore, tubal aging of the egg due to delayed fertilization does cause embryonic abnormalities, particularly triploidy, but it does not seem to be a cause of aneuploidy.

3. pH disturbances

Ingalls and Shimada (1974) have recently proposed that aneuploid offspring may result from pH disturbances in the fallopian tubes of older women. They have determined that a pH of 6.7 to 6.9 causes a broad spectrum of chromosomal anomalies in cultured lymphocytes from adult blood. However, no studies have been published on the pH levels in fallopian tubes or on the effect of pH disturbances on gametes.

If pH changes in the fallopian tube did cause aneuploidy, non-disjunction would occur at the second meiotic division since the oocyte is ovulated at metaphase II (Zuckerman, 1962). But non-disjunction generally occurs at the first meiotic division. Using fluorescent markers, Sasaki and Hara (1973) demonstrated that seven out of eight cases of trisomy 21 occurred at the first meiotic division

of the oocyte. Therefore it seems unlikely that pH disturbances are a significant cause of non-disjunction.

4. Autoimmune disease

In 1963, Engel and Forbes first determined that chromosome non-disjunction occurs more frequently in families with a genetic predisposition to autoimmunity. A high prevalence of thyroid autoantibodies have been demonstrated in children with Down's syndrome (Engel, 1967) and their mothers (Fialkow, 1964). Since the prevalence of autoimmune disease increases with age, Burch (1969) proposed that non-disjunction during meiosis was caused by an autoagressive attack on the germ cells. This autoagressive attack would occur more frequently in older women. This hypothesis could account for the cases of familial clustering of chromosomal abnormalities since a predisposition to some autoimmune diseases is inherited (Hecht et al., 1964). But there is no experimental evidence to support this theory and the correlations between non-disjunction and autoimmunity are present chiefly in young, not old, mothers.

B) Theories of non-disjunction due to aging of the ovum during the dictyotene stage of meiosis

In mammals, meiosis begins in the fetal ovary and progresses to diplotene. At this stage, meiosis is arrested and does not resume until shortly before ovulation in the adult (Zuckerman, 1965a). This extended diplotene, termed dictyotene, can persist in some oocytes until the end of the reproductive life of the female; that is, up to two years in the mouse and fifty years in man. Since the frequency of aneuploid offspring and embryos increases with maternal age, several people have suggested that changes in the oocyte during the prolonged dictyotene increase the frequency of non-disjunction. The following theories suggest the various mechanisms which might cause non-disjunction.

1. Satellite associations of chromosomes

Satellite associations of the acrocentric D and G chromosomes are often present in mitotic cells (Heneen and Nichols, 1966). The satellites of the acrocentric chromosomes have nucleolar organizers (Ferguson-Smith, 1964) and their association is generally regarded as a holdover from

their proximity to the nucleolus. Since the D, E, and G chromosomes are most frequently involved in non-disjunction in live-born children, failure of the nucleolus to disperse has been proposed as a cause of non-disjunction.

Polani et al. (1960) suggested that the duration of the dictyate cycle might cause a slower dispersion of the nucleolus in older oocytes, interfering with chromosome pairing and chiasma formation in D and G chromosomes. But this hypothesis is invalid, since chromosome pairing and chiasma formation occur during prophase in the fetal ovary before dictyotene.

A modification of this theory has been proposed by Evans (1967). He believes that the nucleolus may fail to disperse and persist into metaphase I, preventing separation of the bivalents associated with it.

Many studies have been done on the frequency and type of nucleolar associations in different populations. If satellite associations are causing non-disjunction in man, then in the D group, chromosome 13 should be present in associations more frequently than chromosome 14 or 15, and in the G group, chromosome 21 more often than chromosome

22 because chromosome 13 and 21 are more frequently involved in non-disjunction. Cooke (1972) has determined that chromosome 13 is present in nucleolar associations more frequently than chromosome 14 or 15. However, Nakagome (1973) has shown that satellite associations do not occur more frequently in chromosome 21 than in chromosome 22. Moreover, the parents of children with Down's syndrome do not have more chromosome 21 associations than chromosome 22 (Curtis, 1974). It is important to note that the above reasoning is based on the assumption that chromosomes 13 and 21 are also the most frequent trisomies in pre-natal stages and recent evidence from spontaneous abortions demonstrates that this may not be true. In any case, all of these studies have been performed in mitotic tissue, and the results may be quite different in meiotic tissue. To date, there have not been any reports on associations between the satellite chromosomes in the oocytes of humans or experimental mammals.

Because of reports of clustering of Down's syndrome, some people have suggested that viruses can cause non-disjunction (Stoller and Collman, 1965; Robinson et al., 1969). Evans (1967) believes that viruses may reduce the capacity

for dissolution of the nucleolus because DNA viruses replicate within the nucleus and an increase in the size of the nucleolus is often seen with virus infection.

If the nucleolus is involved in causing non-disjunction, it would explain why the D and G chromosomes occur so frequently in trisomic conditions. But it would not explain non-disjunction of the E chromosomes. Yunis (1965) suggested that the D, E, and G chromosomes are most common in trisomic conditions because they are largely genetically inert, since large portions of these chromosomes replicate late in the cell cycle. Franceschini et al. (1973) pointed out that chromosomes 13, 18, and 21 have much larger areas of fluorescence after treatment with quinacrine than the other members of the D, E, and G groups of chromosomes. Furthermore, since Sanchez and Yunis (1974) have recently shown that Q bands due to quinacrine fluorescence, are sites of repetitive DNA, this implicates the genetic inertness of the specific chromosomes most common in trisomic conditions. Therefore, a loss or gain of these chromosomes would be less deleterious than the loss of other chromosomes and trisomic embryos involving these chromosomes would be more likely to survive to term.

Therefore the evidence supporting the theory of nucleolar association of satellite chromosomes is really restricted to mitotic cells. Further studies in oocytes are needed to determine if nucleolar associations persist into metaphase I.

2. X irradiation

There have been several studies showing a possible association between maternal irradiation and the incidence of aneuploid offspring (Uchida and Curtis, 1961; Alberman et al., 1972). But they have generally been retrospective studies complicated by ascertainment bias.

In 1968, Uchida et al. reported the results of a prospective study on chromosomal anomalies among the children of irradiated mothers. They found that women exposed to abdominal irradiation ran an increased risk of producing aneuploid children, especially late in reproductive life. Advanced maternal age seemed to increase the ability of X rays to cause non-disjunction.

Uchida and Lee (1974) studied the effect of low-dose X irradiation on the oocytes of young female mice. They found a small increase in the frequency of non-disjunction during the first meiotic division in the oocytes.

Yamamoto et al. (1973b) studied the effects of low-dose X irradiation on the production of heteroploid fetuses in young and old female mice. They found a spontaneous rate of aneuploid fetuses in both young and old mothers and the frequency of aneuploid fetuses was significantly higher in older female mice. Radiation increased the frequency of aneuploid fetuses but only in old female mice. Radiation did not cause a significant increase of aneuploidy in young mice. Hence, there must be some underlying mechanism, other than radiation, which causes an increase of non-disjunction with maternal age. Once this process has affected the rate of non-disjunction in older females, radiation can then cause a further increase.

3. Production of univalents in oocytes

Mather (1938) and others have pointed out that a decrease in the frequency of crossing-over and chiasmata is associated with an increase in non-disjunction of chromosomes. A minimum of one chiasma per bivalent is necessary to ensure that chromosomes remain paired and segregate normally at anaphase I of meiosis (Maguire, 1974). Loss of chiasmata has been shown to result in precocious

univalent production in plants (Thomas and Rajhathy, 1966), insects (Shaw, 1971), mice (Purnell, 1973), and man (Pearson et al., 1970). The univalents have then led to anomalous segregation of chromosomes and to aneuploid gametes (Carpenter and Sandler, 1973; Sharma and Reinbergs, 1974). Slizynski (1960) first suggested that the increase of aneuploid offspring with maternal age in humans might be explained by a loss of chiasmata during the long dictyotene stage of meiosis.

Henderson and Edwards (1968) found a significant decrease in the chiasma frequency in the oocytes of old female mice of three different strains——CBA, CBA/T6T6, and C57BL. The location of the chiasmata was more terminal and an increase in the frequency of univalents was also observed in older females. In male mice, a comparable decline in chiasma frequency or increase in univalents was not seen. Henderson and Edwards proposed that univalents were produced in the older females during the prolonged dictyotene stage of meiosis and that none were seen in older males because spermatogenesis is a continuous process. Henderson and Edwards also observed univalents in some human oocytes but the numbers were too small to be

statistically significant. However, they suggested that a similiar reduction in chiasma frequency with subsequent univalent production could explain the increased incidence of trisomy in older women.

In 1973, Luthardt et al. confirmed these results in two different strains of mice, C57 BL/6J and ICR. They demonstrated a significant decrease in chiasma frequency and an increase in univalents with age. They also discovered that univalent production was nonrandom and involved small chromosomes predominantly. This is not surprising since small chromosomes have fewer chiasmata (Slizynski, 1960) and a progressive loss of chiasmata would result in univalent formation first in the small chromosomes. It is interesting to note that the three types of trisomy seen in humans all involve small chromosomes and that the incidence of trisomy 13, 18, and 21 varies inversely with the mean chiasma frequency. Trisomy 21, Down's syndrome, has the lowest chiasma frequency and is the most common trisomy (Lange et al., 1975).

Henderson and Edwards (1968) and Luthardt et al. (1973) proposed that the univalents seen at metaphase I in the old female mice led to non-disjunction and production

of aneuploid gametes. But the univalents could be subject to any one of several modes of behaviour during the ensuing anaphase: random segregation, failure to migrate and exclusion from the main nucleus by micronuclei formation, misdivision of the centromere resulting in isochromosome formation, or normal segregation (John and Lewis, 1965). Detailed chromosome counts of metaphase II oocytes would be required to determine the fate of the univalents.

Since this theory of maternal age related non-disjunction seemed the most plausible and the most amenable to experimentation, the present study was undertaken to determine if the univalents observed at metaphase I do result in non-disjunction in older female mice.

II. MATERIALS AND METHODS

Source and maintenance of mice

Virgin female CBA mice, 4 - 5 weeks old were obtained from the Jackson Laboratory (Bar Harbor, Maine). The animals were aged in the Zoology Vivarium at the University of British Columbia and were housed 4 mice per cage. The animal rooms were kept on a 12 hour light (8 a.m. to 8 p.m.) and dark cycle. The mice were allowed to eat Purina lab chow and drink water ad lib.

Procedure for obtaining oocytes

Oocytes were obtained without the use of gonadotropic hormones to induce superovulation since recent reports have indicated that these hormones can increase the frequency of chromosome abnormalities (Fujimoto, Pahlavan, and Dukelow, 1974; Boué and Boué, 1973). The mice were killed by cervical dislocation. The ovaries were dissected out and placed into an embryological watchglass containing 2 ml. of oocyte culture medium under 2 ml. of equilibrated paraffin oil. Subsequent manipulations were performed under a Zeiss stereodissecting microscope

at a magnification of 12 times for work with the ovaries and 30 times for work with the oocytes. The ovaries were cleaned of any adhering fallopian tubes or fat and transferred to new media. The oocytes were then liberated by puncturing the follicles with a 25-gauge sterile hypodermic needle. All liberated oocytes were washed twice by transferring them via a mouth-controlled Pasteur micropipette to two other embryological watch glasses each containing 2 ml. of medium under paraffin oil. Any adhering follicle cells were removed by sucking the oocyte in and out of the micropipette. Any undamaged oocytes possessing an intact germinal vesicle were selected for culturing. When the oocytes were not being directly manipulated, they were kept at 37°C in a 5% CO_2 atmosphere. The maximum time span from the death of the mouse to the placement of the oocytes in the final culture medium was one hour.

Procedure for culturing oocytes

The medium used to culture the oocytes was a chemically defined Krebs-Ringer salt solution containing sodium pyruvate, albumin and antibiotics as described by Donahue (1968) (cf. Appendix I). The medium was sterilized by positive pressure millipore filtration. The

culture system consisted of microdroplets of medium under sterile, equilibrated paraffin oil in a 35 x 10 mm. Falcon plastic tissue culture dish (Brinster, 1963). The paraffin oil was equilibrated with the culture medium. Between 5 and 10 oocytes were injected into each microdroplet. The oocytes were cultured in a humidified 5% CO_2 incubator at 37°C .

Procedure for oocyte fixation and chromosome preparation

After 17 - 18 hours in culture, the oocytes were transferred to an embryological watch glass containing hypotonic solution (1% sodium citrate) for 10 minutes. The number of oocytes with a polar body was recorded. The oocytes were placed in the centre of a clean slide and the excess hypotonic solution was drawn off. Four drops (each 20λ) of a 3:1 mixture of ethanol-glacial acetic acid were applied to the slide. The slides were then gently blown dry (Tarkowski, 1966). A maximum of 5 oocytes were placed on each slide. The preparations were stained for centromeric bands using the basic method of Arrighi and Hsu (1971) with a few modifications. The procedure was as follows:

1. .2 N HCl at room temperature for 30 minutes.
2. Three rinses in distilled water. Air dry.
3. Pancreatic RNase (100 ug ml in 2 x SSC, pH 7.25) at 37°C in a moist chamber for 45 minutes.
4. Two rinses in each of 2 x SSC (pH 7) 70% ethanol, 95% ethanol. Air dry.
5. .07 N NaOH at room temperature for 50 seconds.
6. Two rinses each in 70% ethanol and 95% ethanol. Air dry. The NaOH must be removed as rapidly as possible to prevent further denaturation. The alcohol wash must be kept separate and not be reused in other parts of the staining procedure.
7. 2 x SSC (pH 7) at 65°C for 16 hours.
8. Two rinses each in 70% and 95% ethanol. Air dry.
9. Stain for 15 - 30 minutes in a Giemsa solution prepared by the following formula: 50 ml. distilled water, 1.5 ml. .1M citric acid, adjust pH with .2M Na_2HPO_4 to pH 7, 1.5 ml. pure methanol, 5 ml. Giemsa stock solution (Gurr). This solution must be prepared immediately before use as it forms a precipitate after a few hours.
10. Two rinses in distilled water. Air dry. Mount in permount.

The chromosomes of some oocytes (about 10%) required restaining for 3 hours to achieve the desired intensity.

Several other simpler and faster staining procedures were attempted but no other technique produced good centromeric bands consistently.

Procedure for analysing oocytes

The slides were coded so that maternal age was not known at the time of analysis. The slides were analysed in groups of approximately 20, containing oocytes from mice of different ages. The number of oocytes on each slide was recorded so that all oocytes or remnants of oocytes were accounted for during the analysis. The chromosome number was determined using the oil immersion lens of a Zeiss photomicroscope. Oocytes were not included in the analysis if the chromosomes were spread more than one microscopic field. All oocytes with an abnormal number of chromosomes were photographed. Only oocytes with an unambiguous number of chromosomes were used for calculations of non-disjunction frequencies; therefore, many oocytes were discarded because the chromosomes were overlapped, too contracted, too spread apart, or the C bands were not clear enough to distinguish between chromatids and chromosomes. However,

all oocytes in which a diploid or haploid number (± 2 chromosomes) could be discerned were used to calculate the incidence of oocytes with 40 chromosomes. Fisher's exact test was used for the statistical analysis.

III. RESULTS

Collection and in vitro maturation of oocytes

A total of 1402 oocytes obtained from 174 CBA females were set up in culture. After 17 - 18 hours, an average of 80.5% of the oocytes had progressed to metaphase II.

The mice were grouped into three different maternal age classes based on litter size data in CBA females. The litter size of CBA mice increases and reaches a maximum at 150 days, then decreases until 240 days, and after this falls to a very low level until all oocytes are depleted at approximately 330 days. (Jones and Krohn, 1961). As shown in Table I (p. 36), the maternal age had a striking effect on the mean number of oocytes obtained per mouse and on the percentage of oocytes maturing in vitro.

It was very difficult to obtain oocytes from mice in the oldest age group. The ovaries contained very few oocytes and many were atretic. As shown in Table I, the oldest maternal age group required almost three times the number of mice used in the youngest age group since the mean number of oocytes obtained per mouse decreased from

14.7 in young females (60-150 days) to 9.6 in middle-aged females (151-240 days) to 4.4 in old females (241-330 days).

The percentage of oocytes maturing in vitro also decreased with maternal age. Eighty-seven per cent of oocytes from young mice progressed to metaphase II. However, only 80.7% of the oocytes from the middle aged group matured in vitro. This was significantly different from the young mice ($p = .01$, Fisher's exact test). The frequency of maturation decreased further to 71.9% in the old maternal age group. This was significantly different from the middle aged mice ($p = .004$) and the drop from 87% in the young mice to 71.9% in the old was highly significant ($p < .000001$).

Therefore, even at this early stage of development, oocytes from older females are less viable than those from younger females. This must occur because of a defect in the oocyte itself since all oocytes were handled in the same manner and cultured in the same medium.

Chromosomal analysis of oocytes

Chromosome preparations which yielded exact chromosome counts were obtained in 473 oocytes (33.7% of the total 1402). These oocytes were used in calculations of aneuploid

frequencies. Chromosome preparations which could be counted to the diploid or haploid number ± 2 chromosomes were used to calculate the frequency of diploid oocytes. This was possible in 877 oocytes (62.6% of the total). Failure of oocyte maturation, loss of oocytes during fixation and poor chromosome quality accounted for the remaining oocytes. Examples of analysed oocytes as well as discarded oocytes are shown in Figures 1 - 9 (p. 38 - 46).

The centromeric banding was very useful in determining the number of chromosomes, particularly when the chromosomes were crowded as in Figure 4. However, it was not possible to karyotype the metaphase II chromosomes and thereby identify a missing or extra chromosome. Mouse mitotic chromosomes are very difficult to karyotype because they are all telomeric and there is very little difference in size. Meiotic chromosomes pose added problems because they vary greatly in morphology and size and the chromatids are convoluted and spread far apart.

The chromosomal analysis of the oocytes is shown in Table II (p. 37). A very small number of oocytes had less than 18 chromosomes. These were assumed to be due to artificial loss and were discarded from the analysis. No

oocytes were found with more than 21 chromosomes (other than the diploid oocytes).

Eleven oocytes (2.3%) lost two chromosomes. There was no significant difference among the maternal age groups and these oocytes probably resulted from artificial chromosome loss during oocyte fixation.

Thirty-four oocytes (7.2%) lost one chromosome. An example of a hypoploid oocyte is shown in Figure 2. The middle maternal age group had 10.7% oocytes with 19 chromosomes and this was significantly different ($p = .04$) when compared to the old maternal age group which had only 3.8% oocytes with 19 chromosomes.

Neither the youngest nor the oldest mice had any oocytes with 21 chromosomes. But the middle maternal age group had six (4.9%) hyperploid oocytes; this was significantly different when compared to the young ($p = .006$) and the old ($p = .01$) maternal age group. These six hyperploid oocytes were obtained from six different mice and they were not confined to one certain time or season or group of mice. (The mice were ordered from the Jackson laboratory in three groups). It is highly unlikely that oocytes with 21 chromosomes resulted from the addition of

one "wandering" chromosome from another oocyte. Chromosomes from different oocytes varied greatly in size, shape, and centromeric banding, and since only five oocytes were placed on each slide, they were generally well separated from each other. Therefore, the six hyperploid oocytes probably resulted from non-disjunction during the first meiotic division. Examples of hyperploid oocytes are shown in Figures 3 and 4.

Theoretically, non-disjunction should produce an equal number of oocytes with 19 and 21 chromosomes. From Table II, it is obvious that the frequency of oocytes with 19 chromosomes exceeds the frequency of oocytes with 21 chromosomes. The preponderance of hypoploid oocytes may be due to different causes:

1. Hyperploid nuclei may be preferentially emitted into the first polar body (and therefore not be detected at metaphase II).
2. One chromosome may be lost due to blowing during oocyte fixation.

This last reason seems the most plausible since oocytes with less than 19 chromosomes were found but none

were found with more than 21. Other investigators have also observed an excess of hypoploid oocytes (Rohrborn, 1972; Hansmann, 1974; Uchida and Lee, 1974). The frequency of oocytes with 19 chromosomes is significantly higher in the middle maternal age group. Presumably this increase occurs because this class is composed of hypoploid oocytes resulting from non-disjunction (the reciprocal event of the hyperploid oocytes in the same age group) as well as chromosome loss.

Since it is not possible to get an accurate estimate of the frequency of hypoploid oocytes due to non-disjunction, a conservative estimate of the non-disjunction rate can be obtained by doubling the hyperploid rate. Thus the overall rate of non-disjunction was 2.6% but it was restricted to the middle maternal age group with a non-disjunction rate of 9.8%.

The overall frequency of oocytes with 40 chromosomes was 1.7%. Examples of diploid oocytes are shown in Figures 5 and 6. There was no significant difference in the frequency of diploid oocytes among the different maternal age groups. These diploid oocytes probably resulted from failure of first polar body formation. Other possible

interpretations include:

1) Two normal oocytes which are in close proximity during chromosome preparation. But the chromosomes of the two oocytes would need to have exactly the same size, shape, and C banding. Since chromosomes from different oocytes are so variable, this is unlikely. Also, if this occurred, two polar bodies would be expected in the same area and no polar bodies were ever found near the diploid oocytes.

2) Proximity of the chromosomes of the oocyte and the extruded first polar body. However, the chromosomes of the first polar body are generally not distinct; they are usually represented by condensed degenerate chromatin. An example of an oocyte with a typical polar body is shown in Figure 7.

The fifth column of Table II shows the frequency of normal oocytes in the different maternal age groups. There is no significant difference between the young (86.9%) and the old maternal age group (91.3%). However, the middle age group has a significantly lower frequency of normal oocytes (77.9%) than either the young group ($p = .05$) or the old group

($p = .003$).

Thus, although the oldest age group has the least number of oocytes per mouse and the poorest rate of *in vitro* maturation (Table I), the oocytes that do reach metaphase II have the highest frequency of a normal chromosome complement.

TABLE I

Effect of maternal age on the mean number of oocytes obtained per mouse and on *in vitro* oocyte maturation.

MATERNAL AGE IN DAYS	NUMBER OF MICE	MEAN NO. OOCTYES‡ OBTAINED/MOUSE	% OOCYTES PROGRESSING TO MET II
Young 60-150	32	14.7	*87.0
Middle 151-240	59	9.6	+80.7
Old 241-330	83	4.4	71.9
TOTAL	174	8.1	80.5

* Significantly different when compared to middle maternal age group ($p = .01$) and to old maternal age group ($p < .000001$).

\+ Significantly different when compared to old maternal age group ($p = .004$).

‡ Undamaged oocytes possessing a germinal vesicle.

TABLE II

Chromosomal analysis of 473 metaphase II oocytes from mice of different ages.

MATERNAL AGE IN DAYS	TOTAL NO. OOCYTES ANALYSED	18 (n-2)	19 (n-1)	20 (n)	21 (n+1)	40 (2n)	⊛TOTAL NO. DIPLOID & HAPLOID (± 2) OOCYTES
Young 60-150	191	3.1% (6)	7.9% (15)	86.9%	0	1.1% (4)	353
Middle 151-240	122	1.6% (2)	*10.7% (13)	+77.9%	‡4.9% (6)	2.1% (6)	280
Old 241-330	160	1.9% (3)	3.8% (6)	91.3%	0	2.0% (5)	244
TOTAL	473	2.3% (11)	7.3% (34)	86.0%	1.3% (6)	1.7% (15)	877

* Significantly different when compared to old maternal age group ($p = .04$), Fisher's exact test.

\+ Significantly different when compared to young maternal age group ($p = .05$) and to old maternal age group ($p = .003$).

‡ Significantly different when compared to young maternal age group ($p = .006$) and to old maternal age group ($p = .01$).

⊛ Used for calculation of the frequency of 2n (40) oocytes.

(cf. text p. 30)

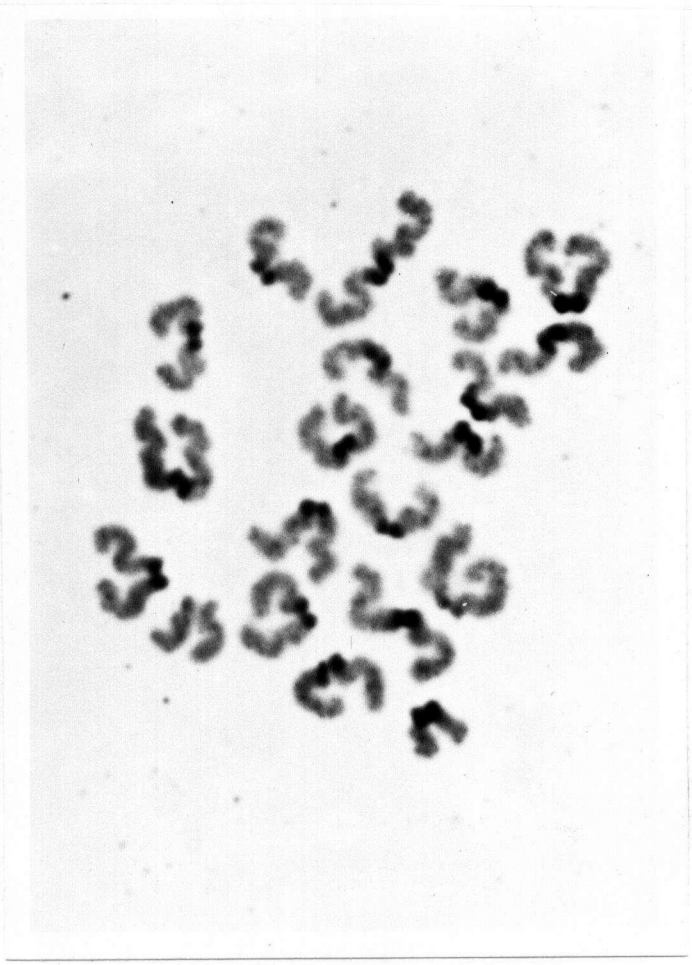

Figure 1. Second meiotic metaphase showing a normal complement of 20 chromosomes. Reproduced at 3100 X.

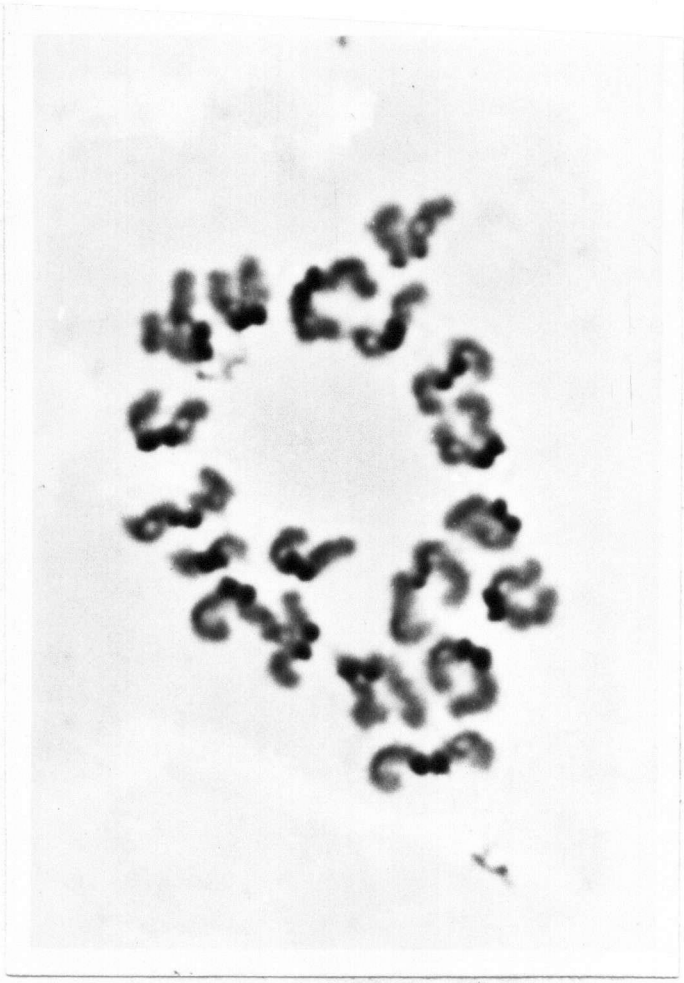

Figure 2. Second meiotic metaphase with 19 chromosomes. Reproduced at 3200 X.

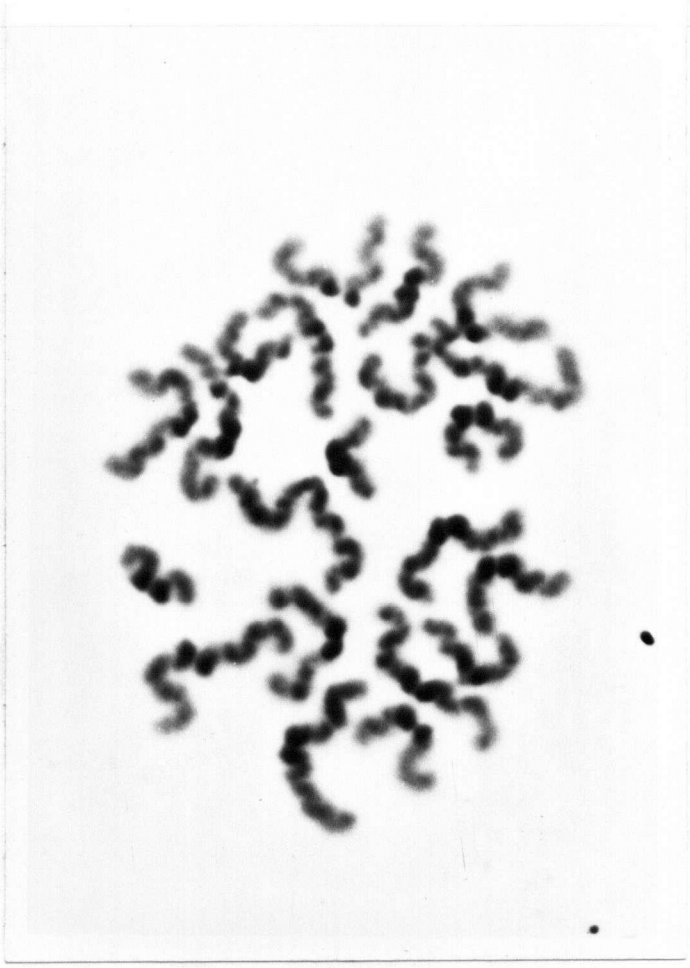

Figure 3. Second meiotic metaphase with 21 chromosomes. Reproduced at 2400 X.

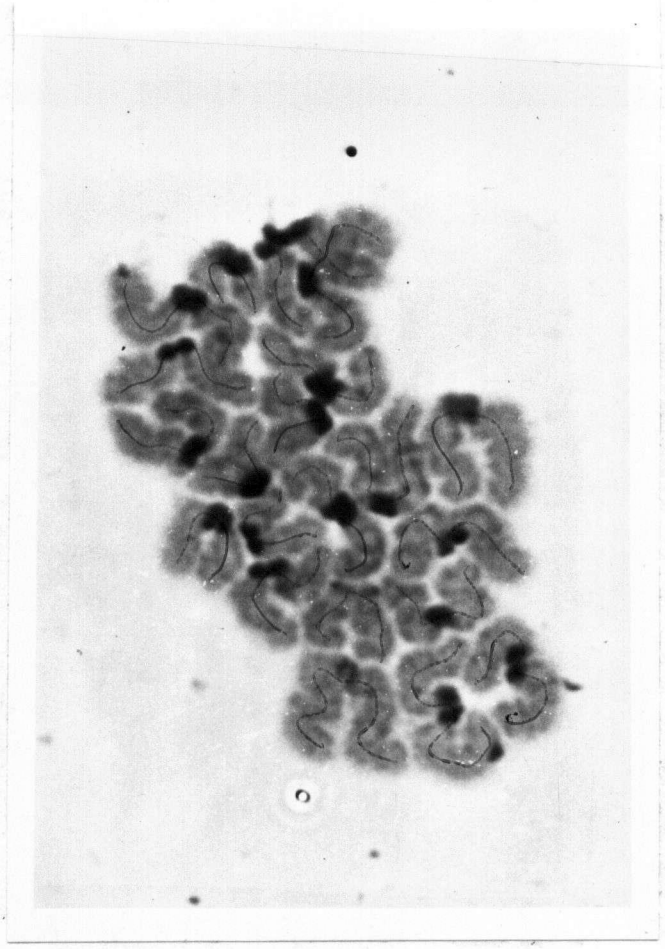

Figure 4. Second meiotic metaphase with 21 chromosomes. The axes of the chromatids are drawn for clarification. The chromosomes are crowded and fuzzy, but they can still be counted utilizing C bands. Reproduced at 3000 X.

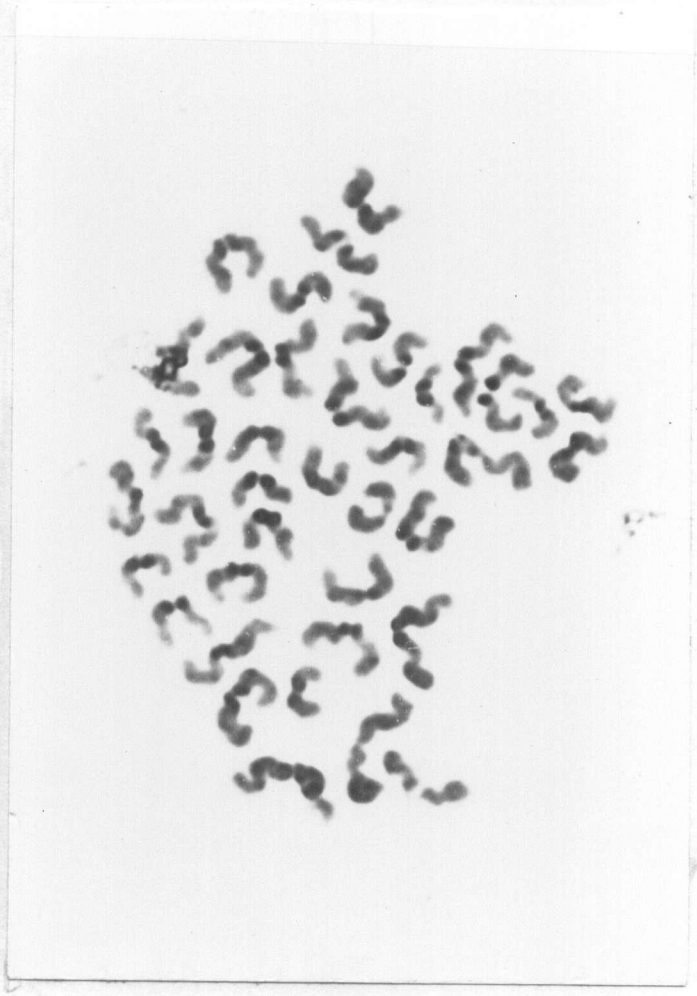

Figure 5. Second meiotic metaphase with 40 chromosomes. Reproduced at 2200 X.

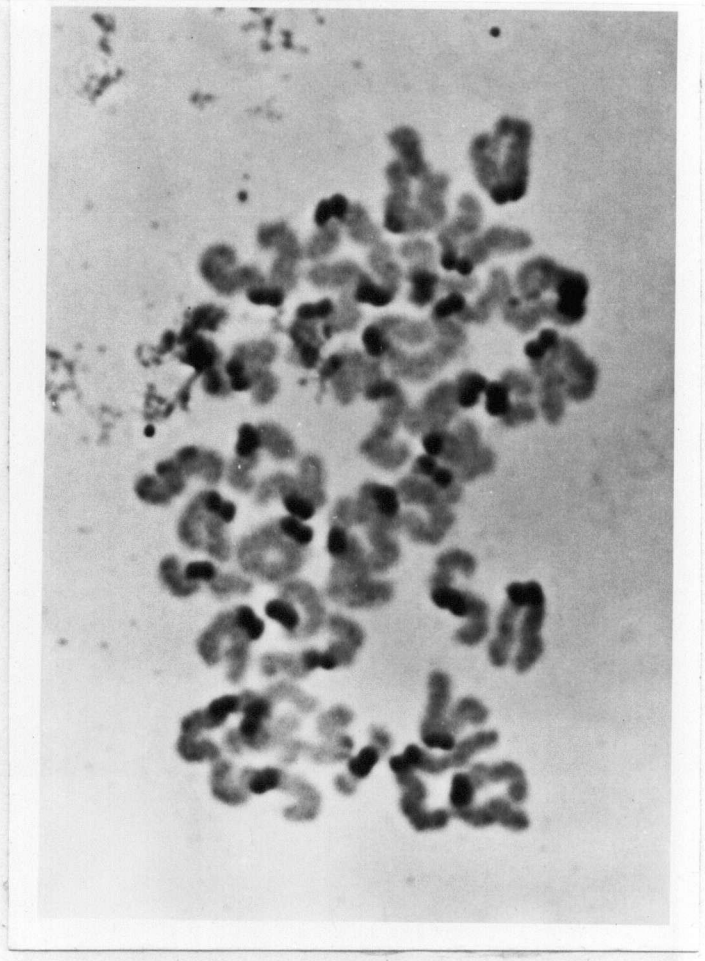

Figure 6. Second meiotic metaphase with 40 chromosomes. Reproduced at 2800 X.

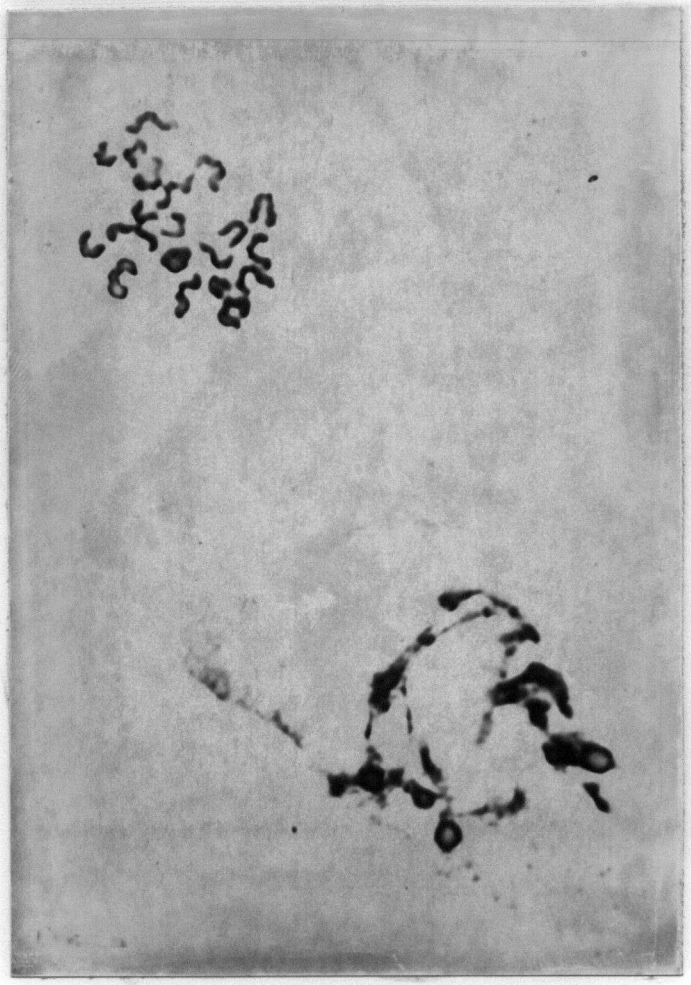

Figure 7. Second meiotic metaphase with its polar body. Reproduced at 780 X.

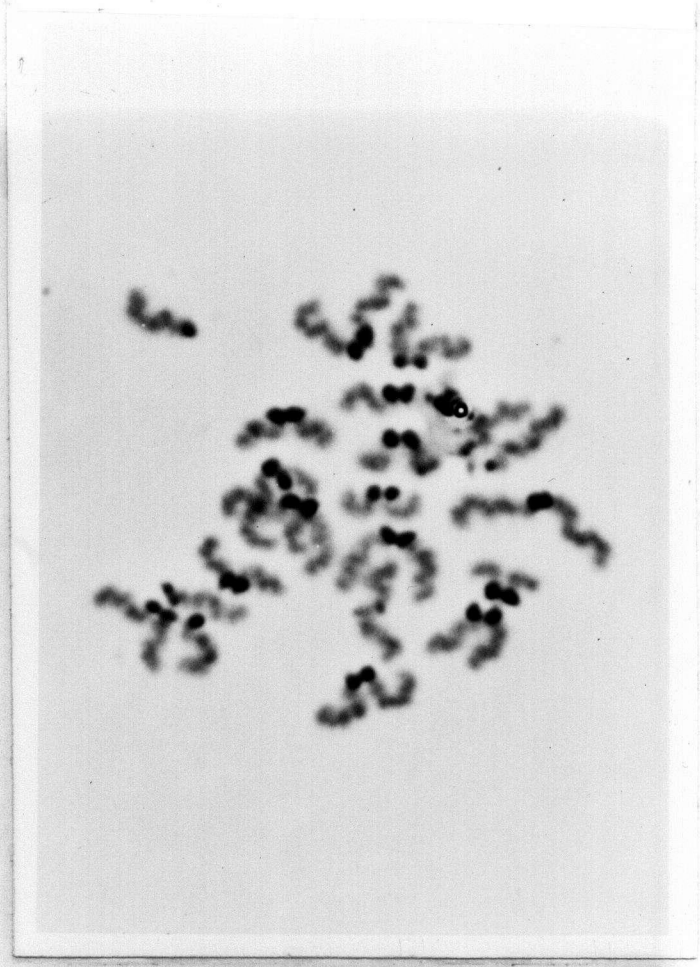

Figure 8. Second meiotic metaphase which could not be analysed because the chromosomes are too crowded together and the C bands are not clear enough. Included in calculations of diploid oocyte frequency. Reproduced at 2900 X.

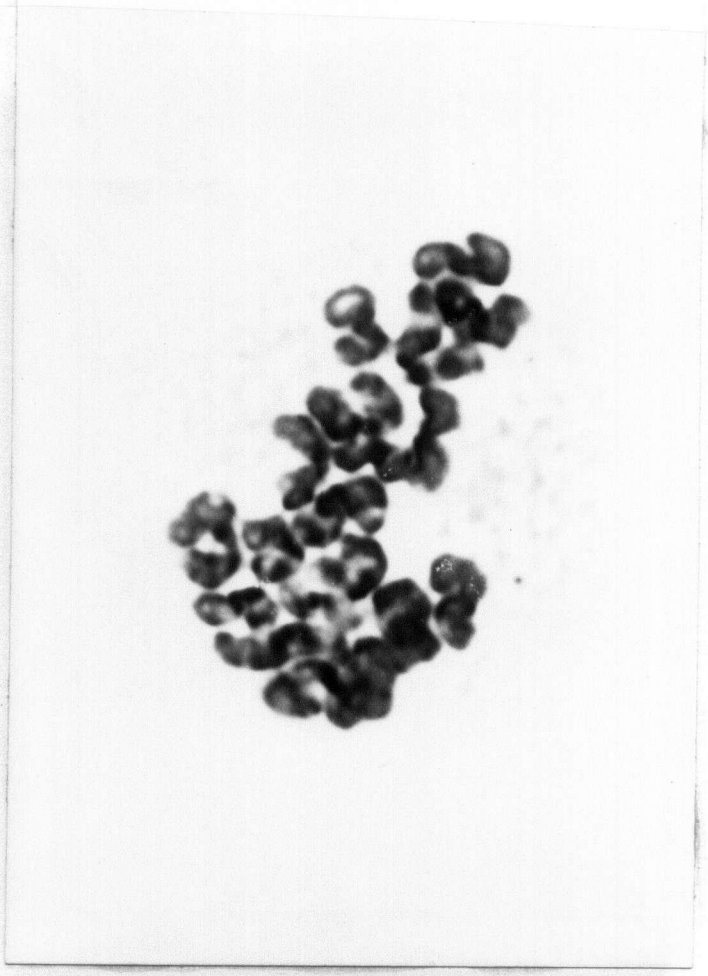

Figure 9. Second meiotic metaphase which could not be analysed because of the presence of some chromatids and debris. Included in calculations of diploid oocyte frequency. Reproduced at 3400 X.

IV. DISCUSSION

In vitro oocyte maturation

Pincus and Enzmann (1935) originally observed that the mechanical release of rabbit oocytes from the ovarian follicles into various culture media resulted in spontaneous breakdown of the germinal vesicle and the continuation of meiosis to the metaphase II stage. Spontaneous maturation of oocytes has subsequently been described in numerous mammalian species (Donahue, 1972a) and occurs in chemically defined as well as in biological media (Biggers, 1972). There have been several attempts to explain this phenomenon but very little is known about oocyte maturation *in vivo* or *in vitro*. Follicle cells have been implicated in the inhibition of oocyte maturation in rabbits (Foote and Thibault, 1969) and in pigs (Tsafriri and Channing, 1975). This hypothesis is attractive because it could explain the arrest of meiosis *in vivo* and the resumption of meiosis as soon as the oocyte is free of the follicle cells, either *in vivo* or *in vitro*. However, oocytes with investing follicle cells have matured successfully *in vitro* in several species

(Kennedy and Donahue, 1969; Robertson and Baker, 1969) and Cross and Brinster (1970) have shown that mouse oocytes cultured with follicle cells have an increased frequency of maturation. Follicle cells are capable of converting various substrates to pyruvate, which the oocyte requires as an energy source (Donahue and Stern, 1968). Since oocytes of several species have been cultured with follicle cells, they cannot be inhibiting maturation. Therefore the mechanism which causes oocytes to mature *in vitro* remains unknown.

Normality of oocyte development in vitro

Several studies have been done to determine if oocyte maturation *in vitro* is comparable to development *in vivo* and if normal embryonic development can proceed. Thibault (1973) demonstrated that rabbit oocytes matured *in vitro* progress to metaphase II in exactly the same length of time as control oocytes matured *in vivo* after coitus. Calarco *et al*. (1972) found no significant ultrastructural differences between mouse oocytes maturing *in vivo* and *in vitro*. Early embryonic development is also normal in rabbit and mouse embryos fertilized and grown *in vitro* (van Blerkom and Manes, 1974; Cross and Brinster, 1970); in addition

normal adult rabbits and 15 day-old mouse fetuses have been produced from oocytes matured and fertilized *in vitro* and transferred to pseudopregnant foster mothers (Thibault, 1973; Cross and Brinster, 1970). Thus, experiments to date show that *in vitro* oocyte maturation is similar to *in vivo* maturation and does not cause an increase in abnormal development.

Comparison of results to other studies

1. Diploid oocytes

The overall incidence of oocytes with 40 chromosomes in this study was 1.7% and this was not maternal age dependent. If a diploid oocyte were fertilized by a normal sperm, a triploid embryo would result. Other studies of mouse embryos have also shown that triploidy is not dependent on maternal age (Yamamoto *et al.*, 1973a; Gosden, 1973). Carr (1971b) has shown that triploidy does not increase with maternal age in humans. Donahue (1970) found anincidence of only .02% diploid oocytes in CF-1 mice. But he found that 1.2% of first cleavage division mouse embryos were triploid; these were caused equally by dispermy and digyny (Donahue, 1972b). Yamamoto *et al.* (1973a)

found an overall incidence of 1% triploidy in 10.5 day CF-1 mouse fetuses. Gosden (1973) found 4.2% triploidy in 3.5 day CBA/HT6 mouse embryos. Presumably, these differences reflect the different gestational stages examined and the different mouse strains used. It is difficult to estimate the frequency of triploid embryos in humans since they cannot be studied unless the embryos are aborted. Carr (1967b) has determined that triploidy occurs in 4% of spontaneous abortuses and in 19% of abortuses with chromosome abnormalities. However, the frequency of triploidy in humans is probably higher in earlier stages of gestation since the frequency of chromosome abberations decreases with gestational age (Boué, 1972). Triploid embryos are generally aborted and only a few human triploids have survived to term (Niebuhr, 1974). In the mouse, triploid embryos are eliminated during the first few days after implantation (Wroblewska, 1971).

2. Aneuploid oocytes

Several investigators have studied the frequency of aneuploidy during meiosis and early development in young mice. Using a hybrid strain, Uchida and Lee (1974) found

no spontaneous aneuploidy in metaphase II oocytes. Donahue (1972b) found no hyperploidy during the first cleavage division of CF-1 embryos. Similarly, Gosden (1973) and Yamamoto et al. (1973a) did not find any trisomy in CBA/HT6 embryos or CF-1 fetuses from young female mice. The present study agrees with these observations; no oocytes were found with 21 chromosomes in the young CBA mice. However, Hansmann (1974) has found that 2.4% of metaphase II oocytes from young C3H female mice have 21 chromosomes; and Phillips and Kaufman (1974) have observed 2.9% hyperdiploidy in oocytes from young (C3H x 101)F_1 mice. This higher frequency of hyperdiploidy may reflect a higher spontaneous rate of non-disjunction in C3H mice. Genetic differences can alter the frequency of aneuploidy. Fishberg and Beatty (1951) discovered that the mutant silver (si) in mice causes a high spontaneous frequency of heteroploid embryos. At 4½ days gestation 14.3% of embryos are heteroploid if the mother is homozygous for the silver mutation. If both parents are silver, the frequency of heteroploid embryos is higher. Phillips and Kaufman (1974) have found a sex-linked mutant (FxO) which causes non-disjunction of the X chromosome in approximately one-third of oocytes. Human

families with a high frequency of aneuploid offspring have also been recorded (Hecht et al., 1964). Since very few human oocytes or early embryos have been studied, it is difficult to estimate the frequency of aneuploidy at a comparable stage in humans. However the frequency of aneuploidy is definitely higher in humans than in mice since 35% of spontaneous abortions are heteroploid and 66% of the heteroploidy is due to aneuploidy (Carr, 1971a).

The effect of age on non-disjunction in mice has been analysed in only two studies. Gosden (1973) studied 3.5 day embryos from CBA/HT6 mice and found 5.2% trisomic embryos in the old females. Yamamoto et al. (1973a) determined that 1.9% of 10.5 day fetuses from old CF-1 females were trisomic. Both studies demonstrated a significant increase of non-disjunction with maternal age. In this study the frequency of oocytes with 21 chromosomes (which would lead to trisomy if fertilized by a normal sperm) was 4.9% in the middle aged female mice. But there was a complete lack of hyperploid oocytes in old females. This may be explained by the peculiarities of the mouse strain used.

Characteristics of CBA mice

CBA mice were chosen for this study because of their short reproductive lifespan (10 - 11 months) (Jones and Krohn, 1961) and because of the high frequency of univalents in the oocytes from old females (Henderson and Edwards, 1968). Henderson and Edwards found that 75% of the oocytes from old CBA females had univalents. Therefore, it was felt that if non-disjunction resulted from the univalents, it would be most evident in oocytes from old CBA females. But the CBA strain is very peculiar. Jones and Krohn (1961) studied four different strains of mice and found that CBA females have the smallest endowment of oocytes at birth; the most rapid depletion of oocytes; and they are the only strain in which the ovary is completely depleted of oocytes long before death. The smaller endowment of oocytes makes it much more difficult to culture large numbers of oocytes; even in young females, a maximum of only 20 oocytes per mouse can be collected. The number of oocytes decreases with age and in old CBA females very few oocytes can be obtained (cf. Table I). The small number of oocytes is not because

virgin mice were used in this study since Jones and Krohn (1961) have demonstrated that there is no significant difference in the rate of depletion of oocytes in virgin or multiparous mice.

Possible explanations for the lack of hyperploidy in the oocytes from old CBA mice

It may be possible that only normal oocytes are being selected for culture from old CBA females. But if this is true, one would have to postulate that oocytes which have many univalents, or are damaged in some other way, preferentially become atretic. Since 87% of oocytes from young females mature in culture compared to only 71.9% in old females, a higher frequency of univalents may prevent defective eggs from reaching metaphase II. If this is the case, a threshold effect must be postulated ——i.e. oocytes from young females have no univalents and no aneuploidy; oocytes from middle aged females have some univalents and these result in non-disjunction leading to 4.9% hyperploidy; oocytes from old females may reach a threshold in which some oocytes have so many univalents that they either become atretic or fail to progress

to metaphase II.

Alternatively, the failure of maturation may not be due to the higher number of univalents but to the type of univalents seen in old CBA females. Henderson and Edwards (1968) classified univalents as loosely-associated, tightly-associated, and true univalents (no association). Although the frequency of loose and tight univalents increased with maternal age, true univalents were only seen in the oldest maternal age group. Therefore, loosely or tightly associated univalents may cause incorrect attachment of spindle fibres resulting in non-disjunction. Spindle fibres may not be able to attach to true univalents and meiosis might then be arrested in the oocytes from old CBA female mice.

Evidence that a reduced chiasma frequency and increased univalent frequency can lead to meiotic arrest has been presented in one male mouse (Purnell, 1973) and ten men (Hulten et al., 1974). In every case the males had a high frequency of univalents and were sterile because of a block in spermatogenesis. Hulten et al. (1974) investigated one patient in detail using electron microscopy and found that cells at diakinesis showed signs of arrest:

centrioles were located at the centre of the group of chromosomes and no microtubular bundles were found either around the centrioles or the centromeres; a large number of vacuoles and swelling mitochondria were also observed in these cells. Therefore, abnormal oocytes from older females may be eliminated before metaphase II, when they are normally ovulated, so that only normal oocytes are seen at this stage.

Thung (1961) has observed that in old female mice oocytes are often retained in the follicle and not ovulated so that corpora lutea containing oocytes are formed. Anovulatory cycles are also common in women approaching the menopause (Riley, 1964). Therefore, there may be some mechanism which detects abnormal oocytes, or oocytes which have been arrested at some stage before metaphase II, and prevents the ovulation of these oocytes.

Relation of chromosomal abnormalities to the decline in fertility in old female mammals

It is obvious that although there is an increase in the frequency of chromosome abnormalities with maternal age, this increase is not great enough to entirely account

for the large drop in litter size in older mammals. In this study, the age group with the highest frequency of chromosome abnormalities had an estimated frequency of 9.8% aneuploidy (2 x 4.9%) and 2.1% triploidy or approximately 12% of oocytes with numerical chromosome anomalies. Gosden (1973) found that 12.1% of 3.5 day embryos from old female mice had abnormal chromosome constitutions. Since litter size can drop by 50% in old mice (Jones and Krohn, 1961), there must be other factors responsible for the decrease. The retention of oocytes in their follicles in old mice may also contribute to the decline in litter size and hormonal function may be impaired in the old mice (Gosden, 1974b).

Discussion of results in relation to the theories of non-disjunction

The results presented in this paper do not directly confirm or discredit any of the theories of non-disjunction presented in the introduction. However, since the mice were not exposed to radiation and the oocytes were not aged by delayed ovulation or fertilization, it is not likely that these causes are responsible for the

non-disjunction observed in the middle age group of mice. The increase of meiosis I non-disjunction with maternal age observed in metaphase II oocytes in this study does not correspond directly to the increase of univalents with age seen in the studies of Luthardt et al. (1973) or Henderson and Edwards (1968). If an increase in univalents with maternal age is causing non-disjunction, then a threshold effect must be postulated to explain the lack of hyperploidy in metaphase II oocytes of old CBA female mice. The evidence that a threshold for the number or type of univalents may exist is presented above. Since CBA mice are peculiar both in their reproductive pattern and their high frequency of univalents in old females, it would be very interesting to determine the frequency of non-disjunction with maternal age in metaphase II oocytes in other strains of mice. To date, no one has published any data on the effect of maternal age on non-disjunction in oocytes.

A unified theory of maternal age related non-disjunction

The univalent theory of non-disjunction is attractive because it provides a mechanism by which

non-disjunction can occur; that is, if bivalents become unpaired or only loosely paired, spindle fibres may not be able to attach correctly and non-disjunction may result. Since univalents have been shown to result in aneuploid gametes in other organisms (Carpenter and Sandler, 1973; Sharma and Reinbergs, 1974) it seems likely that the univalents seen in older mice could explain the increase of aneuploidy. Other theories lack an actual mechanism which could cause non-disjunction. For example, theories of tubal or intrafollicular aging of the ova or radiation damage do not really explain how these factors could affect the chromosomes and thereby cause non-disjunction. However, there is some evidence that various factors such as temperature and radiation can cause an increase in the frequency of non-disjunction. These other factors may be affecting the non-disjunction rate by the mechanism of univalent production. Univalents are produced with advancing maternal age in mice as the frequency of chiasmata decreases. The spontaneous reduction of chiasmata and increase of univalents with maternal age could be further accelerated by environmental factors.

Radiation has been shown to cause a terminalization of chiasmata, a decrease in chiasma frequency, and an increase in univalents in plants (Ramulu, 1973). Since radiation has also been shown to increase the frequency of non-disjunction in mice (Yamamoto et al., 1973b; Uchida and Lee, 1974), the mechanism may be to further decrease the chiasma frequency occurring spontaneously and accelerate univalent production. It may be necessary for precocious terminalization of chiasmata to begin before radiation can further affect the process since Yamamoto et al. (1973b) found that radiation could only increase the rate of non-disjunction in old female mice not in young mice.

An increase in temperature in Schistocera gregaria also causes a terminalization of chiasmata, a decrease in chiasma frequency and an increase in univalents (Henderson, 1962). Grell (1971) has determined that elevated temperature causes an increase in non-disjunction of the sex chromosomes as well as an increase in noncrossover X tetrads in Drosophila melanogaster. Therefore, it again seems likely that any heat induced non-disjunction would occur by the mechanism of univalent production. No experiments have been performed on the effect of increased

temperature on non-disjunction in mammals since it would be difficult to increase the temperature of a warm-blooded animal. However, the finding that heat can induce non-disjunction in lower organisms raises the possibility that physiological changes in temperature, perhaps incident to disease, may contribute to various types of aneuploidy by increasing the number of univalent chromosomes. The seasonal clustering of mongolism and other types of trisomy reported in the literature (Stoller and Collman, 1965; Nielsen and Friedrich, 1969) may be due to infections causing an increase in body temperature.

The slight increase of non-disjunction reported with aging of ova (Butcher and Fugo, 1967; Vickers, 1969) may also be due to univalent formation. Rodman (1971) determined that in some aging mouse oocytes, sister chromatids had disjoined prematurely; and Yamamoto and Ingalls (1972) found chromatid separation in aged hamster oocytes. Therefore any aging of the oocyte may further increase the frequency of univalents leading to non-disjunction.

Thus environmental factors known to affect non-disjunction frequencies may all be operating by their effect on univalent production——increasing the number of univalents

formed due to advancing maternal age.

Variation in chiasma frequency and univalent production can be due to genetic as well as environmental factors. Selection for genetic strains with high or low mean chiasma frequencies has been accomplished in Drosophila melanogaster (Chinnici, 1971) and Schistocerca gregaris (Shaw, 1974). Shaw (1974) determined that 60% of the total variance in chiasma frequency in Schistocerca is due to genetic factors. Genetic mutants which fail to form chiasmata have also been isolated in plants (Sharma and Reinbergs, 1974), mice (Purnell, 1973) and humans (Pearson et al., 1970). A genetic variation in chiasma frequency could explain the clustering of aneuploidy in some families (Hecht et al., 1964). A genetically determined low frequency of chiasmata would cause a greater susceptibility to non-disjunction since fewer environmental factors and less aging would be required to reduce the number of chiasma to the extent that some chromosomes lost all chiasmata and became unpaired.

An inherent low frequency of chiasmata might be part of the cause of Down's syndrome babies born to young mothers. A large number of studies have shown that the

mean age of mothers of Down's syndrome children is 6 to 8 years higher than the population mean (Penrose and Smith, 1966). But each study shows two bumps in the curve ——— one close to the population mean and a larger one in the mid-thirties. These bumps are usually large enough to make the curve bimodal. Therefore Down's syndrome has been stated to be caused by A, a maternal age-independent class and by B, a maternal age-dependent class. Since class A has the same mean as the population, Penrose and Smith (1966) have suggested that Down's syndrome in class A is due to translocations, mosaicism, carrier parents, secondary non-disjunction, and specific genes inducing non-disjunction. The specific genes could be lowering the chiasma frequency, increasing the formation of univalents and thereby causing non-disjunction. An increase in maternal age would not be expected in any of these causes. Class B, the maternal age-dependent group, would then be caused by aging of the oocyte during the prolonged dictyotene stage.

Therefore although several environmental, genetic, and aging factors can affect the frequency of non-disjunction by varying degrees, the mechanism of action

may be due to a single cause, namely univalent production and subsequent unbalanced segregation of the chromosomes.

Models for the decrease in the frequency of chiasmata with maternal age

Henderson and Edwards (1968) suggested that decreasing chiasma frequency with age could be explained either by chiasma terminalization or by a "production line" hypothesis. Terminalization or movement of chiasmata to the distal ends of chromosomes has been observed in plants and insects (Mather, 1938). If terminalization occurred during dictyotene, chiasmata could be progressively lost leading to the formation of univalents, especially in the smaller chromosomes which have fewer chiasmata (Slizynski, 1960). The fact that chiasmata become more terminal in older female mice supports this view. Also, heat and radiation have been shown to cause non-disjunction in lower organisms and both also result in terminalization of chiasmata (Henderson, 1962; Yamamoto et al., 1973b). The main objection to the view that terminalization causes the observed decrease of chiasmata is that it is difficult to envisage a molecular mechanism accounting for movement

of chiasmata to the chromosome ends. So Henderson and Edwards (1968) proposed that the decrease in chiasma frequency in old females was due to differences in chiasma frequencies in the fetal ovary. To explain this a "production line" in the ovary would be necessary. That is, oocytes formed early during oogenesis would possess higher chiasma frequencies than those formed later. The oocytes would subsequently be ovulated in the same sequence accounting for the reduction in chiasma frequency in older mice. Oocytes are formed over a period of weeks in humans (Ohno et al., 1962) and 4 - 5 days in mice (Borum, 1961), allowing sufficient time for gradients in chiasma frequencies to develop. Gradients in nutritional or developmental factors are known to affect chiasma frequencies in plants (Couzin and Fox, 1974; Rees and Naylor, 1960). The decline in the recombination frequency between the genes pallid and fidget with maternal age in mice (Bodmer, 1961) was interpreted by Henderson and Edwards as being favourable to the "production line" hypothesis since recombination occurs during prophase in the fetal ovary, i.e. before dictyotene. Therefore, if gradients causing a decrease in chiasma frequency occurred in the fetal ovary,

a decrease in recombination would be expected in old female mice. However this decrease in recombination frequency with maternal age in mice has only been shown for these two genes. Reid and Parsons (1963) did not find a statistically significant decrease in recombination frequency between the genes leaden and fuzzy with maternal age. This hypothesis of sequential oocyte development in mice could be tested by labelling the DNA while oocytes are being formed in the fetal ovary and determining if those oocytes formed last are conserved into old age.

No formal proof exists for the "production line" model but neither is there any direct evidence for the loss of chiasmata by terminalization during the porlonged dictyotene stage. Therefore, the cause for the reduction in chiasma frequency and increase in univalents with maternal age in mice is not known.

V. CONCLUSIONS

The frequency of numerical chromosome abnormalities in metaphase II oocytes from aging CBA mice was determined. The mice were divided into three different age groups, based on litter size data in CBA females. The frequency of diploid oocytes (which would result in triploid embryos) was 1.7% and did not vary with maternal age. Other studies have reported rates of triploidy varying from .02% to 4.2% depending on the mouse strain and stage of development. There has not been a reported increase of triploidy with maternal age in other studies. Oocytes with 21 chromosomes were used as an index of non-disjunction since chromosomes may be lost during slide preparation, leading to an elevated frequency of oocytes with 19 chromosomes. The overall frequency of oocytes with 21 chromosomes was 1.3% but these were all concentrated in the middle age group of mice with 4.9% hyperploidy. There have been no other published studies of meiosis I non-disjunction observed at metaphase II in aging female mice which could be compared to these results. Two studies of aneuploidy in mouse embryos and fetuses have shown an increase with maternal age, but no

comparable drop in the frequency of aneuploidy in the oldest age group. The lack of hyperploidy in the oocytes from old CBA females may be due to peculiarities of CBA mice. Therefore similar studies in other strains of mice are required to determine if this lack of hyperploidy in old females is strain specific. However, it would be necessary to study much older female mice than in this project since CBA mice have an extremely short reproductive lifespan.

Several theories of non-disjunction have been discussed, particularly Henderson and Edward's theory of univalent formation. The results of this study do not directly confirm any of the theories unless a threshold effect is proposed to explain the lack of hyperploidy in the oocytes of old female mice. Evidence from this study and also other studies is presented to show that a threshold for the type or number of univalents may exist. Once the threshold level has been surpassed, meiosis may break down so that oocytes do not reach metaphase II. This would lead to a lower maturation rate of oocytes, but a higher frequency of chromosomal normality in the old females.

A unified model of non-disjunction is presented in which the various environmental, genetic, and aging

factors known to affect the frequency of non-disjunction, act by an underlying mechanism of univalent formation. Evidence supporting this view is drawn from lower organisms as well as mammals.

The similar maternal aging effects upon the frequency of aneuploid embryos and offspring in mice and humans makes further research on mouse oocytes a necessity until the time when human oocytes are more readily available. Non-disjunction rates in oocytes should provide the best estimate of true rates since abnormal embryos are progressively lost during development. Although human oocytes are difficult to obtain in large numbers, a long-term study of chiasma and univalent frequencies at metaphase I and non-disjunction frequencies at metaphase II should aid in understanding the phenomenon of age-related non-disjunction.

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APPENDIX I

Krebs-Ringer Medium for Oocyte Culture

Constituents	Gram/Litre
NaCl	6.96
Na Pyruvate	.028
KCl	.356
$CaCl_2$	.189
KH_2PO_4	.162
$MgSO_4 \cdot 7H_2O$	.294
$NaHCO_3$	2.106
Pen/Strep	100U/ml/50ugm/ml
Bovine Serum Albumin	1.00
Phenol Red	.01

Medium for equilibrating paraffin oil is made as above but without pyruvate or albumin.