Cytogenetic analysis of ethanol-induced meiotic aneuploidy.

Gerard Thomas O'Neill

Doctor of Philosophy.

University of Edinburgh.

1989.

To Claire.

THE UNIVERSITY *of* EDINBURGH

CONTENTS

		Page
Contents		i
Preface		iii
Acknowledgements		iv
Abstract		v
Chapter 1	Introduction	1
Chapter 2	Materials and Methods	32
Chapter 3	Hyaluronidase-induced parthenogenetic activation of F1 hybrid oocytes: Influence of postovulatory ageing on the chromosome constitution of 1PN parthenogenones	48
Chapter 4	Ethanol-induced parthenogenetic activation of F1 hybrid oocytes <u>in vitro</u>	63
Chapter 5	Ethanol- and Avertin-induced parthenogenetic activation <u>in vivo</u>	86
Chapter 6	The ovulation and fertilisation of primary and secondary oocytes in LT/Sv mice	99

Chapter 7	Ethanol-induced parthenogenetic activation of LT/Sv oocytes *in vitro*	118
Chapter 8	Ultrastructural analysis of the second meiotic division in hyaluronidase and ethanol activated F1 hybrid oocytes	133
Chapter 9	The influence of an intragastric injection of a dilute ethanol solution on the segregation of meiotic chromosomes in F1 hybrid female mice	155
Chapter 10	Conclusions and future studies	186
	References	193
Appendix:	Publications	

Preface

The karyograms and diagrams in this study were prepared with the assistance of Mr. J. Cable and the staff of the Faculty of Medicine Medical Illustration Unit. The semi-thin and ultra-thin sections of mouse oocytes were cut, stained and photographed by Mr. R. D. McDougall. With these exceptions, the work undertaken and presented in this thesis was my own.

Gerard. T. O'Neill

April 1989.

Acknowledgements

This thesis is the result of work undertaken in the Department of Anatomy, University of Edinburgh between November 1985 and October 1988. The project was supported by the Faculty of Medicine Bonnar Scholarship Award.

I am indebted to my supervisor Professor M.H. Kaufman for his constructive advise and unlimited enthusiasm during this period of study.

I wish to express my gratitude to Dr. E.P. Evans for guidance with G-band analysis. I wish to thank the staff of both the Faculty of Medicine Animal Area and the Department of Bacteriology Animal House for their care and management of the animals used in this study. I also thank the staff of the Department of Anatomy for their interest and encouragement.

ABSTRACT

The aim of this investigation was to identify the nature and origin of ethanol-induced meiotic chromosome segregation errors in the oocytes of (C57BL x CBA)F1 hybrid and LT/Sv strain mice. Cytogenetic and ultrastructural analyses of one-cell parthenogenones were undertaken to examine the trisomogenic activity of this agent.

Cytogenetic analyses of *in vitro* hyaluronidase-induced parthenogenones and *in vivo* fertilised one-cell embryos demonstrated that both groups exhibited a low incidence of aneuploidy. However, analysis of the chromosome constitution of *in vitro* ethanol - induced single-pronuclear haploid parthenogenones (1PN) revealed that a significant proportion exhibited aneuploidy. The incidence of aneuploidy was directly related to the duration of ethanol exposure. Karyotypic studies of these parthenogenones found that the smaller chromosomes were more frequently predisposed to malsegregation than other members of the mouse genome. Ultrastructural analyses of ethanol activated oocytes found that this agent induced multipolar meiotic spindles and disrupted chromosome movement during anaphase of the second meiotic division.

Structural abnormalities of individual microtubular filaments were not observed. These combined cytogenetic and ultrastructural studies revealed that ethanol did not interact directly with the microtubular elements of the second meiotic spindle apparatus. These findings indicate that ethanol-induced disruption of the Ca^{2+}-dependent regulation of microtubule dynamics was probably responsible for chromosome malsegregation. The ovulated primary oocytes of LT/Sv strain mice also initiated parthenogenesis when exposed briefly to ethanol <u>in vitro</u>. However, cytogenetic analysis of these parthenogenones revealed that exposure of the first meiotic spindle apparatus to ethanol did not induce chromosome malsegregation.

The intragastric injection of a dilute ethanol solution to (C57BL x CBA)F1 hybrid female mice during the first meiotic division was found to increase the proportion of hyaluronidase-induced 1PN parthenogenones that exhibited aneuploidy. The incidence of ethanol-induced aneuploidy was not influenced by the postovulatory age of the activated oocytes. However, cytogenetic analyses of hyaluronidase activated oocytes that retained both products of the second meiotic division and developed as two-pronuclear diploid parthenogenones (2PN) revealed that the proportion that exhibited aneuploidy did not differ from control values. These findings demonstrated that exposure of the first meiotic spindle apparatus to

ethanol did not induce chromosome malsegregation during the first meiotic division. The 1PN parthenogenones that exhibited aneuploidy following the *in vivo* exposure of primary oocytes to ethanol resulted from chromosome malsegregation that occurred during the completion of the second meiotic division. These results suggest that the development and subsequent normal functioning of the second meiotic spindle apparatus was disrupted by the products of ethanol metabolism as the female mice had metabolised the administered dose of ethanol several hours before parthenogenetic activation occurred

The results from these investigations strongly indicate that the exposure of human oocytes to ethanol, either prior to or at the time of conception, could increase the proportion of human conceptuses that possess an aneuploid chromosome constitution.

CHAPTER 1

INTRODUCTION

CONTENTS

1.1 The incidence of human meiotic aneuploidy

1.2 The origins of human meiotic aneuploidy

1.3 Ethanol-induced reproductive loss and genetic damage

1.4 Ethanol-induced aneuploidy

1.5 Purpose of investigation

1.1 Incidence of human aneuploidy

Epidemiological studies of human reproductive potential have found that 13 - 15% of clinically recognised pregnancies abort spontaneously during the first trimester (Warburton and Fraser, 1964; Freire-Maia, 1982; Czeizel, Bognār and Rochenbauer, 1984; Heidam, 1984). The biochemical recognition of conception, as determined by increased levels of bHCG during the first weeks of pregnancy, have also indicated that the human species is subject to a high incidence of reproductive loss. These studies, although unable to specify the cause of early conceptual loss, have found that 50 - 60% of "biochemically recognised pregnancies" failed to develop to term (reviewed, Grudzinskas and Nysenbaum, 1985).

Cytogenetic analyses of spontaneously aborted foetal and placental tissues have consistently found that 50% - 60% of those that exhibited morphological abnormalities also possessed an abnormal chromosome constitution (Boué, Boué, and Lazar, 1975; 1976; Hassold, et al., 1980a; Lin, De Braekeleer and Jamro, 1985). When the selection of spontaneous abortuses for cytogenetic analysis also included those that did not exhibit distinct morphological abnormalities it was found that a lower proportion exhibited cytogenetic anomalies (Andrews, Dunlop and Roberts, 1984; Procter, Watt and Gray, 1986).

However, the types of cytogenetic anomaly most frequently encountered in each of these studies have been autosomal trisomies (30% - 50%), sex chromosome aneuploidies (15% - 20%) and triploidy (20% - 30%). Spontaneous abortuses with autosomal monosomy have very rarely been encountered (Boué, et al., 1975).

The studies cited above have only been able to determine the incidence of cytogenetic anomalies that permitted development into the early stages of clinically recognised pregnancy. It is recognised that the actual incidence of human aneuploidy at conception is higher than the values indicated by the proportion of spontaneous abortuses and live-born offspring that exhibit this anomaly. Several studies of the developmental potential of a number of monosomic conditions in the mouse have found that these embryos survive only to the pre- or peri-implantation period of gestation (Dyban and Baranov, 1987). These findings strongly indicate that human conceptuses with autosomal monosomy are not recovered during the first trimester because they die during the pre- or very early post-implantation period. The developmental potential of all 19 mouse autosomal trisomies have also been analysed in some detail. All are associated with morphological abnormalities and each one has been found to be lethal. It is established that all develop into the midgestation period but that each condition permits only a specific

and limited degree of further development (Gropp, 1982; Magnuson, 1983; Epstein 1985; Dyban and Baranov, 1987).

In relation to both murine and human trisomic embryos, it has been found that the developmental potential of these conditions was not directly influenced by the size of the extra chromosome present but by its particular genetic constitution. The majority of aneuploid human abortuses exhibit either sex chromosome aneuploidies or trisomies of the autosomal groups D, E and G (Hassold, et al., 1980a, 1980b, Hassold and Jacobs, 1984; Lin, De Braekleer and Jamro, 1985). Furthermore, several human trisomic conditions, notably trisomies 21, 18 or 13, as well as the sex chromosome aneuploidies, are compatible with postnatal development, although only a small proportion of those conceived with these genotypes develop to term. These conditions are also associated with the development of physical and mental disabilities in the liveborn offspring. In contrast, several human autosomal trisomic conditions, notably trisomy 3, 5, 11 and 19 are rarely observed in spontaneous abortions. Trisomy 1 has only been recently observed in an eight-cell stage embryo (Watt, et al., 1987), but this observation has established that all human chromosomes have the potential to undergo malsegregation during meiosis. These studies not only illustrate that the human trisomic conditions express a greater range of developmental capacities than observed in appropriate murine conditions but also that

the actual incidence of these conditions at conception cannot be determined from the cytogenetic analysis of aborted foetal tissues. The incidence of these anomalies during the first trimester is probably more a reflection of their potential for postimplantation development than being a reliable indicator of the incidence at which specific chromosomes are involved in malsegregation.

It has been variously estimated from the results of these cytogenetic analyses of human spontaneous abortuses that the proportion of human conceptuses that exhibit aneuploidy is in the range of 10% (Chandley, 1982) to 50% (Ford, 1975). These estimates have assumed that all chromosomes are equally predisposed to undergo malsegregation, that monosomy and trisomy are complementary events that occur with equal frequency, and that the proportion of aneuploid conceptuses that develop into the postimplantation period are a representative fraction of the actual incidence of aneuploidy at conception. However, these widely differing estimates clearly demonstrate that the incidence of human aneuploidy at conception can only be established from analyses of the chromosome constitution of human oocytes and preimplantation embryos.

Recent cytogenetic studies of human oocytes and preimplantation embryos have strongly indicated that the incidence of human aneuploidy at conception may be as

high as 34% (Plachot, et al. , 1987; 1988). The analysis of the chromosome constitution of human sperm by the "humster technique" (Bandriff, et al., 1985; Jenderny and Röhrborn, 1987; Templado, et al., 1988) has also confirmed that chromosome segregation errors occur more frequently during oogenesis than during spermatogenesis, as only 3-5% of sperm genomes exhibited numerical chromosome anomalies. Cytogenetic analyses of both ovulated oocytes and pre-implantation embryos have also indicated that a higher proportion of malsegregation events involved the smaller members of the human genome. However, in the majority of these studies, karyograms were prepared from chromosome preparations that were "solid" stained with giemsa. Without the use of differential chromosome banding techniques, it was, more often, only possible to associate specific numerical chromosome anomalies with particular chromosomal groups. A more complete karyotypic analysis of early human embryonic material using chromosome banding techniques is consequently required to establish the rate at which the individual autosomes and sex chromosomes are involved in malsegregation. This would undoubtedly assist in the identification of factors that predispose human meiosis to a high incidence of aneuploidy. It would also confirm whether all meiotic chromosomes were randomly involved in malsegregation or if particular chromosomes were predisposed to these events. However, human oocytes, in contrast to those isolated from laboratory maintained

animals, are not freely available for experimental analysis.

Cytogenetic analyses of the ovulated oocytes and fertilised one-cell embryos of several rodent species have consistently found that both aneuploidy and polyploidy arise at the significantly lower frequency of only 1 or 2% (mouse: reviewed, Nihjoff and de Boer, 1981; rabbit: Asakawa, et al., 1988; hamster: Hansmann and Probeck, 1979; Martin, Balkan and Burns, 1983; Sengoku and Dukelow, 1988). There are few detailed cytogenetic analyses of the ovulated oocytes, sperm or preimplantation embryos of other mammalian species. Recent cytogenetic analyses of preimplantation sheep embryos (Long and Williams, 1980) have revealed, however, that the incidence of aneuploidy in this species is only in the order of 4 - 5%. It has been reported that meiosis in the marmoset may be predisposed to to a high incidence of aneuploidy (cited in Chandley, 1982). Preliminary analyses of the chromosome constitution of in vitro fertilised one-cell squirrel monkey embryos have found that 12% exhibited hyperploidy (Asakawa and Dukelow, 1982) However, in this study only a total of 25 embryos were available for analysis and it would, from these observations, be unwise to assume that the primates, as a whole, were subject to a high incidence of meiotic aneuploidy.

The cytogenetics of meiotic maturation and early development have been studied most extensively in the mouse. However, even in this species, the factors that induce meiotic chromosome segregation errors have yet to be identified. Furthermore, it is not known if spontaneous chromosome segregation errors involve the malsegregation of specific chromosomes or whether all chromosomes of the mouse genome are equally predisposed to these events. The only study that has attempted to analyse the specific chromosome constitution of euploid and aneuploid G-banded one-cell mouse embryos (Martin-De Leon and Boice, 1983) is now considered to be misleading as the authors failed to prepare accurate karyograms of the metaphase chromosome spreads (see, O'Neill and Kaufman, 1987a).

1.2 The origins of human aneuploidy

Meiotic chromosome segregation errors may occur during either the first or the second meiotic division. Chromosome polymorphism studies of trisomy 21 abortuses (Niikawa, Merotto and Kajii, 1977; Jacobs and Mayer, 1981) or live-born trisomy 21 offspring (Mikkelson, et al. 1980) and their parents, have found that 60 - 70% of the segregation errors involving this chromosome occurred during the first meiotic division of the oocyte. A further 15% - 20% of Trisomy 21 offspring were the result of maternaly-derived segregation errors that occurred

during the completion of the second meiotic division. Only 10%- 15% of trisomy 21 conditions were found to be of paternal origin and due to chromosome malsegregation that occurred during either the first or the second meiotic divisions of spermatogenesis (see also, Juberg and Mowry, 1983). These observations may, however, only be valid for this particular human chromosome. Subsequent analyses of a number of chromosome polymorphisms in spontaneous abortuses that exhibited trisomy for chromosomes 3, 4, 9, 13, 14, 15, 16, 21, and 22 have revealed that 92.4% of chromosome segregation errors were maternal in origin. The majority of these events occurred during the first meiotic division (Hassold, Chiu and Yamane, 1984). Furthermore, those abortuses that exhibited paternally derived aneuploidy were, most frequently, due to segregation errors that involved chromosomes 13 and 21. The recent use of restriction fragment length polymorphism (RFLP) analysis has also indicated that a significant proportion of monosomy X conditions were the result of chromosome loss from the paternally-derived genome (Hassold, et al., 1985). The results from these studies indicate that several mechanisms may give rise to aneuploid gametes and that several factors may influence the malsegregation of particular chromosomes.

The factors that are responsible for the high incidence of human meiotic aneuploidy have yet to be identified. In

fact, the conditions responsible for the induction of "spontaneous" aneuploidy in any mammalian species have still to be understood. The higher incidence of chromosome segregation errors that originate during the first, rather than the second, meiotic division of the human oocyte highlight that the first and second meiotic divisions are essentially different cytological processes. Furthermore, a proportion of the malsegregation events that arise during the first meiotic division may be induced by specific factors that interfere with cytological events unique to this stage of meiosis. Similarly, the possibility exists that factors which interfere with chromosome segregation at both meiotic divisions may interact with the processes of each division by differing mechanisms.

Maternal ageing is the only factor that is known to increase the incidence of spontaneous abortions that exhibit autosomal trisomy(Hassold, et al., 1980b, Hassold and Chiu, 1985). It is also proposed that this phenomenon, rather than being the sole product of an increased incidence of aneuploidy may be, in part, influenced by a "relaxed" selection against, and thus greater developmental potential in these women of several specific aneuploid conditions (Lippman and Aymé, 1984). However the cytogenetic analysis of human (Angell Templeton and Aitken, 1984), Djungarian hamster (Hummler, Theuring and Hansmann, 1987) and mouse oocytes and

pre-implantation embryos (Martin, Dill and Miller, 1976; Maudlin and Fraser, 1978) have found that advancing maternal age was associated more significantly with an increased incidence of aneuploidy. Furthermore, the incidence of all human trisomic abortuses are not equally influenced by this phenomenon. The smaller chromosomes of groups D - G, except chromosome 16, exhibit the strongest age-related increase in meiotic aneuploidy. The incidence of spontaneous abortuses that possessed trisomies of chromosome groups A, B and C were not significantly altered by increasing maternal age. Furthermore, both spontaneous abortuses and offspring that exhibited monosomy X were more frequently encountered during the earlier years of reproductive life and their incidence decreased with advancing maternal age (Chandley, 1982; Hassold and Jacobs, 1984). Recent studies have also found that a proportion of trisomy 16 anomalies are restricted to the extraembryonic tissues of a normal foetus (Gosden, 1986). This may account for the absence of maternal age-related alterations to the incidence of this commonly encountered trisomic condition. Overall, these studies indicate that a combination of several specific factors, only some of which are influenced by maternal age, disturb the normal cytogenetic events that occur during the first meiotic division.

The majority of clinical and animal studies that have sought to identify the aetiological factors that induce

human aneuploidy have largely attempted to identify the relationship between maternal ageing and human meiotic anomalies. This phenomenon is still the only condition that is known to increase the incidence of aneuploidy (Chandley, 1982; Hassold and Chiu, 1985). A large number of experimental animal studies have clearly indicated that those agents that induce mutagenic or clastogenic abnormalities, or those that interfere with the spindle apparatus of both male or female germ cells, have the potential to produce aneuploid gametes. However, no specific compound or agent has been clearly identified which is capable of increasing the incidence of human aneuploidy (Bond and Chandley, 1983). Epidemiological studies are clearly unable to determine directly the mechanisms and factors that predispose human oocytes to aneuploidy. This will only be achieved from the direct cytogenetic analysis of human meiosis. The majority of human oocytes and preimplantation embryos that have recently been analysed cytogenetically are frequently the "spare" oocytes and embryos from IVF programmes that have either failed to initiate development or those embryos that were not replaced in utero as they were considered to have a limited devlopmental capacity. Furthermore, some of the initial procedures that have been used to stimulate ovulation or assist with the isolation of oocytes and embryos have been found to induce the malsgregation of meiotic chromosomes (Edwards, 1983; Kaufman, 1983a; Jagiello, 1987).

It has been suggested that the induction of aneuploidy was predetermined by developmental anomalies that originated during oogenesis in the foetus (Henderson and Edwards, 1968). It was proposed that these anomalies subsequently induced the formation of univalent chromosomes and that their independent segregation during anaphase of the first meiotic division increased the incidence of gametes with numerical chromosomal anomalies. Univalent chromosomes have been observed in metaphase chromosome preparations of primary oocytes recovered from ageing mice (Luthardt, Palmer and Yu, 1973). However, their presence is now primarily believed to be due to the easier mechanical separation of the more contracted chromosomes of the oocyte in aged female mice. Univalents are usually associated with the smallest chromosomes of the mouse genome and are not usually associated with the induction of aneuploidy (Speed and Chandley, 1983; Sugawara and Mikamo, 1983a, 1986; Liang, et al., 1986).

Recent animal studies have indicated that maternal age-related meiotic chromosome segregation errors may result from anomalies in the hormonal regulation of meiotic resumption. A recent study has demonstrated that unilateral ovariectomy of CBA mice can induce premature "reproductive ageing" and subsequently increase the incidence of aneuploid fertilised one-cell embryos

(Brook, Gosden and Chandley, 1984). This finding has supported the hypotheses that hormonal irregularities due to "reproductive" or "biological" ageing can influence the incidence of aneuploidy (Crowley, et al., 1979; Hayden, Crowley and Wilson, 1980) It has been proposed that hormonal imbalance lengthens the duration of the transition from diakinesis to metaphase I. These authors have considered that this predisposes the meiotic chromosomes to premature chiasmata terminalisation and univalent formation before the chromosomes are aligned within the meiotic spindle apparatus. However, there is little direct evidence that univalents are selectively involved in aneuploidy (Speed and Chandley, 1983). A series of cytological studies of the resumption of meiosis in the oocytes of the Djungarian hamster has found that the bivalents undergo sequential separation during the first meiotic division and that the larger submetacentric chromosomes of this species were the last to separate at the first meiotic anaphase (Hummler and Hansmann, 1985). These chromosomes were also selectively involved in maternal age-related aneuploidy (Hummler, Theuring and Hansmann, 1987). These authors have consequently proposed a model by which aneuploidy is induced when the altered control of meiosis in ageing females interferes with the anaphase separation of the late segregating bivalents and increases the incidence of malsegregation events that involve these chromosomes.

However, the recent use of immunofluorescent localisation techniques to analyse the arrangement of spindle microtubules and chromosome localisation at metaphase and anaphase of the first meiotic division in the primary oocytes of young, ovariectomised and aged CBA mice have failed to identify any fundamental alterations in the structure of the meiotic spindle apparatus that could have the potential to induce aneuploidy (Eichenlaub-Ritter, Chandley and Gosden, 1988). Since the cytogenetic evidence indicates that the malsegregations of meiotic chromosomes are probably not predetermined events, it is possible that future endocrinological analyses may be able to identify individuals who might be particularily susceptible to meiotic anomalies. However, the precise nature of the hormonal anomalies that predispose human meiotic chromosomes to malsegregation have yet to be established.

Several studies have also examined the possibility that the chromosomal organisation of the human genome may be an intrinsic factor that predisposes specific chromosomes to malsegregation (Chandley, 1982; de Boer and Tates, 1983). Ultrastructural analyses of human foetal oocytes have indicated that the nucleolar organising regions of the NOR-bearing prophase chromosomes are embedded in a single fibrillar protein matrix. However, this physical association of the NOR regions was not observed in murine oocytes at the same stage of meiosis (Mirre, Hartung and

Stahl, 1980). These authors have proposed that the persistance of this material could predetermine the onset of maternal age-related aneuploidy if it interfered with chromosome movement during the diakinesis-metaphase period. However, the evidence from the cytogenetic analyses of human spontaneous abortuses has found that the NOR-bearing chromosomes did not exhibit a significant age-related increase in aneuploidy (Hassold, et al., 1980b). The double NOR polymorphism (dNOR) of chromosome 21 is also considered as a another chromosomal factor that predisposes this particular chromosome to malsegregation (Jackson-Cook, et al., 1985). However the proportion of Trisomy 21 offspring that possess this polymorphism is not found to be significant (Jacobs and Mayer, 1981; Hassold, et al., 1987).

There is no epidemiological or cytogenetic evidence that directly associates maternal ageing with an increased incidence of chromosome malsegregation during the second meiotic division. The endogenous factors that induce chromosome segregation errors at fertilisation remain to be established. It has been proposed that malsegregation at this final stage of meiosis results primarily from the reduced ability of postovulatory aged oocytes to effect the balanced segregation of meiotic chromosomes (Juberg, 1983). A reduction in the expected number of offspring has been reported when the ovulated oocytes of several mammalian species were aged in vivo

prior to fertilisation (reviewed, Austin, 1970). The cytogenetic and cytological studies of the developmental potential of fertilised postovulatory aged oocytes have shown, however, that delayed fertilisation either increases the incidence of triploidy (Vickers, 1969) or predisposes the resultant embryos to developmental arrest prior to the first cleavage mitosis (Juetten and Bavister, 1983). Ultrastructural analyses of ovulated mouse oocytes have found that postovulatory ageing significantly increased the proportion that exhibited morphological anomalies in the structure of the meiotic spindle apparatus (Szöllösi, 1971, 1975; Eichenlaub-Ritter, Chandley and Gosden, 1986). However, O'Neill and Kaufman (1988) have found that postovulatory ageing did not significantly increase the proportion of hyaluronidase-induced mouse parthenogenones that exhibited an aneuploid chromosome constitution.

The segregation of chromosomes during mitotic and meiotic anaphase is preceded by the centromeric separation of sister chromatids. This is a sequential genetically regulated process in each species. It has been proposed that both the spontaneous or induced "out-of-phase" separation of chromosomes are factors that increase the risk of aneuploidy (Vig, 1983). Several cytogenetic studies that have examined the sequence of centromeric separation in the lymphocytes isolated from the parents of trisomy 18 and trisomy 21 offspring have reported that

a small proportion of these individuals exhibited "out-of-phase" chromosome separation for these particular chromosomes (Méhès, 1978; Fitzgerald, Archer and Morris, 1986; Gabarrón, Jimenez and Glover, 1986; Bajnóczky and Méhès, 1988). These authors failed to establish whether the trisomic offspring were the result of chromosome segregation errors at the first or the second meiotic division. However, these studies do acknowledge that both meiosis and mitosis are subject to genetic regulation, and that some individuals may be predisposed to aneuploidy as they carry mutations in those genes that regulate the specific events associated with chromosome segregation and separation (Petrinelli, et al., 1984).

The precise nature of the endogenous factors that induce aneuploidy during the second meiotic division of the human oocyte therefore remains to be established. Overall, the conditions that predispose this stage of meiosis, both in human and rodent oocytes, to chromosome malsegregation have largely been neglected. Most investigators have sought instead to identify the nature of those factors responsible for the higher incidence of aneuploidy that arises during the first meiotic division. The difference between the incidence of aneuploidy observed in the chromosome preparations of human ovulated oocytes and that of pre-implantation embryos has indicated that the completion of the second meiotic division in 3% - 5% of fertilised oocytes is

subject to chromosome segregation errors. This value is still several fold higher than the total incidence of aneuploidy observed in the chromosome preparations of oocytes and fertilised one-cell embryos isolated from experimental animal models (Plachot, et al., 1987, 1988). These cytogenetic studies of human material may indicate that, as noted by Ford (1975), a significant proportion of human aneuploidy may be another "disease of civilisation". Although this over-generalised conclusion indicated that the author believed that the high incidence of human aneuploidy was the product of both human reproductive physiology and sexual behaviour, it also acknowledges that the exposure of human gametes to a variety of harmful exogenous agents, not normally encountered by other animals, could be a significant factor in the development of aneuploid human conceptuses.

Numerous cytogenetic studies of both mammalian and non-mammalian meiosis have clearly shown that those environmental agents that interfere with either the genetic constitution or cytoskeletal apparatus of eukaryote cells have the potential to induce the development of aneuploid gametes (Bond and Chandley, 1983). The continued use of animal models to examine the trisomogenic potential of suspect environmental agents is justified as they can assist us in the identification of those agents that should be avoided to reduce the

incidence of human aneuploidy.

1.3 Ethanol-induced reproductive loss and genetic damage

Ethanol is a small lipophilic molecule that exhibits the properties of a weak general anaesthetic and in the form of beers, wines and spirits it is widely consumed in most human societies. Chronic ethanol consumption can damage the normal functioning of all body organs as well as disrupting the regulation of the haemopoietic; and endocrine systems. Persistent ethanol abuse can reduce the reproductive potential of both men and women by the ability of this agent to interfere with the endocrine control of gonadal function. This can induce sterility and testicular atrophy in males or the early onset of the menopause or infertility in females (Gavaler and Van Thiel, 1987). The early onset of the menopause has also been considered as a factor that significantly increases the risk of aneuploidy (Brook, et al., 1984). Furthermore, female alcohol consumption may also inhibit the initiation of embryonic development as ethanol can interfere with the metabolic changes necessary for sperm capacitation in utero prior to fertilisation (Anderson, et al., 1982; Rogers, Cash and Vaughn, 1987).

Ethanol is rapidly transported to both the foetus and amniotic fluid compartment when administered to pregnant female mice by intragastric injection (Kaufman and

Woollam, 1981). A series of clinical and animal studies have clearly established that ethanol and its primary metabolite, acetaldehyde, are potent teratogens. Several epidemiological studies of maternal alcohol consumption during pregnancy have found that even "moderate" alcohol consumption (2-5 ethanol units or drinks/week) can increase the risk of spontaneous abortion, still-births or the delivery of low birth-weight infants (Kline, et al., 1980; Harlap and Shiono, 1980; Wright, et al., 1983). Even infrequent ethanol consumption can be fetotoxic and it has been recommended that women should abstain from alcohol both before and during pregnancy (Hälmesmaki, Raivio and Ylikorkala, 1987). Furthermore, a significant proportion of the children born to alcoholic mothers, or to those who have consumed large quantities of alcohol consisitently during pregnancy, have been found to exhibit a range of specific craniofacial abnormalities and mental retardation. This has been termed the Fetal Alcohol Syndrome (FAS) (Jones and Smith, 1975; Streissguth, et al., 1980).

Several strains of the mouse will readily consume dilute (10%) ethanol solutions in preference to water, and when supplied ad libidum it constitutes 15% - 30% of the calorific content of the animals dietary intake. The voluntary consumption of ethanol by pregnant female mice, either throughout gestation (Chernoff, 1977), or more specifically, during the period of organogenesis (Randall

and Taylor, 1979) is found to induce a dose dependant range of systemic developmental anomalies in the exposed foetuses. Even the administration of a single "binge" dose during early organogenesis was sufficient to induce foetal abnormalities (Sulik, Johnston and Webb, 1981). The induction of similar anomalies following the in vivo exposure of mouse foetuses to acetaldehyde has found that the primary metabolite of ethanol was the more active teratogen (O'Shea and Kaufman 1979, 1981). Although there are distinct differences between human and murine embryogenesis, these studies have clearly demonstrated that the human FAS, and the equivalent anomalies observed in experimental animal studies, represent the teratogenic effects of maternal alcohol abuse on foetal development.

A high incidence of gross structural chromosome anomalies (Obe, et al., 1980; Butler and Sanger, 1981) and incomplete metaphase chromosome preparations (Mitelman and Wadstein, 1978) have been observed in the peripheral lymphocyte chromosome preparations of alcoholics. However, examination of the peripheral lymphocytes, bone marrow or hepatocytes of several rodent species maintained on ethanol supplemented diets have been unable to consistently detect ethanol-induced structural or numerical chromosome anomalies. Various strains of mice differ in their ability to metabolise ethanol to acetaldehyde (McClearn and Rodgers, 1972; Teichert-Kuliszewska, Israel and Cinader, 1988). The

latter may have limited the incidence and occurrence of structural chromosome damage as it is recognised that the clastogenic changes induced by chronic ethanol intake are believed to be largely effected through the activity of acetaldehyde (Obe and Anderson, 1987). The clastogenic properties of alcoholic beverages are also believed to be greater than those of ethanol solutions as they also contain a variety of higher alcohols, esters and alkaloids.

An increased frequency of sister chromatid exchanges (SCE) has also been observed in the peripheral lymphocytes of alcoholics (Butler and Sanger, 1981; Obe and Anderson, 1987) and rodents (mouse: Obe, et al., 1979; rat: Tates, Vogel and Neutebaum, 1980) exposed to this agent. The SCE assay represents the capacity of an agent to induce chromosomal mutations such as small deletions and inversions. It is not known whether these classes of chromosomal error are induced in human germ cells exposed to ethanol. However, a reduction in the number of offspring, an increase in the incidence of developmental anomalies and postnatal death has been consistently observed when male rodents, maintained for several weeks on an ethanol supplemented diet, were mated with unexposed females (mouse: Anderson, Beyler and Zaneveld, 1978; rat: Klassen and Persaud, 1976; Mankes, et al., 1982). These authors have concluded that the increased frequency of resorptions and abnormalities in

foetal morphology were due to the induction of dominant lethal mutations during one of the stages of spermatogenesis. The chromosome constitution of the male germ cells or the male pronuclear set of chromosomes in fertilised one-cell embryos was not analysed in these studies and it is unclear whether ethanol induced the development of aneuploid sperm under these conditions.

1.4 Ethanol-induced aneuploidy

The maturation of the human oocyte is arrested during the later stages of foetal development at the dictyate stage of meiosis. Oocytes usually remain at this stage of meiosis until the appropriate hormonal signals activate the resumption of oocyte maturation and subsequent ovulation. It is at this resting stage and also during the resumption of the first meiotic division or the completion of the second meiotic division that numerous exogenous factors may interfere, either once or cumulatively, with the balanced segregation of the meiotic chromosomes. The exposure of both male and female germ cells to a wide variety of environmental hazards and pollutants is considered to be the second major factor in the aetiology of human aneuploidy (Hoffman, Dellarco and Voytek, 1986). Many of the agents that possess either teratogenic, mutagenic or carcinogenic properties, eg. exposure to ionising radiation, heavy metals, antibiotics

and a broad spectrum of biohazardous compounds, have also the potential to induce aneuploidy in mammalian germ cells. The mechanism by which malsegregation occurs is specific to the biochemical interaction between the agent or compound with the chromosomes or other components of the meiotic spindle apparatus. Aneuploidy may be induced when trisomogens interfere with microtubule polymerisation or act directly on other components of the meiotic spindle apparatus such as the kinetochore proteins. Clastogenic changes may also lead to malsegregation during meiosis through the misalignment or mispairing of novel chromosome arrangements. All stages of oogenesis in the adult mammal have been found to be susceptible to the adverse influence of these agents (Bond and Chandley 1983; Bond, 1987).

It has been recognised, since the mid 1940s, that the compounds that exhibit anaesthetic properties interfere with the cell cycle of mammalian cells. This large diverse group of small lipophilic compounds, which includes ethanol, are recognised to interfere with both the organisation and morphology of cytoskeletal elements and to impair chromosome movement during mitosis (Jackson, 1975; Onfelt, 1986). Only a relatively small number of cytological and cytogenetic studies have investigated the potential of ethanol to interfere with the mechanics of the mitotic spindle apparatus. Ethanol-induced spindle anomalies were reported following

the exposure of the meristematic cells of germinating Allium cepa seeds to low doses of ethanol (0.12% -2%) for several hours (Sax and Sax, 1966). A high incidence of mitotic nondisjunction was achieved when the diploid and haploid conidia of Aspergillus nidulans were germinated in culture media supplemented with 3% - 6% ethanol (Harsanyi, Granek and Mackenzie, 1977; Käfer, 1984). Reliable techniques required to accurately analyse the chromosome constitution of male and female mammalian germ cells and preimplantation embryos have been available for at least two decades. Despite the ample evidence that a direct relationship existed between ethanol exposure and the induction of both structural and numerical chromosome anomalies, it is only relatively recently, that studies were carried out to investigate the capacity of ethanol to interfere with meiotic chromosome segregation.

Ethanol-induced meiotic chromosome segregation errors were first recognised to occur in mammalian gametes when the cytogenetic analysis of ethanol-induced parthenogenones revealed that a significant proportion exhibited aneuploidy. These findings strongly indicated that ethanol interfered with the microtubular elements of the second meiotic spindle apparatus (Kaufman, 1982). In subsequent studies, it was found that the intragastric administraion of a dilute ethanol solution to female mice during the first meiotic division or at the completion of the sperm-activated second meiotic division also

significantly increased the proportion of fertilised one-cell mouse embryos that exhibited aneuploidy (Kaufman, 1983b; Kaufman and Bain, 1984; O'Neill and Kaufman, 1987a). It has also been reported that the administration of a similar dose of ethanol to male mice can increase the incidence of aneuploid second meiotic metaphase chromosome preparations (Hunt, 1987). However, the oral administration of a dilute solution of ethanol to male Chinese hamsters was not reported to increase the incidence of numerical meiotic chromosome anomalies (Daniel and Roane, 1987).

These recent mouse studies have strongly indicated that ethanol induced aneuploidy by interfering with the functioning of the microtubular elements of the first and second meiotic spindles of mammalian gametes. It would also appear that the oocyte is particularly predisposed to the trisomogenic action of ethanol. It has consequently been proposed that the exposure of human oocytes, and possibly male germ cells, to ethanol in vivo during the same stages of meiosis may produce aneuploid gametes and subsequently be responsible for the development of a proportion of the aneuploid embryos that are known to be lost during the early weeks of human gestation (Kaufman. 1985, Kaufman and O'Neill, 1988). However the precise mechanisms by which ethanol induces chromosome malsegregation in male and female germ cells has still to be identified.

1.5 Purpose of investigation

A better understanding of the conditions that predispose the meiotic chromosomes of mammalian oocytes to undergo malsegregation following their exposure to ethanol, as well as the mechanisms by which these events arise, can indicate whether this agent has the potential to induce the development of human aneuploid conceptuses. The analysis of the chromosome constitution of fertilised one-cell embryos accurately detects the presence and incidence of numerical chromosome anomalies that have arisen during meiosis. This method, however, does not provide information on the mechanisms or origins of ethanol-induced meiotic chromosome segregation errors. In the following studies, these aspects of the interaction between ethanol and chromosome segregation in primary and secondary mouse oocytes is examined by the cytogenetic and ultrastructural analysis of several parthenogenetic models and test systems. The use of parthenogenetic models, the methodolgy of which are described by Kaufman (1983c), expands the number and type of experimental conditions under which the trisomogenic potential of ethanol can be investigated. The use of these models to determine the induction of aneuploidy in female germ cells is also in accordance with the criteria established by the Aneuploidy Data Review Committee (Mailhes, Preston

and Lavappa, 1986). In fact, the ability to analyse the mitotic chromosomes of one-cell parthenogenones is a more accurate and efficient means to assess the induction of aneuploidy than that proposed by Mailhes, et al. (1988). These authors recommended that the cytogenetic analysis of ovulated oocytes was amenable to objective investigation. However, this stage of meiosis is widely acknowledged to be technically difficult to analyse. The present methodology, as described below, can facilitate the cytogenetic analysis of chromosome segregation during both the first and second meiotic divisions.

The majority of parthenogenetically activated oocytes develop a single haploid pronucleus following the extrusion of the second polar body. This haploid genome is equivalent to the female-derived pronucleus that would normally develop if fertilisation had occurred. The analysis of the chromosome constitution of this parthenogenetic class can be used to identify the incidence of chromosome segregation errors that occur during both the first and second meiotic divisions. Parthenogenones that exhibit two haploid pronuclei and thus possess a diploid chromosome constitution may also develop if second polar body extrusion fails to occur. The cytogenetic analysis of these parthenogenones permits an examination of both products of the second meiotic division. The analysis of both the incidence and type of numerical chromosomal anomaly observed in the metaphase

preparations of this parthenogenetic class at the first cleavage mitosis can also determine the possible mechanisms by which chromosome segregation errors arise during the first meiotic division.

The specific chromosome constitution of euploid and aneuploid parthenogenones can be determined by G-band analysis of the chromosome spreads prepared at metaphase of the first cleavage mitosis. This form of analysis can determine if all chromosomes of the mouse are randomly involved in malsegregation. Furthermore, it can identify whether factors such as an enhanced degree of heterochromatin, the presence of NOR regions or chromosome size influence the malsegregation of meiotic chromosomes. Such studies can indicate if the genomic organisation of particular chromosomes predisposes them to ethanol-induced malsegregation events or whether ethanol interferes nonspecifically with the meiotic spindle apparatus to facilitate a more random pattern of chromosome malsegregation. The cytogenetic and karyotypic analysis of the oocyte-derived genomes of parthenogenetically activated oocytes also removes the possibility of recording sperm-derived numerical chromosome errors.

The completion of the second meiotic division proceeds more uniformly in a population of parthenogenetically activated oocytes than that achieved following

fertilisation *in vivo*. This aspect of parthenogenetic development facilitates an ultrastructural investigation of chromosomal movement and cytoskeletal reorganisation during the completion of the second meiotic division. In the following investigation, an ultrastructural analysis of the meiotic spindle apparatus of ethanol activated oocytes is undertaken. This can determine the events associated with the reorganisation of the second meiotic spindle during the earliest stages of ethanol-induced parthenogenetic development. It may also identify the nature of ethanol-induced spindle anomalies that increase the incidence of chromosome malsegregation.

The combination of cytogenetic and ultrastructural investigative techniques has the potential to identify the mechanism by which ethanol induces meiotic chromosome segregation errors in the primary and secondary oocytes of the mouse. The parthenogentic models and test systems that have been developed in this investigation can be applied in subsequent studies to the examination of the trisomogenic or clastogenic properties of other suspected agents. However, the primary aim of the following cytogenetic and ultrastructural analyses of ethanol-induced meiotic aneuploidy in the mouse is to assess whether the consumption of alcohol during specific stages of human meiosis presents a serious hazard to human reproductive potential.

CHAPTER 2

MATERIALS AND METHODS

CONTENTS

2.1 Mouse stocks

2.2 Superovulation

2.3. Preparation of embryo culture media

2.4 Parthenogenetic activation

2.4.1 Hyaluronidase-induced activation

2.4.2 Ethanol-induced activation *in vitro*

2.4.3 Ethanol-induced activation *in vivo*

2.4.4 Avertin-induced activation *in vivo*

2.4.5 Cytochalasin D-induced 2PN parthenogenones

2.4.6 Intragastric administration of ethanol

2.4.7 Parthenogenetic activation of LT/Sv oocytes

2.5 Isolation of fertilised one-cell embryos

2.6 Cytogenetic analysis of fertilised one-cell embryos and parthenogenones

2.6.1 Chromosome preparations in the absence of colcemid

2.6.2 Colcemid-arrested one-cell embryos and parthenogenones

2.6.3 Giemsa-banding of metaphase preparations from fertilised one-cell embryos and parthenogenones

2.7 Transmission electron microscopy

2.1 MOUSE STOCKS

(C57BL x CBA) F1 hybrid.

Six-week old female C57BL/6J and male CBA/Ca mice were purchased from Olac (Hull). These animals were caged as individual breeding pairs and the (C57BL x CBA) F1 hybrid offspring were used for these studies. The F1 hybrid offspring were weaned at 21 days post-partum and the female mice were allowed to develop to maturity in stock cages, each housing 15-20 animals. After a further 3 weeks they were transferred to stock cages housing a maximum of 8 females and were used for experimental studies when they were 10-12 weeks old. They had a mean weight of 21.5± 0.99g (n=23).

The majority of F1 male mice were killed at 3 weeks post-partum. A group of 30 12-week old proven fertile male mice were individually housed and maintained as a stud colony. This colony was replaced at 5-6 month intervals.

LT/Sv.

Six breeding pairs of the LT/Sv strain were obtained from MRC Carshalton. The breeding colony was expanded by intersib matings and future breeding pairs were chosen from litters of seven or less offspring. Male mice that were not selected as future breeding animals were killed at 27 days post-partum. The female mice were allowed to

develop to maturity in stock boxes that housed 8 animals. These animals were used for experimental studies when they were 8-12 weeks old.

All animals were given pelleted rodent food (Bantum & King) and tap water ad libidum and the animal house was on a 12h light/dark cycle with lights on at 08.00h.

2.2 SUPEROVULATION

Pregnant mares' serum gonadotrophin (PMSG) and human chorionic gonadotrophin (HCG) were supplied by Intervet, Cambridge. Mature female F1 and LT/Sv mice were administered 5i.u. of PMSG in 0.2ml of Dulbecco's phosphate buffered saline (PBS) by intraperitoneal injection (i.p) to stimulate follicular maturation. The resumption of the first meiotic division was induced 46-48h later by the i.p. injection of 5i.u. of HCG in 0.2ml Dulbecco's PBS. Ovulation occurred 11.5h - 13h after the HCG injection (Edwards and Gates, 1959). The mean number of oocytes ovulated by the F1 females was 27.3± 1.6 (n=47). The mean number of oocytes ovulated by the LT/Sv female mice was 9.7± 0.3 (n=10)

2.3 PREPARATION OF EMBRYO CULTURE MEDIA

Dulbecco's Phosphate Buffered Saline (PBS)

Dulbecco's phosphate buffered saline tablets (Oxoid) were

dissolved in 80-90ml sterile Analar grade water. This was supplemented by the addition of 0.5ml of mineral salt solution (Oxoid) and the final volume was made up to 100ml. At 25°C the PBS solution had a pH of 7.2.

M16 Embryo Culture Medium

The in vitro culture of fertilised one-cell embryos and parthenogenones was conducted in a modified form of the M16 embryo culture medium described by Whittingham (1971). The chemical constituents in 100ml of this medium are given below. The medium was prepared weekly and was stored in 10ml aliquots at 4°C. The bovine serum albumen (BSA) was not added to the M16 medium until shortly before use. The complete medium was then passed through a sterile 0.22um Millipore filter. Parthenogenones and fertilised one cell embryos were incubated in 0.05ml microdrops of M16 medium under filtered light paraffin oil (BDH) in an atmosphere of 5% CO_2 in air at 37°C. The embryo culture plates (60mm x10mm, Falcon) were prepared on the evening before use to equilibrate the M16 medium with the appropriate culture conditions.

Constituents of 100ml of M16 embryo culture medium

Sodium Chloride	0.413g
Potassium Chloride	0.036g
Potassium Dihydrogen Phosphate	0.016g

Magnesium Sulphate	0.029g
Calcium Chloride (dihydrate)	0.025g
Sodium Hydrogen Carbonate	0.211g
Sodium Lactate **	0.32ml
Sodium Pyruvate	0.006g
Glucose	0.100g
Phenol Red	pinch
Bovine Serum Albumen* (BSA)	0.400g

* BSA: Fraction V.(Sigma)

** Sodium Lactate was a 60% syrup (Sigma). The medium was prepared using sterilised Analar grade water and Analar grade salts (BDH).

2.4 PARTHENOGENETIC ACTIVATION

2.4.1 Hyaluronidase-induced parthenogenetic activation

Ten- to 12-week old F1 hybrid female mice were superovulated as described above. The female mice were killed by cervical dislocation at HCG+ 18h - 26h. The oviducts were dissected out and placed in an embryological watchglass containing 1ml of equilibriated M16 medium. In Chapter 4 the cumulus masses were isolated in PBS prior to their exposure to hyaluronidase supplemented M16. The cumulus masses, containing the ovulated oocytes, were released from the oviduct through an incision made by watchmakers forceps in the swollen ampullary region. They were transferred by pasteur

pipette to microdrops of M16 medium supplemented with 3mg/ml of hyaluronidase (Sigma: sheep testis, Type II) and incubated in this medium for 10 - 15 minutes to induce parthenogenetic activation. The cumulus cell-free oocytes were then washed three times in hyaluronidase-free M16 medium and then left to culture for 6h in microdrops of M16 medium in an atmosphere of 5% CO_2 in air at 37°C. This is a modification of the method described by Kaufman (1973a)

At the end of this period the majority of the activated oocytes had extruded a second polar body and developed a single haploid pronucleus (1PN). A proportion of activated oocytes fail to extrude the second polar body and develop as diploid parthenogenones. These parthenogenones exhibit either two haploid pronuclei (2PN) or a single diploid pronucleus (1PND). A fourth class of activated oocyte undergo immediate cleavage (IC) and develop two equal-sized blastomeres, each containing a haploid pronucleus. The four main pathways of parthenogenetic development are represented in Fig 2.1. The activated oocytes were transferred by hand drawn micropipettes (with a central bore of 70-80um) to fresh microdrops of M16 medium.

2.4.2 Ethanol-induced parthenogenetic activation in vitro

Ten- to 12-weeek old F1 hybrid female mice were

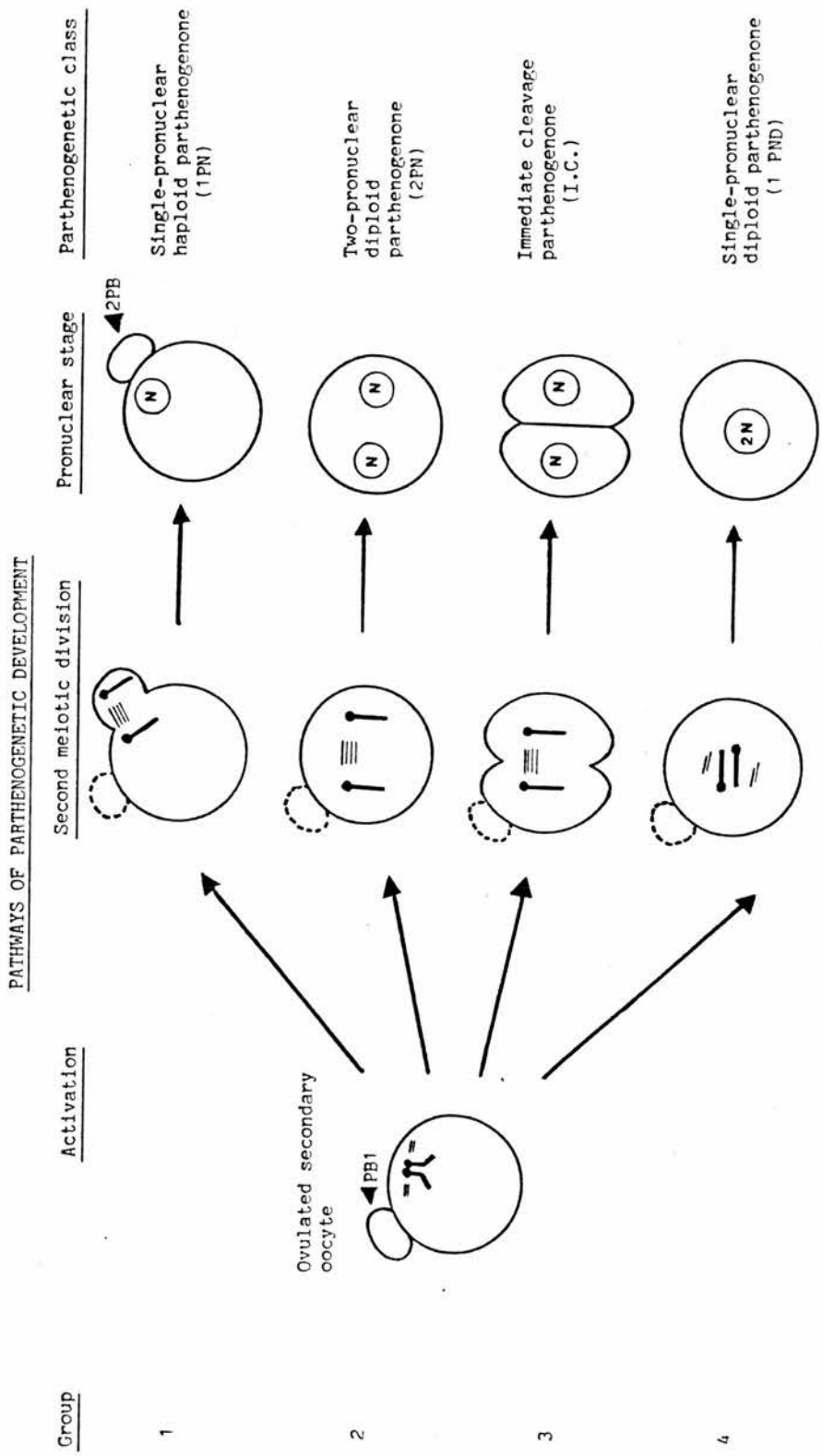


Fig. 2.1 The four pathways of parthenogenetic development observed following the activation of ovulated secondary oocytes isolated from (C57BL x CBA) F1 hybrid and LT/Sv strain mice.

superovulated as described above. The mice were killed at HCG+ 17h and their oviducts were dissected out and transferred to 1ml of Dulbecco's PBS in an embryological watch glass maintained at 37°C. The cumulus masses were released from the ampullary region of the oviduct, transferred by pasteur pipette to 1ml of 7% Analar ethanol in Dulbecco's PBS and incubated in this solution for either 1, 3 or 5 minutes to induce parthenogenetic activation. The cumulus masses were then washed in three changes of ethanol-free Dulbecco's PBS followed by two further washes in M16 medium. The cumulus masses were then transferred to microdrops of M16 medium and incubated for 5-6h at 37°C in 5% CO_2 in air. At the end of this period the cumulus cells were removed following a brief exposure to M16 medium supplemented with 0.5mg/ml of hyaluronidase. The majority of activated oocytes were observed to develop as 1PN parthenogenones. The other classes of parthenogenone were observed at a lower frequency. The activated oocytes were transferred to fresh microdrops of M16 medium. This method is a modification of that described by Kaufman (1982).

2.4.3 Ethanol-induced parthenogenetic activation in vivo.

Ten- to 12-week old female F1 hybrid mice were superovulated as described above. At HCG+ 18h the mice received an i.p. injection of either 0.35ml or 0.50ml of a 25% solution of Analar ethanol in Dulbecco's PBS

to induce parthenogenetic activation (Dyban and Khozhai, 1980). After a period of 6h the mice were killed by cervical dislocation and their oviducts were dissected out and placed in 1ml of equilibrated M16 medium in an embryological watch glass. The cumulus masses were released from the ampullary region and transferred by pasteur pipette to microdrops of M16 medium supplemented with 0.5mg/ml of hyaluronidase to remove the adhering cumulus cells. The majority of the activated oocytes developed as 1PN parthenogenones. The other classes of parthenogenone were observed at a lower frequency. The activated oocytes were transferred to fresh microdrops of M16 medium.

2.4.4 Avertin-induced parthenogenetic activation in vivo

Avertin (Winthrop) is a 1g/1ml solution of tribromoethanol in amylene hydrate. Avertin anaesthetic was prepared by the addition of 0.12ml of Avertin to 9.9ml of hot Dulbecco's PBS. This solution was shaken vigorously as Avertin is not readily water soluble. The standard dose of Avertin anaesthetic for the mouse is 0.02ml/g body weight of this solution.

F1 hybrid female mice were superovulated as described above. At HCG+ 18h the mice received an i.p. injection of a standard (x1.0 Std.), x0.5 Std. or x1.5 Std. dose of Avertin to induce parthenogenetic activation (see,

Kaufman, 1975a). After a period of 6h the mice were killed by cervical dislocation. Their oviducts were removed to 1ml of equilibrated M16 medium in an embryological watch glass. The cumulus masses were released from the ampullary region and transferred by pasteur pipette to microdrops of M16 medium supplemented with 0.5mg/ml of hyaluronidase to remove adhering cumulus cells. The majority of activated oocytes developed as 1PN parthenogenones and these were transferred to fresh microdrops of M16 medium.

2.4.5 Cytochalasin D-induced 2PN parthenogenones

A stock solution was prepared by dissolving 1mg of cytochalasin D (Sigma) in 1ml of dimethyl sulphoxide (DMSO). The incubation of hyaluronidase exposed oocytes for 4h in M16 medium supplemented with 1ug/ml of cytochalasin D inhibited the extrusion of the second polar body. The majority of activated oocytes exposed to cytochalasin D develop as 2PN parthenogenones.

2.4.6 Intragastric administration of ethanol

F1 hybrid female mice were lightly anaesthetised with ether and 1ml of a 12.5% (v/v) solution of ethyl ethanol (Analar grade, BDH) was injected into the stomach via a fine plastic tube. The ethanol solution was administered at either HCG+ 4h, 6h, 8h, 18h or 20h. Analysis of blood samples, using a Lion Alcolmeter (model AE-D1, Lion Laboratories), established that the 1ml dose of the

ethanol solution gave a blood alcohol level of 200-230mg/100ml after 30mins. This concentration of blood alcohol remained at this level for about 2h. After this time the blood levels dropped rapidly and at 6h after the time of administration the blood alcohol concentration did not differ from that observed in female mice that were not exposed to ethanol.

2.4.7. Parthenogenetic activation of LT/Sv oocytes

The ovulated oocytes of LT/Sv strain mice initiate spontaneous parthenogenetic development in vivo and in vitro at a low frequency (Stevens and Varnum, 1974). The exposure of ovulated LT/Sv oocytes to either hyaluronidase or ethanol in vitro as described in 2.4.1 and 2.4.2 induced a high incidence of parthenogenetic activation.

The activated secondary oocytes of the LT/Sv strain developed as 1PN, 2PN, IC, and 1PND parthenogenones when exposed to a parthenogenetic stimulus in vitro. A significant proportion of ovulated oocytes of the LT/Sv strain exhibit the chromosome constitution of primary oocytes. The majority of the activated ovulated primary oocytes of this strain developed a single diploid pronucleus following the extrusion of a polar body (1DPN). A small proportion of ethanol-induced parthenogenones failed to extrude a polar body and developed two diploid pronuclei(2DPN). The special

Fig 2.2 The two pathways of parthenogenetic development observed following the activation of ovulated primary oocytes isolated from LT/Sv strain mice. Parthenogenones of the 2DPN class, illustrated in Group 2, are encountered at a very low frequency.

pathways of parthenogenetic development initiated by the oocytes of LT/Sv strain mice are represented in Fig 2.2.

2.5 ISOLATION OF FERTILISED ONE-CELL EMBRYOS

Female F1 hybrid or LT/Sv strain mice were superovulated as described in 2.2. Two- to 6-hours after the HCG injection the female mice were indivdually caged with F1 male mice of proven fertility. Early the next morning the female mice were examined for the presence of a vaginal plug and this was taken as evidence that mating had taken place. At 6-8h after the expected time of fertilisation, the mice were killed by cervical dislocation, their oviducts were dissected out and transferred to 1ml of equilibrated M16 medium in an embryological watch glass. The cumulus masses were released from the ampullary region and transferred by pasteur pipette to microdrops of M16 medium containing 0.5mg/ml of hyaluronidase to remove adhering cumulus cells. At this stage of development, the cumulus cell-free fertilised oocytes had developed a second polar body and exhibited two pronuclei. The fertilised oocytes were then transferred by hand-drawn micropipettes (with a central bore of 70-80um) to fresh microdrops of M16 and incubated in an atmosphere of 5% CO_2 in air at 37° C.

2.6 CYTOGENETIC ANALYSIS OF FERTILISED ONE-CELL EMBRYOS AND PARTHENOGENONES

Air-dried metaphase chromosome spreads were prepared from fertilised one-cell embryos and parthenogenones using the method described by Tarkowski (1966). Only one chromosome spread was prepared on each precleaned microscope slide. Microscope slides were marked with a small square (0.5cm^2 approximately) on one surface by a diamond pencil and then washed in acidified ethanol (1:100 acetic acid : ethanol). The oocyte was positioned within these limits, on the opposite surface of the slide, to facilitate the later identification of the position of the chromosome spread

2.6.1 Chromosome preparations in the absence of colcemid

Fertilised one-cell embryos were observed to enter metaphase of the first cleavage division at 16-18h after the expected time of fertilisation. Ethanol-induced parthenogenones entered metaphase at 15-16h after activation and hyaluronidase-induced oocytes were in metaphase at 14-15h after activation. The onset of metaphase of the first cleavage division was signalled by the dissolution of the pronuclear membranes. The metaphase chromosome spreads prepared at this stage of development were suitable for both the determination of the numerical chromosome constitution and karyotypic (G-band) analysis of fertilised one-cell embryos and

parthenogenones.

One-cell embryos and parthenogenones at this stage of the cell cycle were removed from the M16 culture medium and were transferred to 0.05ml drops of a hypotonic solution of 0.9% tri-sodium citrate for 15 minutes. A microdrop of hypotonic solution together with an oocyte was then transferred to a precleaned glass microscope slide. The application of a drop of 3:1 methanol: glacial acetic acid fixative from a micropipette dissolved the zona pellucida. The further application of 2-3 drops of fixative permitted the cytoplasm to spread over the premarked area of the microscope slide. The gentle puffing of air from a rubber bulb assisted with the uniform dispersal of the ooplasm. This method is modified from that described by Tarkowski (1966).

2.6.2 Colcemid-arrested fertilised one-cell embryos and parthenogenones

The incubation of fertilised one-cell embryos and parthenogenones at 10h after fertilisation or activation in M16 medium supplemented with 1ug/ml of Colcemid (Gibco) arrested development at metaphase of the first cleavage division. This permitted the preparation of metaphase chromosome spreads several hours after the oocytes had entered metaphase. The chromosome spreads were prepared by the Tarkowski method described above (2.6.1). These preparations were only suitable for the

determination of the numerical chromosome constitution of the embryo or parthenogenone as Colcemid-induced chromosome contraction inhibited the formation of quality G-bands.

2.6.3 Giemsa-banding of metaphase chromosome preparations from fertilised one-cell embryos and parthenogenones

Chromosome spreads of fertilised one-cell embryos and parthenogenones were also prepared during metaphase of the first cleavage mitosis. These preparations were allowed to "age" for 12-16 days before they were processed for G-band analysis. This has been reported to improve the quality of band patterns (Gallimore and Richardson, 1973). The chromosome spreads were analysed by phase contrast optics before staining. This enabled the physical appearance of the chromosomes in each spread to be classified into four distinct types. The following criteria determined the subsequent duration of trypsin exposure:

Type 1 were dense and cylindrical in appearance and incubation in trypsin solution for 30sec. was required to produce G-bands. The Type 2 and 3 chromosome spreads were less dense and flatter in appearance and were incubated for 20 and 15 sec., respectively, in the trypsin solution. The cytoplasm either overlayed or was adjacent to the chromosomes in Type 4 chromosome spreads and these frequently did not produce G-bands following trypsin

exposure.

Slides were immersed in 2 x SSC (17.53g/L sodium chloride; 8.82g/L tri-sodium citrate) at 60°C for 2h. They were then washed in distilled water and incubated in a 0.15-0.2g/100ml solution of trypsin (Difco; 1:250) in saline at 25° C for 15-30 sec. The duration of trypsin digestion was determined by the physical appearance of the pre-treated metaphase chromosomes. Those spreads that were classified as Type 1 or 4 were exposed to the trypsin solution for 30 sec., Type 2 and 3 for 20 and 15sec. repectively. The slides were then washed in saline and stained in 3% Giemsa's stain in Giemsa buffer (Gurr) at pH 6.8 for 1.5h.

G-banded and "solid" stained metaphase chromosome spreads were photographed using Kodak 1525 Technical Pan film. Karyograms of G-banded metaphase preparations were prepared according to the Committee for the Standardized Genetic Nomenclature for Mice (1972) and Nesbitt and Francke (1973).

2.7.1 TRANSMISSION ELECTRON MICROSCOPY

The following method was used for processing non-activated, ethanol- and hyaluronidase activated oocytes isolated from F1 hybrid mice for transmission

electron microscopy.

Cumulus cell-free oocytes were transferred from M16 medium to an embryological watch glass that contained 1ml of 3% glutaraldehyde in 0.1M phosphate buffer, pH 7.2 at 4°C and were left to fix in this solution for 1h. The oocytes were then washed in 0.1M phosphate buffer containing 0.5% sucrose and subsequently postfixed in 2% osmium tetroxide. The oocytes were dehydrated through a graded alcohol series and then removed to BEEM capsules containing a 1:1 solution of epoxypropane and araldite for 1h. The oocytes were transferred to araldite and retained in this medium at 20°C for 24-48h before they were finally embedded in fresh araldite. Three oocytes were embedded in each capsule.

Semithin sections were cut at a nominal thickness of 0.7um and were stained with 1% toluidine blue/1% pyronin B in a 0.8% borax solution (Ito and Winchester, 1963). Ultrathin sections were stained with lead citrate and uranyl acetate. Sections were viewed on a Philips EM 301 electron microscope at 60kV. Ultrathin sections were photographed using Kodak 4489 electron microscopy film.

2.8 Statistical Analysis: The Chi-square test (X^2).

The X^2 test was used to test whether the quantitative values obtained for (i) the frequencies of parthenogenetic activation, (ii) the proportionate incidence of the four classes of parthenogenone and (iii) the incidence of spontaneous and induced aneuploidy in the control and test conditions were significantly different or due to chance. The formula for the determination of the X^2 value is:

$$X^2 = \sum \left[\frac{(O - E)^2}{E} \right]$$

with O and E being observed and expected values.

The X^2 test ascertained the probability (p) that the deviation between two sets of data, such as control and test values for the conditions listed in (i), (ii) and (iii) were due to chance or the influence of the test conditions. The probability (p) was determined from standardised X^2 value tables at 1 degree of freedom. If the deviation between the two sets of values was equel to or less than 5% (p .05), the difference was not considered to be due to chance but due to the influence of experimental factors. When the p value was greater than .05 it was considered that the deviation between two values was due to chance and that the values were not significantly different.

CHAPTER 3

HYALURONIDASE-INDUCED PARTHENOGENETIC ACTIVATION OF F1 HYBRID OOCYTES: INFLUENCE OF POSTOVULATORY AGEING ON THE CHROMOSOME CONSTITUTION OF 1PN PARTHENOGENONES

CONTENTS

3.1 Introduction

3.2 Methods

3.3 Results

3.3.1 Chromosome constitution of 1PN parthenogenones at metaphase of the first cleavage mitosis

3.3.2 Chromosome constitution of fertilised one-cell embryos at metaphase of the first cleavage mitosis

3.3.3 Effects of postovulatory ageing on the incidence of the 1PN pathway of parthenogenesis

3.4 Discussion

3.1 INTRODUCTION

In young mature female mice maintained under controlled environmental conditions, the spontaneous ovulation of oocytes occurs at about the midpoint of the dark cycle during proestrus (Braden, 1957). When female mice at this stage of the estrous cycle are caged with proven fertile males it has been observed that mating approximately coincides with the expected time of ovulation. This behaviour therefore ensures that freshly ovulated oocytes are fertilised by recently capacitated sperm.

Numerous studies involving a variety of mammalian species have demonstrated that postovulatory ageing of oocytes prior to fertilisation, termed delayed mating or delayed fertilisation, was responsible for a significant decrease in the number of live-born offspring (reviewed Austin, 1970). Delayed fertilisation *in vivo* has been observed to increase the incidence of dispermic and digynic triploidy (Vickers, 1969; reviewed Austin, 1970). These classes of embryo develop as an increasing proportion of aged oocytes either fail to extrude the second polar body or possess a reduced ability to inhibit polyspermy (Braden, Austin and David, 1954). Postovulatory ageing prior to *in vitro* fertilisation has also been reported to increase the incidence of triploidy (Fraser and Dandekar, 1973; Santalo, Estop and Egozcue, 1988). The genomic imbalance that results from triploidy has been reported to arrest

development at midgestation in several strains of the mouse (Niemierko, 1981, Kaufman and Speirs, 1987) and is recognised to be but one of the developmental anomalies that account for the reduction in litter size following delayed mating. Prolonged postovulatory ageing of rodent oocytes has also been found to induce a low incidence of spontaneous parthenogenesis (Marston and Chang, 1964; reviewed Austin, 1970; Kaufman, 1983c) and to reduce the incidence of embryonic development beyond the pronuclear stage of the first cell cycle when aged rodent oocytes were fertilised in vitro (Juetten and Bavister, 1983; Smith and Lodge, 1987).

Ultrastructural analyses of ovulated mouse oocytes have indicated that postovulatory ageing induces the displacement of the meiotic spindle apparatus from its close alignment with the avillous zone of the plasma membrane to a more central location within the oocyte. The cortical granules, that normally lie subjacent to the villous region of the plasma membrane, have also been observed to migrate from this position to a more central location within ageing ovulated oocytes (Zamboni, 1970; Szöllősi, 1971; 1975). The central migration of the meiotic spindle apparatus has been considered to be one of the primary reasons for the reduced incidence of polar body extrusion in sperm and parthenogenetically activated postovulatory aged oocytes (Kaufman, 1975b). More recent immunocytochemical analyses of the cytoskeletal

organisation of fertilised and ageing oocytes have found that the close alignment between the meiotic spindle apparatus and the plasma membrane is primarily maintained by the presence of actin filaments located within the avillous zone of the oocyte (Maro, Johnson, Webb and Flach, 1986; Webb, Howlett and Maro 1986). Degenerative changes in the spatial organisation of microtubules within the meiotic spindle have also been observed in a significant proportion of postovulatory aged oocytes (Eichenlaub-Ritter, Chandley and Gosden, 1986). These authors have proposed that defects in the organisation of the meiotic spindle apparatus in ageing oocytes may serve to increase the incidence of chromosome segregation errors during the completion of the second meiotic division and that the limited developmental potential of a proportion of fertilised aged oocytes may be due, in part, to their aneuploid chromosome constitution.

A preliminary analysis of the chromosome constitution of one-cell mouse embryos that developed <u>in vivo</u> following delayed mating (Donahue and Karp, 1973) indicated that postovulatory ageing prior to fertilisation did not appear to be associated with a significant increase in the incidence of aneuploidy. A small proportion of the postovulatory aged mouse oocytes from mated females were also found to have initiated parthenogenetic development <u>in vivo</u>. This was due to the ability of hyaluronidase, present in the ejaculate, to activate a proportion of the

aged oocytes that failed to develop as fertilised one-cell embryos (see, Kaufman 1973a).

Aim of investigation

A high proportion of ovulated oocytes that have aged in vivo for several hours may initiate parthenogenetic development when briefly incubated in tissue culture medium supplemented with hyaluronidase (Kaufman, 1973a; O'Neill and Kaufman, 1988). A high proportion of these parthenogenones extrude a second polar body and subsequently develop a single haploid pronucleus. The cytogenetic analysis of this class of haploid parthenogenone at metaphase of the first cleavage mitosis can establish whether increasing postovulatory age predisposes the completion of the second meiotic division to errors in chromosome segregation. The experimental approach employed in the present study enables the precise postovulatory age of the oocyte at the time of activation to be controlled and excludes the possibility of recording sperm-derived aneuploidy. This wholly parthenogenetic analysis also removes the problem, encountered by Donahue and Karp (1973), of having to separately account for both sperm-mediated and parthenogenetic pathways of development.

3.2 METHODS

Hyaluronidase-induced parthenogenetic activation

Ten- to 12-week old F1 hybrid female mice were superovulated as described in Chapter 2.2. At HCG+ 18h, 20h, 22h, 24h and 26h the mice were killed by cervical dislocation. The cumulus masses were isolated and exposed to hyaluronidase for 10 - 15 mins to induce parthenogenetic activation as described in section Chapter 2.4.1. The activation frequency was determined 6h later and the 1PN parthenogenones were transferred to fresh microdrops of M16 medium. After a further 4h - 5h the 1PN parthenogenones were transferred to microdrops of M16 supplemented with 1ug/ml of Colcemid to arrest development at metaphase of the first cleavage division.

Fertilised one-cell embryos

An additional group of F1 hybrid female mice were superovulated as described above and caged individually with F1 hybrid males of proven fertility. Early the next morning the females were examined for the presence of a vaginal plug and this was taken as evidence that mating had occurred. At 6h - 8h after the expected time of fertilisation, the female mice were killed by cervical dislocation. The fertilised one-cell embryos were isolated from the ampullary region of the oviduct to incubate in microdrops of M16 medium as described in

Chapter 2.5. After a period of 6h, the fertilised one-cell embryos were transferred to M16 medium supplemented with 1ug/ml of Colcemid.

Metaphase chromosome preparations from fertilised one-cell embryos and parthenogenones

Chromosome spreads were prepared from the metaphase-arrested parthenogenones and fertilised one-cell embryos using the Tarkowski method (1966) described in Chapter 2.6.2.

3.3 RESULTS

3.3.1 Effects of postovulatory ageing on the incidence of the 1PN pathway of parthenogenesis

The influence of increasing postovulatory age on the frequency of hyaluronidase-induced parthenogenetic activation is presented in Table 3.1. The lowest incidence of activation (34.9%) was observed when oocytes were exposed to hyaluronidase at HCG+ 18h. A significant increase in the activation frequency (87.6%) was obseved when oocytes were exposed to hyaluronidase at HCG+ 20h. The highest incidence of parthenogenetic activation (95.4%) was achieved when oocytes were exposed to hyaluronidase at HCG+ 24h. Hyaluronidase activation at HCG+ 26h was associated with a reduced incidence of parthenogenesis but activated oocytes exhibited the highest incidence of the IC pathway of parthenogenesis.

The 1PN parthenogenones were the most commonly encountered developmental class of parthenogenone in each of the groups studied (Fig 3.1). A decrease in the incidence of this developmental pathway (from 91.9% to 64.3% of the activated oocytes examined) was observed as the postovulatory age of the oocytes increased from HCG+ 18h to HCG+ 26h. The decreased incidence of this class was associated with a proportionate increase in the

TABLE 3·1

The effect of increasing postovulatory age on the incidence of hyaluronidase-induced parthenogenetic development

Group	Postovulatory age (h)	No. of oocytes	No. of activated oocytes	Activation %age	Class of parthenogenone 1PN	2PN	IC	1PND
1	18	284	99	34.85	91	8	0	0
2	20	169	148	87.57	109	34	3	2
3	22	254	209	82.28	153	50	5	1
4	24	175	167	95.42	109	48	9	1
5	26	280	216	77.14	139	39	36	2

Group 1: the incidence of 1PN development is significantly different
from Group 3 : X^2 =14.2 p 0.01
and Group 5 : X^2 =16.1 p 0.01

Group 3: the incidence of 1PN development is significantly different
from Group 5 : X^2 = 3.8 $p < 0.05$

Group 4: the frequency of parthenogenetic activation is significantly different
from Group 2 : X^2 = 6.8 $p < 0.01$
and Groupp 3 : X^2 = 16.5 $p < 0.01$

Group 3: the frequency of parthenogenetic activation is not significantly different
from Group 2: X^2 = 2.1 p 0.2 - 0.1

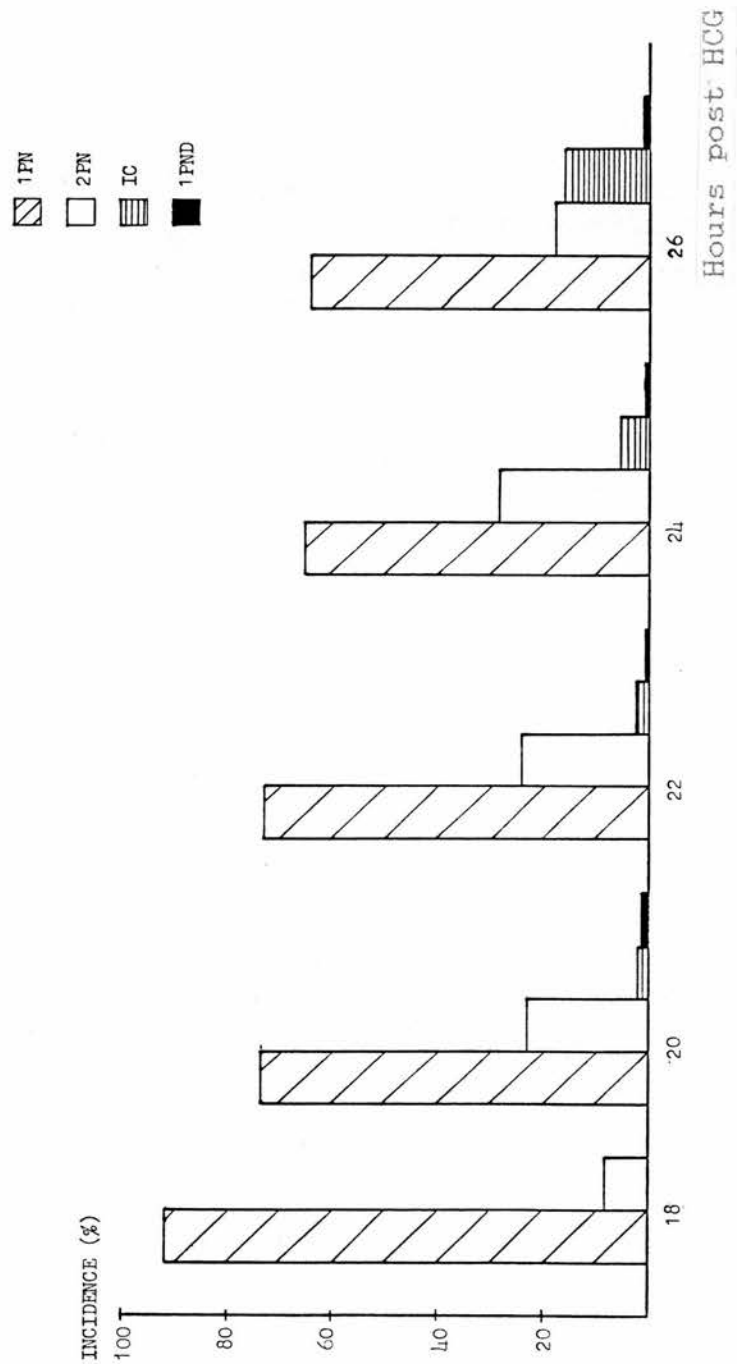


Fig. 3.1 The proportionate incidence of the four pathways of parthenogenetic development observed following the hyaluronidase-induced activation of F1 hybrid oocytes at 18h - 26h after the HCG injection.

incidence of parthenogenones in which polar body extrusion failed to occur.

3.3.2 Chromosome constitution of 1PN parthenogenones at metaphase of the first cleavage mitosis

The analysis of the chromosome constitution of 1PN parthenogenones that developed following the hyaluronidase-induced activation of ovulated oocytes at HCG+ 18h, 20h, 22h, 24h, and 26h is presented in Table 3.2. The incidence of aneuploidy observed in these five groups was 2.7%, 4.0%, 3.7%, 2.8% and 4.1%, respectively. These values do not differ significantly from each other. This clearly demonstrates that the increasing postovulatory age of the oocyte at the time of activation does not increase the incidence of aneuploidy. The majority of the aneuploid chromosome preparations (84.6%) involved the loss or gain of only one chromosome. The overall ratio of hypohaploidy to hyperhaploidy was 1:1.6. Representative euploid and aneuploid chromosome spreads from this study are illustrated in Fig 3.2.

3.3.3 The chromosome constitution of fertilised one-cell embryos at metaphase of the first cleavage mitosis

The chromosome constitution of the fertilised one-cell embryos at metaphase of the first cleavage mitosis is presented in Table 3.3. The relatively higher incidence

TABLE 3·2

Chromosome constitution of single pronuclear haploid parthenogenones (1PN) at metaphase of the first cleavage division

Group	Postovulatory age (h)	No. of chromosome spreads	No. not analysable	Chromosome constitution 19	20	21	22	24	Frequency of aneuploidy (%)
1	18	83	10	0	71	1	1	0	2.73
2	20	81	6	2	72	1	0	0	4.00
3	22	94	12	0	79	2	0	1	3.65
4	24	75	4	1	69	1	0	0	2.81
5	26	80	8	2	70	1	0	0	4.11

Group 1: the incidence of aneuploidy is not significantly different from Group 5: $X^2 = 0.2$ $p = 0.7\text{-}0.5$

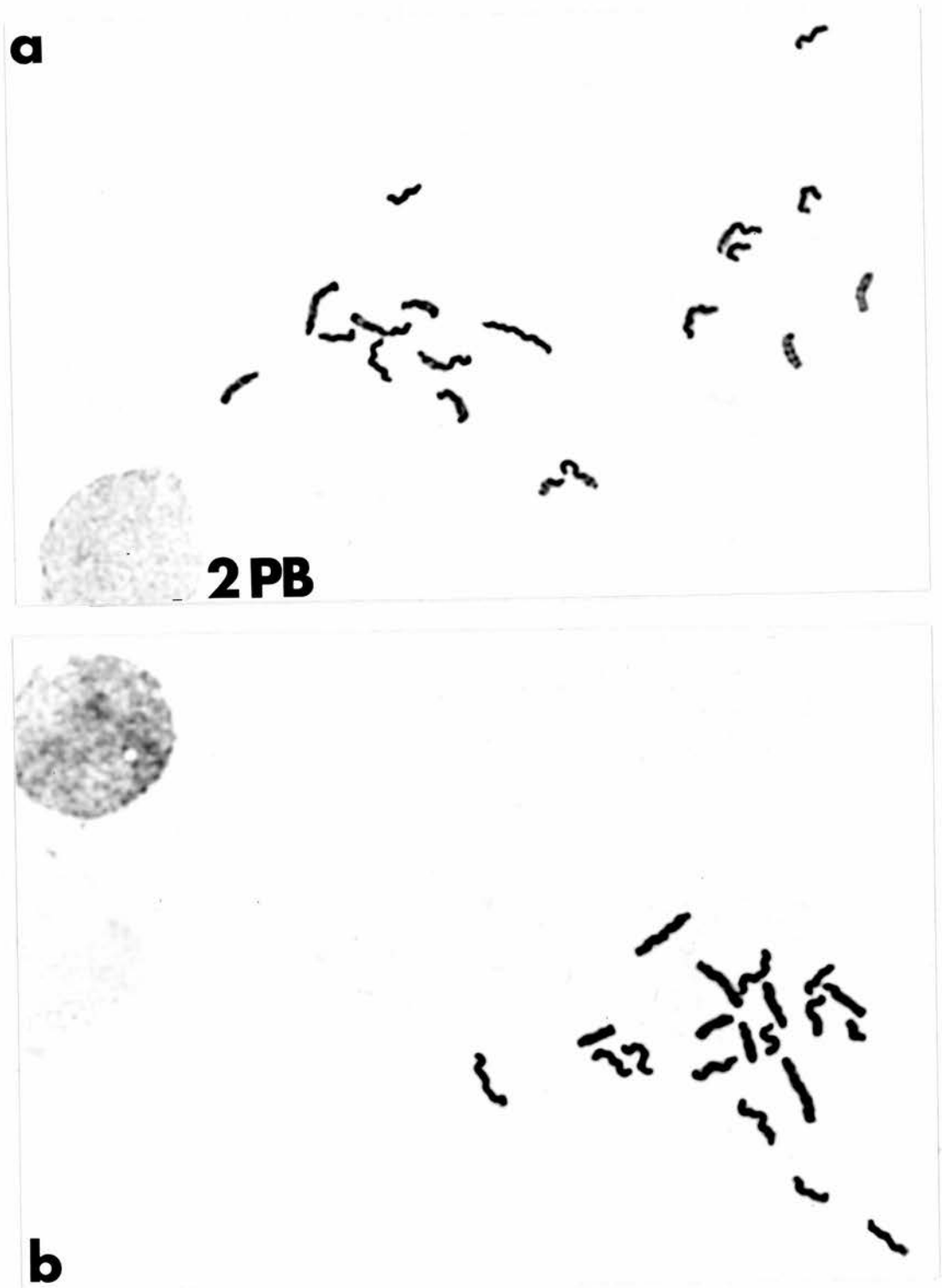


Fig 3.2 Representative air-dried chromosome spreads from hyaluronidase activated 1PN parthenogenones at metaphase of the first cleavage mitosis that exhibit (a) 19, (b) 20, (c) 20 and (d) 21 chromosomes respectively. The nucleus of the second polar body (2PB) is visible in each preparation. Giemsa stain.

c
2PB

d

TABLE 3·3

Chromosome constitution of fertilised one-cell embryos from F1 hybrid female mice analysed at metaphase of the first cleavage mitosis.

Group	Number of chromosome preparations	Number of nonanalysable preparations	Chromosome Constitution			Number of triploid embryos	Adjusted incidence of aneuploidy excluding triploidy (%)
			19/20	20/20	21/20		
1	364	51	8	298	2	5	1·3

The adjusted incidence of aneuploidy in Table 3·3 Group 1 is not significantly different to that observed in Table 3·1 Group 1 - 5. $X^2 = 3·2$, $P = 0·1 - 0·05$

of hypodiploidy over hyperdiploidy encountered in the present study would seem to indicate that a proportion of these chromosome preparations may have resulted from mechanical loss incurred during the fixation procedure. As it is generally believed that the incidence of hyperdiploid spreads is a more reliable indicator of malsgregation events, the incidence of aneuploidy has been recalculated using the following formula:

$$\frac{\text{No. of hyperhaploid preparations}}{\text{Total No. of chromosome spreads}} \times 2$$

The determination of an adjusted level of aneuploidy using this formula has found that 1.3% of the one-cell embryos exhibited an aneuploid chromosome constitution. This value was considerably lower than the incidence of aneuploidy observed in 1PN parthenogenones but the difference was not found to be significant (X^2: P = 0.1 - 0.05). This analysis has also demonstrated that 1.5% of the fertilised one-cell embryos exhibited a triploid chromosome constitution.

3.4 DISCUSSION

The majority of hyaluronidase activated oocytes developed as 1PN parthenogenones. The proportionate incidence of this developmental pathway decreased significantly as the postovulatory age of activated oocytes increased from HCG+ 18h to HCG+ 26h. This relationship between postovulatory age, parthenogenetic activation and the subsequent development of several distinct classes of parthenogenone has previously been reported (Kaufman, 1975b; 1983c). Numerous studies have clearly demonstrated that the extrusion of the second polar body in mouse and hamster oocytes is dependent on the close alignment of the second meiotic spindle apparatus with an overlying actin-rich and microvillus-free zone of the plasma membrane (mouse: Szöllösi, 1971, 1975; Wasserman and Fujiwara, 1978; Sato and Blandau, 1979; Longo and Chen, 1985; Maro, Johnson, Webb and Flach, 1986; Webb, et al., 1986; hamster: Okada, Yanagimachi and Yanagimachi, 1986). Immunocytochemical analyses of the postovulatory age-related changes that occur in the organisation of actin filaments in mouse oocytes have demonstrated that in an increasing proportion of oocytes the actin-rich cortical zone becomes disorganised and facilitates the migration of the meiotic spindle to a more central location in the oocyte (Webb, et al, 1986). In this study it has been found that the activation of postovulatory

aged oocytes, especially those isolated at HCG+ 24h and HCG+ 26h favoured the development of parthenogenones in which both products of the second meiotic division were retained within the oocyte. Second polar body extrusion is also inhibited when activated oocytes are incubated in culture medium supplemented with cytochalasin B (Bałakier and Tarkowski, 1976) or cytochalasin D (Chapter 9) as these agents inhibit the polymerisation of actin filaments.

Preliminary studies to establish the optimum conditions for hyaluronidase activation found that lower doses (0.5 - 1.0 mg/ml) than that used in the present analysis removed the adhering cumulus cells from the oocyte but did not consistently induce parthenogenetic activation. Parthenogenetic activation has also been observed following the brief exposure of ovulated oocytes to pronase, an enzyme that has the potential to dissolve the zona pellucida. Although trypsin exposure also removes adhering cumulus cells from ovulated oocytes it fails to induce parthenogenetic activation in the mouse (Kaufman, 1983c). Ultimately, both hyaluronidase and pronase must evoke a series of electrophysiochemical changes within the exposed oocytes, possibly homologous with those that occur at fertilisation, that facilitate the induction of parthenogenesis. These events may be initially mediated by the rapid dispersal of the cumulus cells from the zona pellucida, but the precise mechanism by which these

events induce activation has yet to be determined.

Cytogenetic studies of unfertilised ovulated oocytes and one-cell fertilised embryos from several strains of mice (reviewed: Nijohff and de Boer, 1981; Dyban and Baranov, 1987) have revealed that the incidence of spontaneous meiotic chromosome segregation errors are in the order of 0.2-2.6%. In the present study, the cytogenetic analysis of fertilised one-cell embryos has revealed that the "baseline" incidence of spontaneous aneuploidy was about 1.3%, and that the majority of the aneuploid embryos had either lost or gained only one chromosome. The analysis of the 1PN parthenogenones revealed that this group had a higher overall incidence of aneuploidy. Aneuploid parthenogenones with a complement of 22 and 24 chromosomes were occasionally observed. Although this is indicative of a greater degree of disorganisation in the postovulatory aged spindle, the difference in the incidence of aneuploidy between the fertilised and parthenogenetically activated embryos was not found to be significant.

The use of chromosome polymorphism studies to determine the parental origin of several autosomal trisomic conditions (Niikawa, Merotto and Kajii, 1977; Mikkelsen, et al., 1980; Juberg and Mowry, 1983; Hassold, Chiu and Yamane, 1984) have indicated that the majority of aneuploid conceptuses develop as a result of

chromosome segregation errors during the first meiotic division of the oocyte. Chromosome segregation errors during the completion of the second meiotic division of human oocytes are estimated to account for a further 10% of autosomal trisomic conceptuses. It has been proposed that the postovulatory ageing of human oocytes prior to fertilisation may induce chromosome segregation errors at the second meiotic division, and that this is a significant factor in the origin of human aneuploidy (German, 1968; Juberg, 1983; Juberg and Mowry, 1983). This does not appear to be consistent with the present findings, which have demonstrated that the incidence of aneuploidy in activated aged mouse oocytes did not differ significantly from that observed in fertilised one-cell mouse embryos. The present findings from this parthenogenetic model indicate that delayed fertilisation of human oocytes is probably not a significant factor in the origin of aneuploidy. However, it should also be considered that the meiotic spindle apparatus of the ageing human oocyte may be exposed to a number of exogenous factors prior to fertilisation that later reduce the ability of a fertilised oocyte to effect the balanced segregation of chromosomes or to regulate pronuclear development. Under these conditions postovulatory ageing of human oocytes could <u>indirectly</u> increase the risk of aneuploidy. However, it appears more likely that postovulatory ageing prior to fertilisation is one of the factors responsible for the induction of

human triploid conceptuses (Kaufman, 1988). It is recognised that about 20% of spontaneous abortuses exhibit a triploid chromosome constitution (Linn, De Braekeleer and Jamro, 1985) and that the incidence of this anomaly at conception may be as high as 8%.

This study has also demonstrated that parthenogenesis per se does not significantly increase the incidence of chromosome segregation errors during the completion of the second meiotic division and that this parthenogenetic stimulus can be used as a control in the following studies concerned with the analysis of the influence of ethanol on the segregation of chromosomes at the first or second meiotic division.

CHAPTER 4

ETHANOL-INDUCED PARTHENOGENETIC ACTIVATION OF F1 HYBRID OOCYTES in vitro

CONTENTS

4.1 Introduction

4.2 Methods

4.3 Results

4.3.1 Observations on the proportionate incidence of the main pathways of parthenogenetic development

4.3.2 Chromosome constitution of 1PN parthenogenones at metaphase of the first cleavage mitosis

4.3.3 Karyotypic analysis of hyaluronidase- and ethanol-induced parthenogenones

4.4 Discussion

4.1 INTRODUCTION

Ovulated mouse oocytes also exhibit a high incidence of parthenogenetic activation following their brief exposure to a dilute solution of ethanol *in vitro*. As observed following hyaluronidase-induced activation, four distinct pathways of parthenogenetic development can be identified at the pronuclear stage of the first cell cycle. The majority of ethanol activated oocytes develop as 1PN parthenogenones (Kaufman, 1982; O'Neill and Kaufman, 1989a).

The incidence of spontaneous chromosome segregation errors, which occur at either the first or second meiotic division, has been found to be in the range of 0.6% - 2.6% in the oocytes of mouse strains that do not possess chromosomal rearrangements (reviewed: Nijhoff and de Boer, 1981; Dyban and Baranov, 1987). In the previous study, the incidence of aneuploidy observed in the metaphase chromosome spreads of F1 hybrid fertilised one-cell embryos at metaphase of the first cleavage mitosis was found to be in the region of 1.3%. Cytogenetic analyses of hyaluronidase activated 1PN parthenogenones have also demonstrated that parthenogenetic activation, *per se*, is not usually associated with a significant increase in chromosome malsegregation during the completion of the second

meiotic division (Chapter 3; O'Neill and Kaufman, 1988). However, analysis of the chromosome constitution of ethanol-induced 1PN parthenogenones at metaphase of the first cleavage division has revealed that a significant number exhibited an aneuploid chromosome constitution (Kaufman, 1982; O'Neill and Kaufman, 1989b).

It has been postulated that ethanol disorganises the balanced segregation of meiotic chromosomes by interfering with the normal functioning of the microtubular elements of the spindle apparatus (Kaufman, 1985; Kaufman and O'Neill, 1988). Aneuploid oocytes (Mailhes and Yuan, 1987) and embryos can also be experimentally induced when spindle-active agents such as colchicine (McGaughey and Chang, 1969; Sugawara and Mikamo, 1980; Hummler and Hansmann, 1985; Tease and Fisher, 1986) or vincristine sulphate (Danford and Parry, 1986) are administered to female rodents in low doses during the first or at the completion of the second meiotic division. These compounds bind stoichiometrically with tubulin dimers to form a stable complex. This complex inhibits tubulin polymerisation and disrupts the dynamic equilibrium between microtubule assembly-disassembly and consequently promotes the net depolymerisation of microtubules. However there is no evidence to date that ethanol induces structural changes in the morphology of individual spindle elements.

All compounds that possess anaesthetic properties have the potential to induce mitotic spindle dysfunction (Jackson, 1975; Onfelt, 1986) but the mechanism by which anaesthetics interact with the mitotic spindle apparatus, and also the precise stage of the cell cycle at which disruption occurs, is dependent on the chemical nature of the compound. The inhalational anaesthetics which share a number of the physiological effects of ethanol have been reported to disrupt the integrity of microtubules when cultured cells and isolated neural tissue were exposed to these agents in vitro (Halothane: Allison, et al., 1970; Hinkley & Samson, 1972; Hinkley and Telser, 1974; nitrous oxide: Brinkley and Rao, 1973; Cox, Rao and Brinkley, 1977). These studies have found, however, that while nitrous oxide interferes with prometaphase chromosome movement, Halothane induced microtubule depolymerisation at a later stage of the cell cycle. It has also been observed that Avertin anaesthesia can increase the incidence of meiotic chromosome segregation errors (Kaufman, 1975c) and decrease the number of viable implants (Kaufman, 1977) when this agent is administered to female mice before conception occurs. However, the mechanism of ethanol-induced spindle disorder has still to be determined.

Aims of Investigation

This study examines the relationship between the frequency of parthenogenetic activation, the

proportionate incidence of the various pathways of parthenogenetic development and the duration of ethanol exposure in vitro. These studies permit an indirect analysis of this agent's potential to induce disordered cytokinetic events during the completion of the second meiotic division in activated oocytes.

Ethanol-induced parthenogenesis also serves as a model to analyse the ability of an intragastric injection of a dilute ethanol solution to induce chromosome segregation errors during the completion of the second meiotic division in fertilised mouse oocytes. The influence of ethanol on the incidence of chromosome malsegregation during the completion of the second meiotic division is examined by an analysis of Giemsa-banded metaphase chromosome spreads from ethanol-induced parthenogenones at metaphase of the first cleavage mitosis. A karyotypic analysis of the metaphase chromosome spreads of ethanol-induced parthenogenones has the potential to indicate whether numerical chromosomal anomalies are the result of true segregation errors or if a proportion are due to the induction of chromosome breaks and the formation of chromosome fragments. It can also identify whether specific chromosomes are more frequently associated with aneuploidy or if segregation errors are purly random events. This investigation of the developmental pathways and chromosome constitution of ethanol activated oocytes provides us with a valuble

model to investigate the processes associated with the induction of meiotic chromosome segregation errors during the completion of the second meiotic division.

4.2 METHODS.

Ethanol-induced activation

F1 hybrid female mice were superovulated as described in Chapter 2.2 and were killed by cervical dislocation at HCG+ 17h. Parthenogenetic activation was induced following the incubation of ovulated oocytes in a 7% solution of ethanol in Dulbecco's PBS for either 1min., 3min. or 5min., as described in Chapter 2.4.3.

Hyaluronidase-induced activation: control

An additional group of F1 hybrid female mice were superovulated as described above and were killed at either HCG+ 18h or HCG+ 20h by cervical dislocation. The ovulated oocytes were incubated in microdrops of M16 containing 3mg/ml of hyaluronidase for 10 - 15 minutes to induce parthenogenetic activation, as described in Chapter 2.4.2.

Preparation of metaphase chromosome spreads from hyaluronidase and ethanol-induced parthenogenones

Metaphase chromosome spreads were prepared according to the method described in Chapter 2.6.2 and were G-banded as described in Chapter 2.6.3.

4.3 RESULTS

4.3.1 Observations on the proportionate incidence of the main pathways of parthenogenetic development

The incidence of the various pathways of parthenogenetic development observed following the isolation of ovulated oocytes at HCG+ 17h and their incubation for either 1, 3 or 5 min. in a 7% solution of ethanol are presented in Table 4.1, while comparable values for ovulated oocytes isolated at HCG+ 18h and HCG+ 20h and exposed to culture medium containing hyaluronidase are presented in Table 4.2. The highest overall frequency of parthenogenetic activation in the present study was observed following hyaluronidase activation at HCG+ 20h (78.9%). A lower frequency of activation, in the range of 63.7 - 69.3%, was observed following ethanol-induced activation but these values are higher than those observed following hyaluronidase activation at HCG+ 18h.

The majority of ethanol activated oocytes developed as 1PN parthenogenones (Fig 4.1). An increase in the duration of ethanol exposure from 1min. to 3min. was not associated with a significant decrease in the proportionate incidence of the 1PN class (Table 4.1; $X^2 = 2.3$, $p = 0.2\text{-}0.1$). However, the proportionate incidence of the 1PN parthenogenones was significantly reduced from

Table 4·1

The proportionate incidence of the pathways of parthenogenetic development observed following the exposure of ovulated oocytes from (C57BL x CBA)F1 female mice to a 7% solution of ethanol in PBS at HCG + 17h

Group	Duration of activation stimulus (min)	Total number of ovulated oocytes	Total number of activated oocytes	Activation frequency (%)	Pathway of parthenogenetic development at the pronuclear stage			
					1PN	2PN	IC	1PND
1	1	497	321	64.6	285	24	8	4
2	3	969	673	69.5	574	83	4	12
3	5	1864	1188	63.7	801	325	37	25

Group 1 is not significantly different from Group 2; $X^2 = 2.3$, $p = 0.2\text{-}0.1$

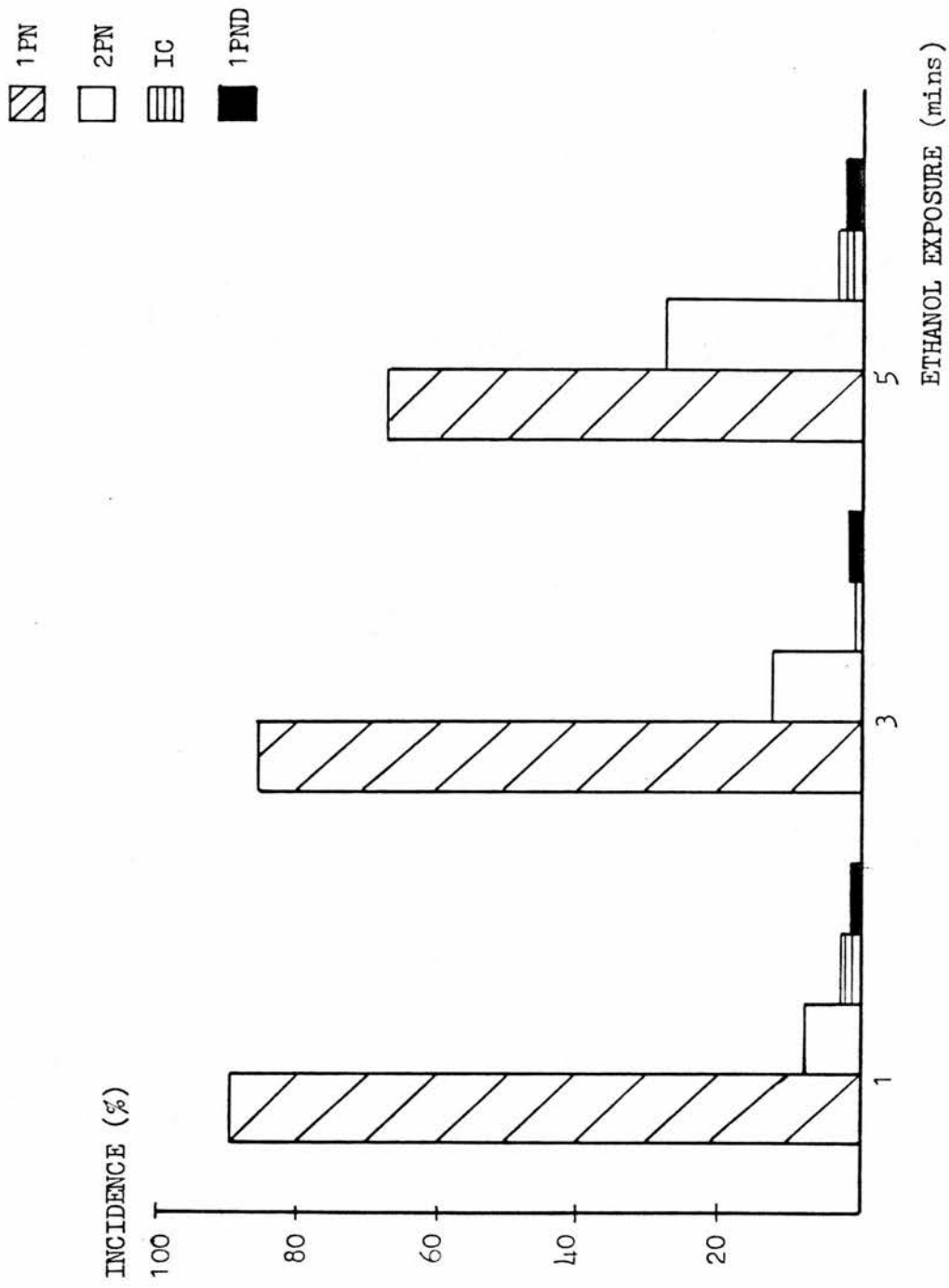


Fig. 4.1 The proportionate incidence of the four pathways of parthenogenetic development observed following the brief exposure of ovulated F1 hybrid oocytes to a 7% ethanol solution for either 1, 3, or 5 mins. at HCG+ 17h.

TABLE 4·2

The proportionate incidence of the pathways of parthenogenetic development observed when ovulated oocytes from (C57BL x CBA)F1 female mice were exposed to hyaluronidase

Group	Postovulatory age (h)*	Total number of ovulated oocytes	Total number of activated oocytes	Activation frequency (%)	Pathway of parthenogenetic development at the pronuclear stage			
					1PN	2PN	1C	1PND
1	18	401	155	38.7	146	9	0	0
2	20	692	546	78.9	434	100	9	3

* Time of isolation after the HCG injection. Ovulation occurs at HCG + 11-12h

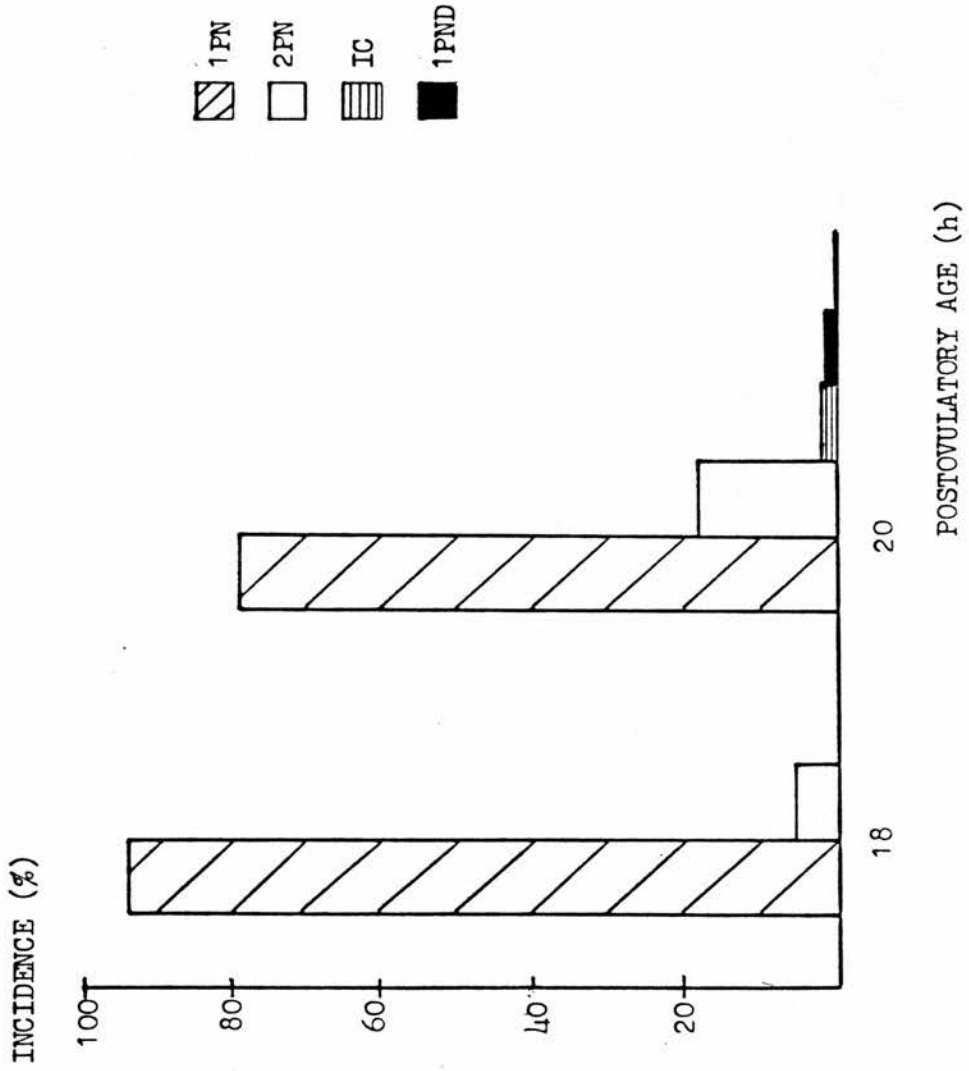


Fig. 4.2 The proportionate incidence of the four pathways of parthenogenetic development observed following the exposure of F1 hybrid oocytes to hyaluronidase for 10 - 15 mins. at either HCG+ 18h or HCG+ 20.

85.3% to 67.4% as the period of ethanol exposure increased from 3 min. to 5 min.. This decrease in the incidence of 1PN development was related directly to an increase in the incidence of 2PN, 1PND and IC parthenogenones. The majority of the activated oocytes that failed to extrude a polar body developed as 2PN parthenogenones.

The majority of the ovulated oocytes that initiated parthenogenetic development following exposure to hyaluronidase developed as 1PN parthenogenones (Fig 4.2). An increase in the postovulatory age of oocytes at the time of their activation from HCG+ 18h to HCG+ 20h was also associated with a significant decrease in the proportionate incidence of the 1PN class. The IC and 1PND classes of parthenogenone were not observed following activation at HCG+ 18h. The 2PN parthenogenones were the most common class observed when activated oocytes failed to extrude the second polar body and retained both genomic products of the second meiotic division.

4.3.2. Chromosome constitution of 1PN parthenogenones at metaphase of the first cleavage division

The chromosome constitution of ethanol and hyaluronidase activated 1PN parthenogenones is presented in Tables 4.3 and 4.4, respectively. The chromosome spreads that are recorded as non-analysable represent those in which the

Table 4·3

The chromosome constitution of single-pronuclear haploid parthenogenones at metaphase of the first cleavage division following activation in a 7% solution of ethanol in PBS at HCG + 17h

Group	Duration of activation stimulus (min)	Total number of chromosome preparations	Number of preparations not analysable	Chromosome constitution							Aneuploidy (%)
				18	19	20	21	22	23	24	
1	1	100	7	1	9	79	4	0	0	0	15.1
2	3	275	17	5	17	218	15	2	1	0	15.5
3	5	506	46	25	41	342	37	8	5	2	25.7

Group 1 is not significantly different from Group 2; $X^2 = 7.7 \times 10^{-3}$. $p = 0.99\text{-}0.98$
Group 2 is significantly different from Group 3; $X^2 = 6.5$, $p = .02 - .01$

presence of overlapping chromosomes prevented an accurate analysis being made of the number of chromosomes present, and those that were obviously hypohaploid due to chromosome scattering incurred during the chromosome spreading procedure. The overall ratio of hypohaploidy to hyperhaploidy observed following ethanol-induced activation was 1.0 : 0.8. As it is possible that a small proportion of hypohaploid spreads arise from the mechanical loss of chromosomes during the spreading technique, and that hyperhaploid counts represent a more reliable indicator of errors in chromosome segregation, an adjusted value for the incidence of aneuploidy has been determined from the following equation:

$$\frac{\text{Total number of hyperhaploid chromosome spreads}}{\text{Total number of chromosome spreads}} \times 2$$

The cytogenetic analysis of hyaluronidase activated 1PN parthenogenones at HCG+ 20h indicates that the control or "background" incidence of aneuploidy is low (total: 4.3%, adjusted: 3.3%). A significantly higher incidence of aneuploidy (total: 15.1%, adjusted: 8.6%) was observed when oocytes were activated following 1min. exposure to ethanol. When oocytes were exposed to ethanol for either 3 min. or 5 min. the incidence of aneuploidy was 15.5% (adjusted: 13.9%) and 25.7% (adjusted: 22.6%), respectively, and these values were significantly different from each other ($X^2 = 6.5$, $P = .02 - .01$). Although the incidence of aneuploidy does not increase

Table 4.4

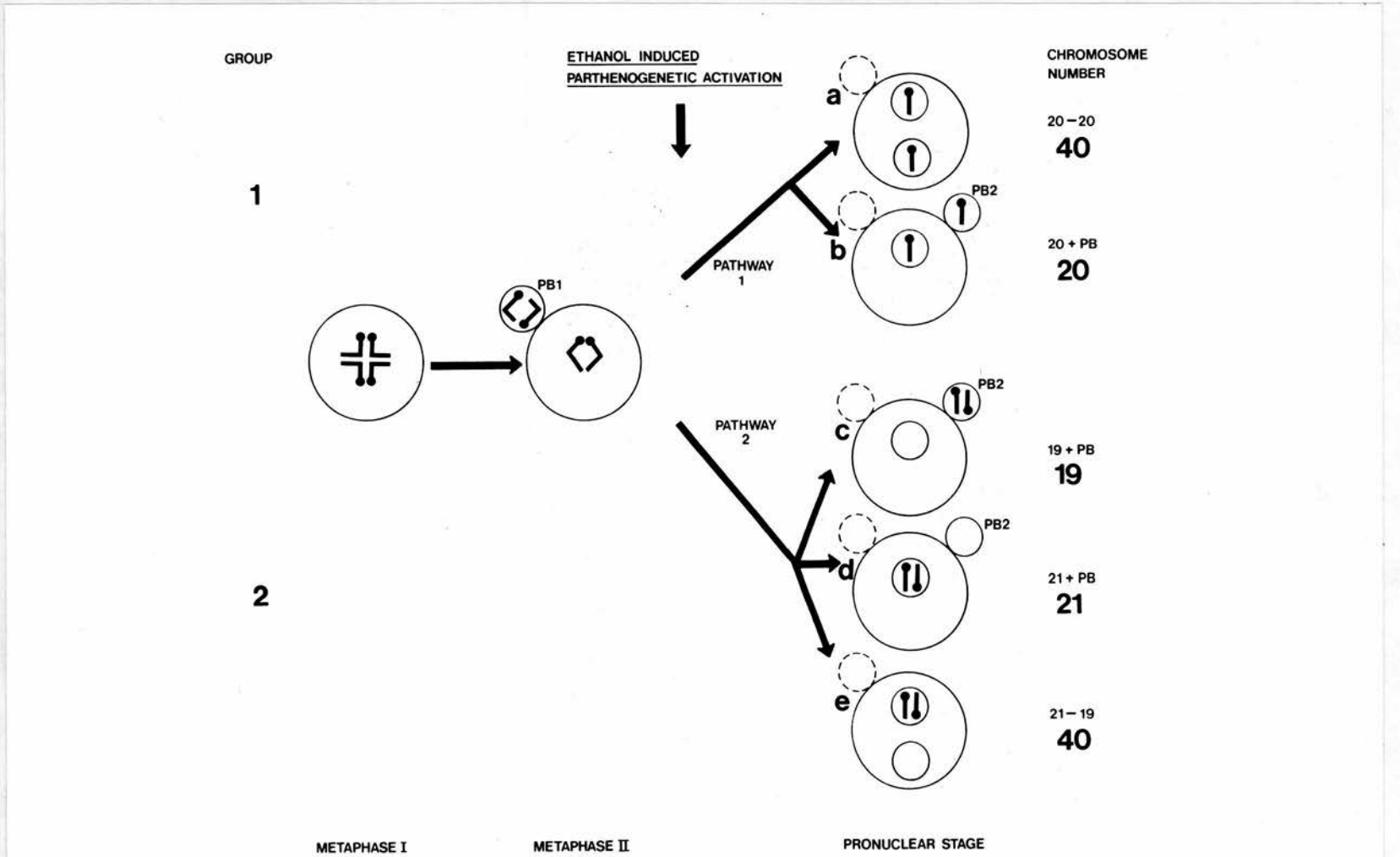
The chromosome constitution of single-pronuclear haploid parthenogenones at metaphase of the first cleavage division following hyaluronidase activation at HCG + 20h

Group	Postovulatory age (h)*	Total number of chromosome preparations	Number of preparations not analysable	Chromosome constitution					Aneuploidy (%)
				18	19	20	21	22	
2	20	328	27	2	6	288	4	1	4.3

* see Table 4·2

Table 4·3 Group 1 is significantly different from Table 4·4 Group 2; $X^2 = 10.64$, $p < 0.01$

Fig. 4.3 Development of ethanol-induced 1PN and 2PN parthenogenones following the in vitro exposure of secondary oocytes to a dilute ethanol solution. Pathways 1a and 1b illustrate the chromosome constitution of parthenogenones at metaphase of the first cleavage mitosis following the balanced segregation of chromosomes at the completion of the second meiotic division. In the parthenogenones that develop along pathways 2c and 2d, exposure to ethanol has induced chromosome segregation errors during the completion of the second meiotic division. This results in the formation of aneuploid haploid parthenogenones with complements of either 19 or 21 chromosomes. The parthenogenones that have developed along pathway 2e have also undergone malsegregation but both genomic products of the second meiotic division have been retained within the activated oocyte. It has a complete chromosome constitution that is euploid and diploid. However, one pronucleus is hypohaploid, the other is hyperhaploid.


GROUP
ETHANOL INDUCED
PARTHENOGENETIC ACTIVATION
CHROMOSOME NUMBER
1
2
PB1
PATHWAY 1
PATHWAY 2
a
b
c
d
e
PB2
PB2
PB2
20 – 20
40
20 + PB
20
19 + PB
19
21 + PB
21
21 – 19
40
METAPHASE I
METAPHASE II
PRONUCLEAR STAGE

significantly when the duration of ethanol exposure increases from 1min. to 3min., (total; X^2 = 7.7 x10 $^{-3}$, P= 0.99 - 0.98: adjusted; X^2 = 0.83, P= 0.5 - 0.3) the incidence of malsegregation events that involved more than one chromosome increased from 7.1% to 20.0%. When the duration of exposure was increased from 3min. to 5min. the percentage of malsegregation events that involved more than one chromosome was increased to 36.0%. Thus an increase in the duration of ethanol exposure clearly increases the probability that more than one chromosome is involved in each nondisjunctional event. The development of aneuploid and euploid parthenogenones is represented in Fig. 4.3.

4.3.3 Karyotypic analysis of hyaluronidase- and ethanol-induced parthenogenones

The karyotypic analysis of G-banded metaphase chromosome preparations from ethanol-induced 1PN parthenogenones has indicated that not all chromosomes of the mouse genome are equally predisposed to malsegregation at the second meiotic division (Fig 4.4 - 4.8). The specific chromosome constitution of the aneuploid 1PN parthenogenones that exhibited G-band patterns is presented in Table 4.5. The results presented in Table 4.6 indicate that the chromosomes 5, 6, 7, 8, 9, 14, and 19 were less frequently involved with malsegregation

Table 4.5

The incidence of specific chromosome malsegregation observed in ethanol-induced 1PN parthenogenones at metaphase of the first cleavage mitosis

Chromosome	Incidence of Hyperhaploidy	Hypohaploidy	Total
1	4	0	4
2	2	2	4
3	1	3	4
4	4	2	6
5	0	1	1
6	1	1	2
7	0	0	0
8	0	3	3
9	1	1	2
10	2	3	5
11	2	4	6
12	3	4	7
13	2	2	4
14	3	0	3
15	6	2	8
16	2	2	4
17	6	0	6
18	5	5	10
19	0	1	1
X	3	1	4

Expected incidence (E) of malsegregation for each chromosome is 4

$$E = \frac{\text{Number of hyperhaploid + hypohaploid events}}{\text{Number of chromosomes in euploid 1PN parthenogenone}} \qquad \frac{(47 + 37)}{(20)}$$

Table 4.6

The specific chromosome constitution of ethanol-induced aneuploid 1PN parthenogenones.

Chromosome constitution of hyperhaploid 1PN parthenogenones	Incidence
1 min. ethanol exposure	
21, X; + 1	1
21, X; + 10	1
3 min. ethanol exposure	
21, X; + 1	1
21, X; + 2	1
21, X; + 4	1
21, X; + 11	1
21, X; + 12	1
21, X; + 15	3
21, X; + 17	2
21, X; + 18	1
22, X; + 6, 16	1
5 min. ethanol exposure	
21, X; + 1	1
21, X; + 4	2
21, X; + 12	1
21, X; + 13	2
21, X; + 14	2
21, X; + 15	1
21, X; + 16	1
21, X; + 17	2
21, X; + 18	2
21, X; + X	1
22, X; + 14, 15	1
22, X; + 15, 17	1
22, X; + 18, X	1
23, X; + 1, 3, 9	1
23, X; + 10, 11, 18	1
24, X; + 2, 4, 12, 17	1

Chromosome constitution of hypohaploid 1PN parthenogenones	Incidence
1 min. ethanol exposure	
19, X; - 12	1
19, X; - 18	1
3 min. ethanol exposure	
19, X; - 3	1
19, X; - 6	1
19, X; - 10	1
19, X; - 11	1
19, X; - 15	1
19, X; - 16	1
19, X; - 18	1
18, X; - 2, 4	1
18, X; - 2, 8	1
18, X; - 3, 18	1
18, X; - 11, 19	1
5 min. ethanol exposure	
19, X; - 3	1
19, X; - 4	1
19, X; - 5	1
19, X; - 8	2
19, X; - 10	1
19, X; - 11	1
19, X; - 12	3
19, X; - 13	1
19, X; - 15	1
19, X; - 16	1
19, X; - 18	1
18, X; - 9, 18	1
18, X; - 10, 11	1
18, - 13, X	1
Hyaluronidase-induced aneuploid 1PN parthenogenones	
18, X; - 11, 16	1
18, X; - 12, 16	1

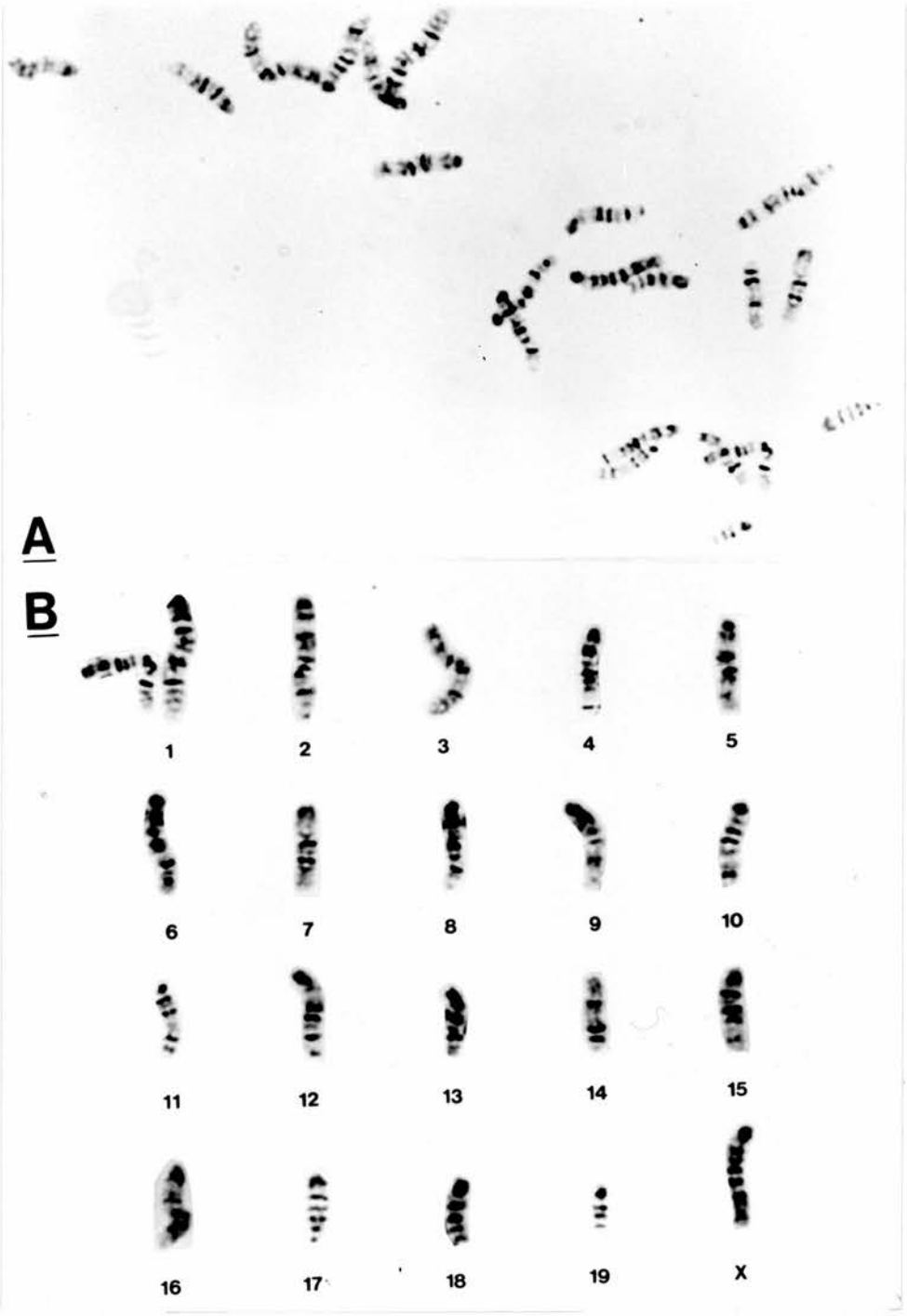


Fig 4.4 (A). Giemsa-banded, air-dried chromosome spread from a hyperhaploid (N = 21) ethanol-induced 1PN parthenogenone at metaphase of the first cleavage mitosis. (B). Karyotypic analysis demonstrates that this preparation is disomic for chromosome 1.

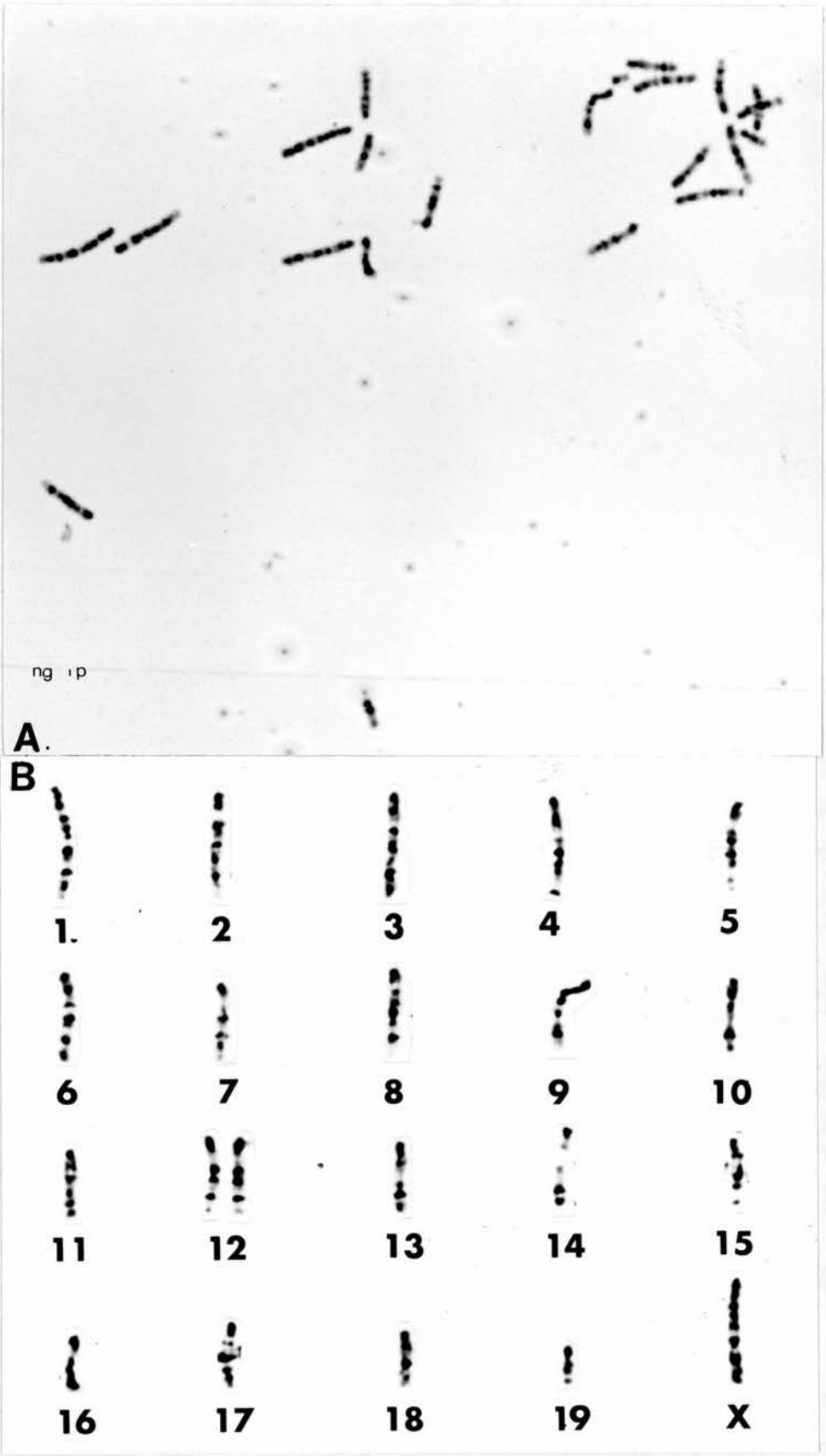


Fig 4.5 (A). Giemsa-banded, air-dried chromosome spread from a hyperhaploid (N = 21) ethanol-induced 1PN parthenogenone at metaphase of the first cleavage mitosis. (B). Karyotypic analysis demonstrates that this preparation is disomic for chromosome 12 and that chromosome 14 exhibits a break at band 14D.

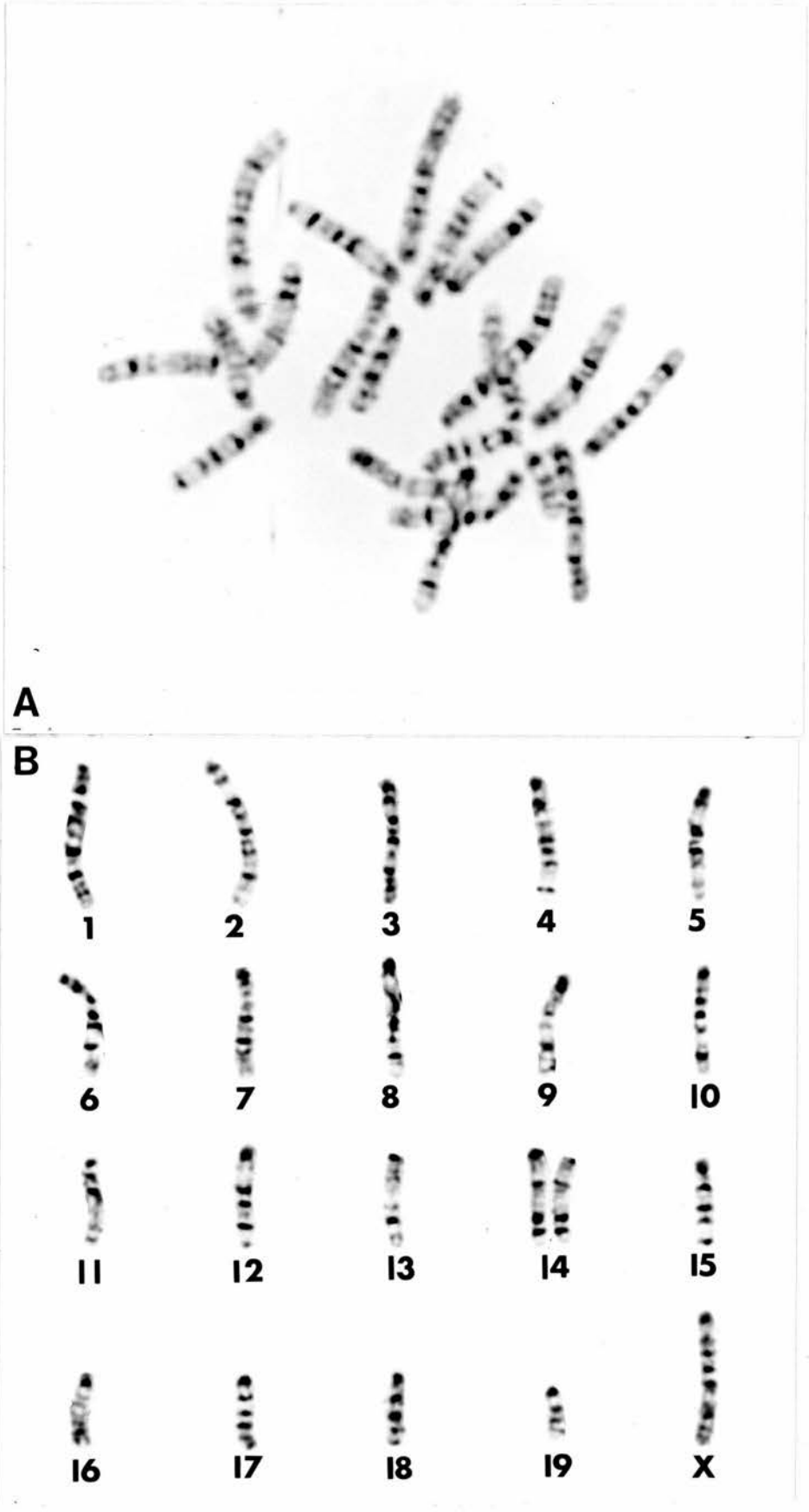


Fig 4.6 (A). Giemsa-banded, air-dried chromosome spread from a hyperhaploid (N = 21) ethanol-induced 1PN parthenogenone at metaphase of the first cleavage mitosis. (B). Karyotypic analysis demonstrates that this preparation is disomic for chromosome 14.

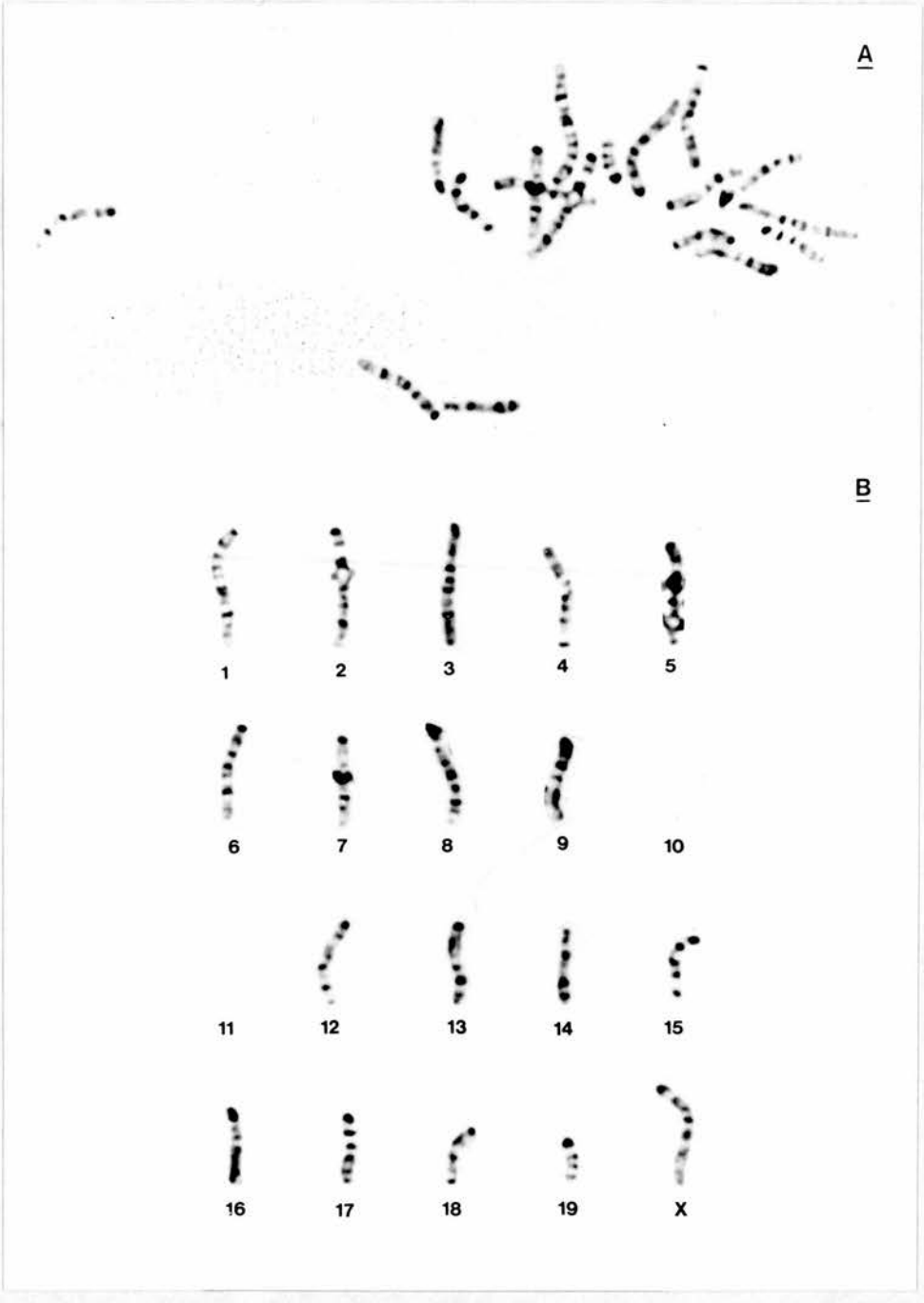


Fig. 4.7 (A). Giemsa-banded, air-dried chromosome spread from a hypohaploid (N = 18) ethanol-induced 1PN parthenogenone at metaphase of the first cleavage mitosis. (B). Karyotypic analysis demonstrates that this preparation is nullosomic for chromosomes 10 & 11.

Fig. 4.8 Giemsa-banded chromosome preparation from a hyperhaploid (N = 24) ethanol-induced 1PN parthenogenone at metaphase of the first cleavage mitosis. This preparation was found to be disomic for chromosomes 2, 4, 12, & 17.

events than expected. However, a complementary number of hypohaploid metaphase preparations nullisomic for chromosome 14 were not observed. Several chromosomes, notably chromosome 4 and the majority of the smaller chromosomes of the mouse genome (10, 11, 12, 15, 17 and 18) were observed in aneuploid metaphase preparations at a higher frequency than expected if chromosome segregation errors were completly random events. All malsegregation events that involved chromosome 17 were observed in hyperhaploid metaphase preparations, whereas chromosome 11 was more frequently associated with hypohaploidy. The remaining chromosomes 1, 2, 3, 13, 16 and X were involved in malsegregation at a frequency equivalent to that expected for random chromosome error. However, a complementary number of hypohaploid chromosome spreads that were nullisomic for chromosome 1 were not observed.

The karyotypic analysis of the G-banded metaphase chromosome preparations of hyaluronidase activated oocytes at HCG+ 20h was unable to identify the disomic chromosomes in the hyperhaploid parthenogenones. The two hypohaploid preparations that exhibited 18 chromosomes were found to be nullisomic for chromosomes (11, 16) and (12, 16).

Karyograms prepared from 57 randomly chosen metaphase

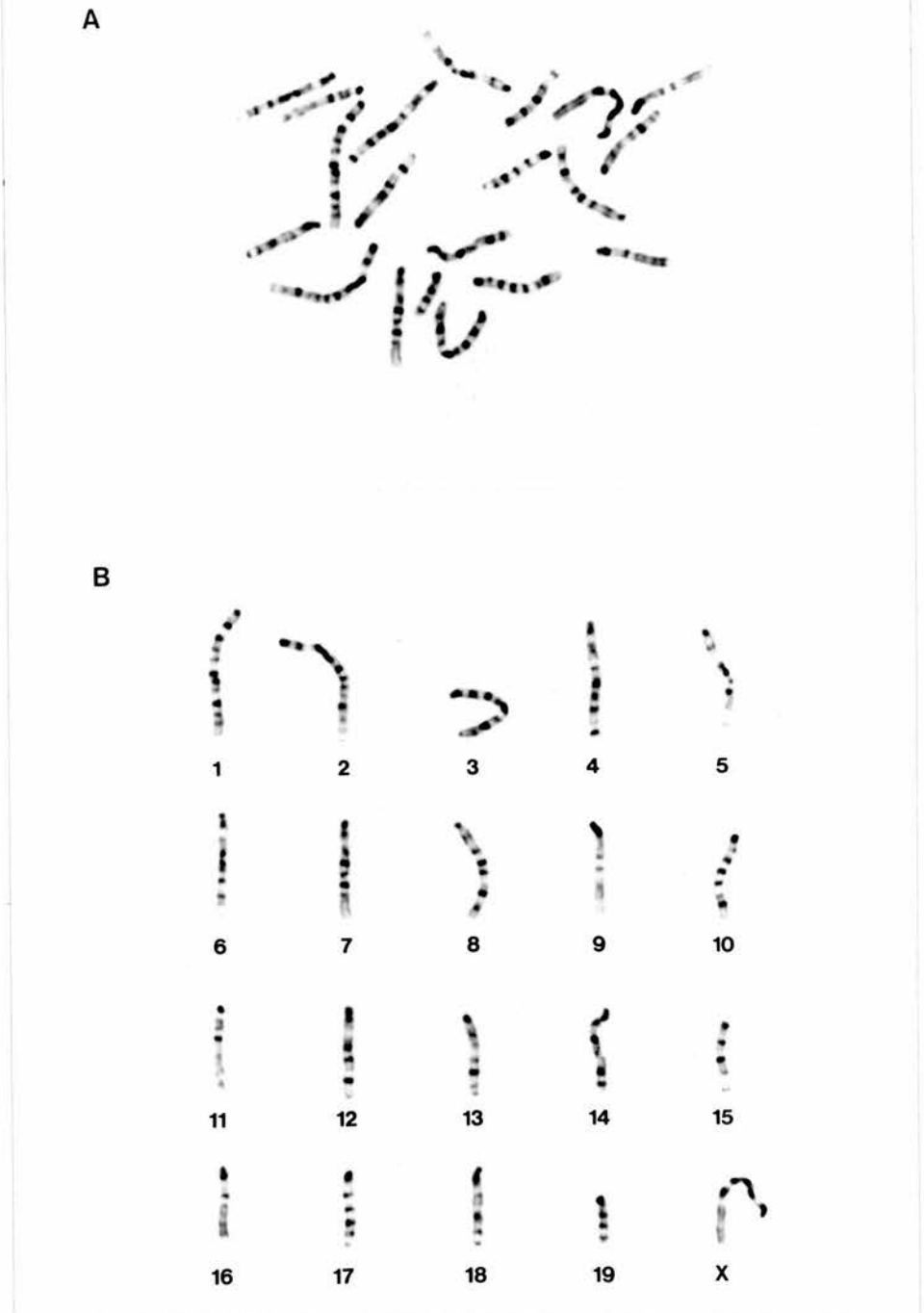
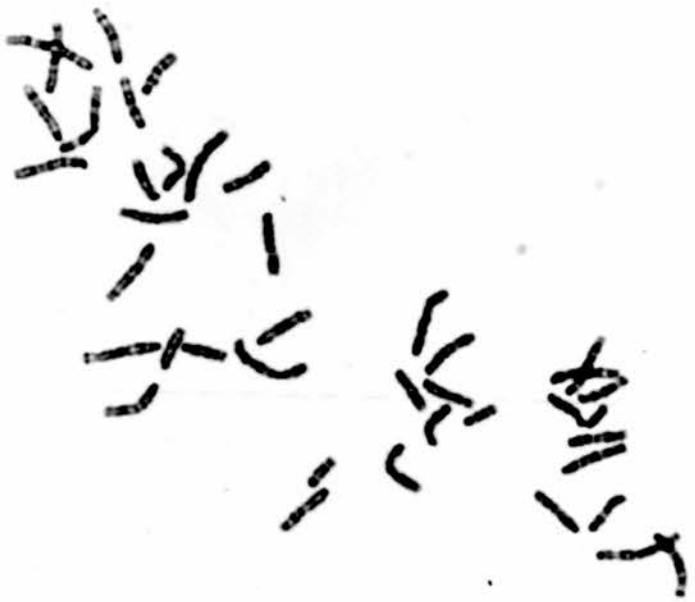


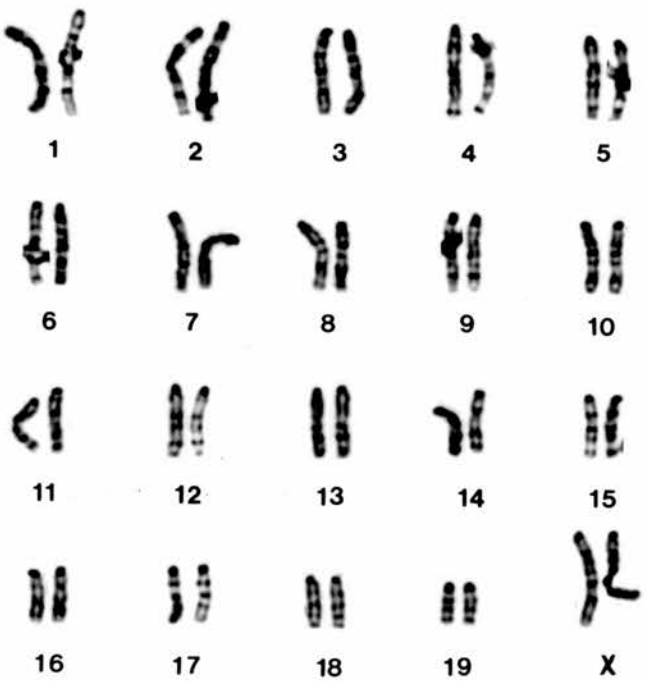
Fig. 4.9 (A). Giemsa-banded, air-dried chromosome spread from a haploid (N = 20) ethanol-induced 1PN parthenogenone at metaphase of the first cleavage mitosis. (B). Karyotyic analysis demonstrates that this preparation is both balanced and euploid.

Fig. 4.10 (A). Giemsa-banded, air-dried chromosome spread from an ethanol-induced 2PN parthenogenone at metaphase of the first cleavage mitosis. This 2PN parthenogenone has the apparent pronuclear chromosome constitution of (18 - 22). This situation could arise following ethanol-induced chromosome malsegregation during the completion of the second meiotic division. It is also equally possible that the complementary chromosome constitution of the two pronuclear sets was due to the displacement of chromosomes incurred during the fixation procedure. The pronuclear chromosome set with eighteen chromosomes also possesses both chromosomes 19. This chromosome has rarely been observed to be involved with chromosome malsegregation. This would further support the conclusion that the apparent complementary aneuploid pronuclear chromosome sets have arisen from chromosome displacement during the fixation procedure. (B). Karyotypic analysis illustrates that this 2PN parthenogenone has a balanced diploid chromosome constitution.



A

B



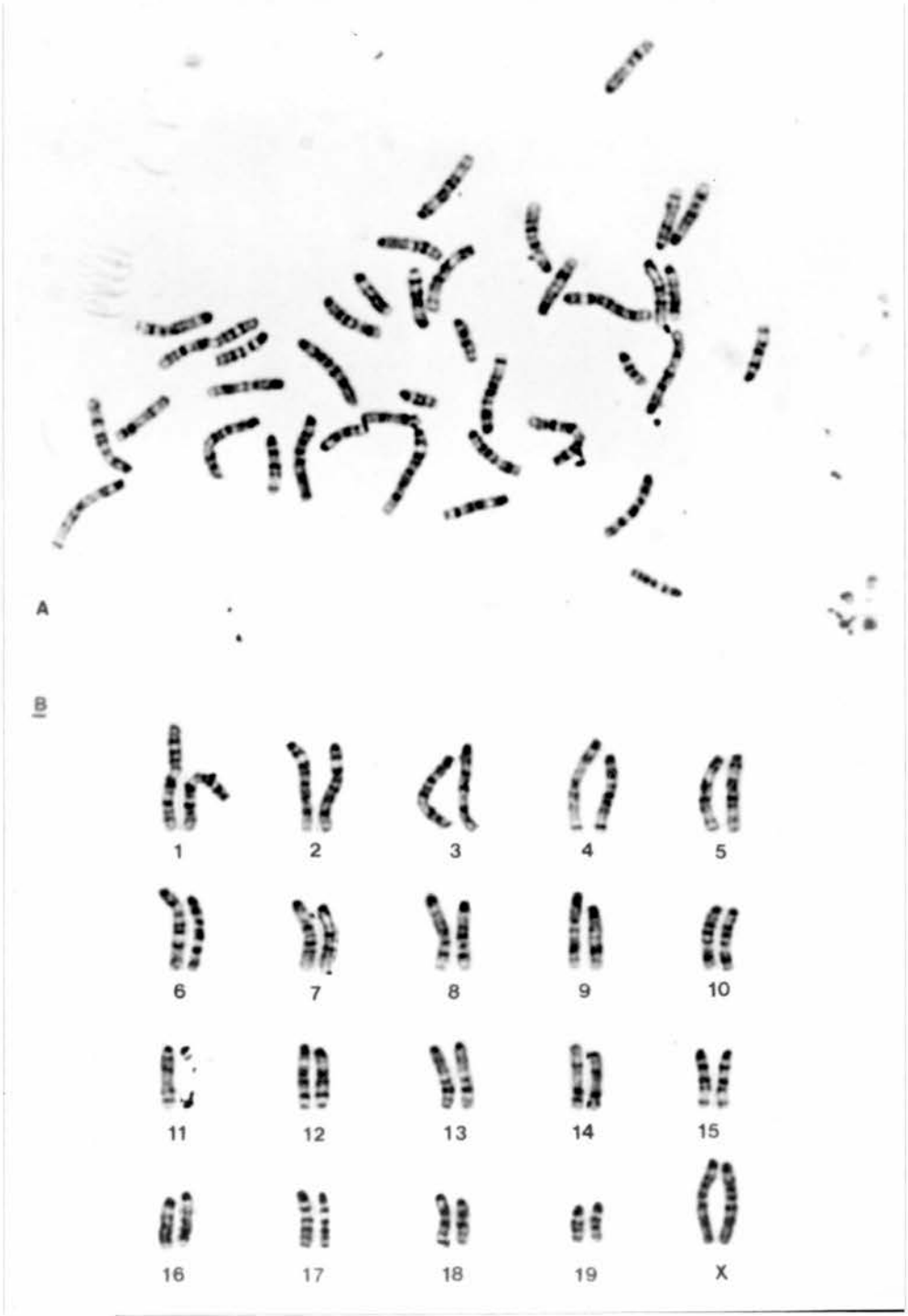


Fig. 4. 11. Giemsa banded, air-dried chromosome spread from an ethanol-induced 2PN parthenogenone. This metaphase chromosome preparation does not exhibit two distinct haploid chromosome sets. (B) Karyotypic analysis demonstrates that this 2PN parthenogenone has a balanced diploid chromosome constitution.

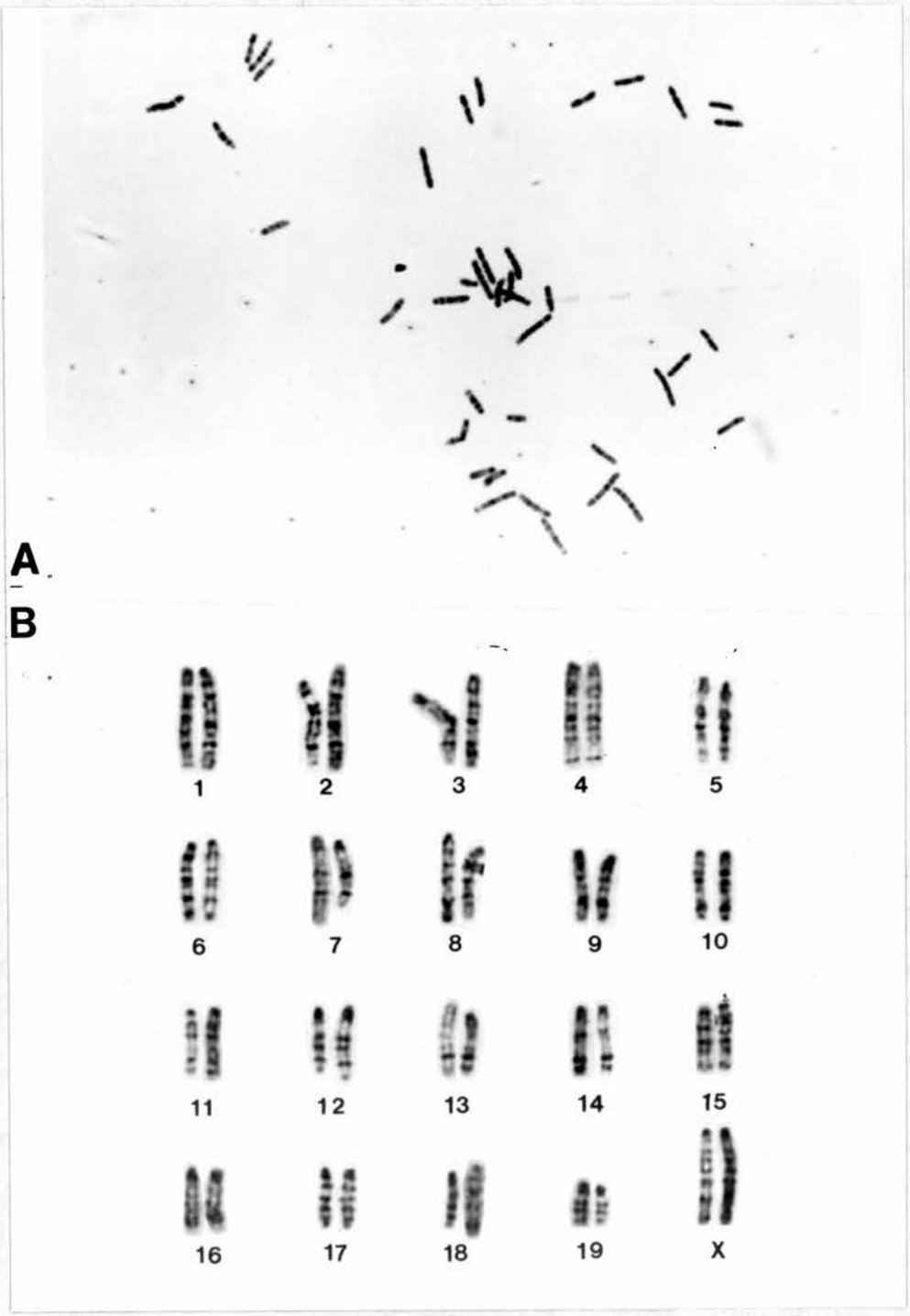


Fig. 4. 12. Giemsa-banded, air-dried chromosome spread from an ethanol-induced 2PN parthenogenone at metaphase of the first cleavage mitosis. (B). Karyotypic analysis illustrates that this 2PN parthenogenone is heterozygous for a terminal deletion at band F of chromosome 7.

spreads that exhibited the euploid number of chromosomes has established that all were genetically balanced (Fig 4.9). This indicates that double malsegregation events, in which one chromosome pair migrate to one pole while another pair migrate to the opposite pole to provide an apparantly euploid complement, either did not occur or were very rare events. These observations would also indicate that hyperhaploid parthenogenones are not reduced to the euploid number by the mechanical loss of chromosomes during the spreading procedure.

An analysis of the chromosome constitution of 44 2PN parthenogenones that initiated development following exposure to ethanol in vitro for 5 min. revealed that the majority (42) exhibited the euploid number of chromosomes at metaphase of the first cleavage mitosis. Two hypodiploid chromosome preparations exhibited 36 and 38 chromosomes, respectively, but the chromosomes that were involved in the malsegregation events could not be identified due to the poor quality of the G-bands. The chromosomes of each pronuclear set were frequently mixed at the boundary between the two metaphase groups (Figs 4.10 & Fig. 4.11) and it was not possible to identify whether displaced chromosomes were the result of segregation errors or chromosome mixing during the preparation of metaphase preparations. One 2PN parthenogenone was observed to be heterozygous for a deletion at the distal end of chromosome 7 (Fig. 4.12).

4.4 DISCUSSION

This investigation has demonstrated that the exposure of recently ovulated mouse oocytes to a dilute solution of ethanol, for either 1, 3 or 5 min., induced a high incidence of parthenogenetic activation. A significant alteration to the frequency of activation was not influenced by the duration of ethanol exposure. It appears that the period of ethanol exposure used in these studies is equal to and greater than the threshold value necessary to induce parthenogenesis, but remaining brief enough not to induce the degeneraton of the exposed oocytes. The considerable differance between the frequency of ethanol-induced activation at HCG+ 17h and that observed following hyaluronidase exposure at HCG+ 18h strongly indicates that ethanol is the more potent parthenogenetic stimulus and that the mechanisms by which these two agents initiate the resumption of the second meiotic division may be dissimilar. Numerous analyses of the electrophysiological changes in fertilised hamster oocytes have found that transient changes in the intracellular concentration of Ca^{2+} ions is one of the earliest events associated with the sperm-mediated oocyte activation (Igusa and Miyazaki, 1986; Miyazaki, et al., 1986). The exposure of cells in tissue culture to ethanol, or other anaesthetic compounds, also has the capacity to induce the efflux of calcium ions from

cytoplasmic organelles (Onfelt, 1986). However, it has been observed that the patterns of Ca^{2+}-stimulated photoemissions from ethanol activated and fertilised aequorin-permeated oocytes were dissimilar (Cuthbertson, Whittingham and Cobbold, 1981) and the Ca^{2+}-mediated electrophysiological changes in ethanol activated oocytes were not found to be homologous with those that occur during fertilisation (Eusebi and Siracusa, 1983). Ethanol-induced activation, in contrast to the events associated with fertilisaton, presumably induces a series of nonspecific electrophysiological changes that result in the parthenogenetic activation of a high proportion of exposed oocytes.

The majority of ethanol activated oocytes were observed to develop as 1PN parthenogenones. However, the proportionate incidence of this developmental class decreased as the duration of exposure to the activation stimulus increased from 1min. to 5min. This decrease in the incidence of the 1PN pathway of development was primarily associated with a significant increase in the proportionate incidence of the 2PN class of parthenogenone. These findings clearly demonstrate that the duration of ethanol exposure is capable of regulating the proportionate incidence of the four main developmental pathways of parthenogenesis. A similar decrease in the proportionate incidence of the 1PN class was observed both in the present study, and when oocytes

of increasing postovulatory age were exposed to hyaluronidase (Kaufman, 1973a; O'Neill and Kaufman, 1988; Chapter 3). The recent use of immunofluorescent localisation techniques has demonstrated that postovulatory ageing of mouse oocytes induces the dispersal of the actin filaments associated with the avillous zone of the plasma membrane and facilitates the central migration of the meiotic spindle apparatus (Webb, et al, 1986). These filaments are actively involved in the formation of the second polar body. The higher incidence of diploid parthenogenones and digynic triploid fertilised one-cell embryos that develop following postovulatory ageing are due primarily to the inability of dispersed actin filaments to organise the cytokinetic events associated with polar body formation. The proportion of ethanol activated oocytes that failed to extrude a second polar body was significantly greater than that observed following hyaluronidase activation at HCG+ 18h. This would indicate that the normal cytokinetic events associated with the extrusion of the second polar body are disrupted, or at least interfered with, following exposure to this agent. Second polar body formation has also been inhibited when activated oocytes were incubated in culture medium supplemented with either cytochalasin B (Bałakier and Tarkowski, 1976) or cytochalasin D (Chapter 9) for 3-4h following exposure to a parthenogenetic stimulus. It appears likely that under the experimental conditions employed in this study,

ethanol exposure interferes with the organisation of the actin filaments at the avillous zone of the plasma membrane to increase the proportion of activated oocytes that fail to extrude the second polar body.

In both this study and in Chapter 3, analysis of the chromosome constitution of hyaluronidase-induced 1PN parthenogenones have found that parthenogenesis itself does not significantly increase the incidence of chromosome segregation errors during the completion of the second meiotic division (O'Neill and Kaufman, 1988). In this study, a significant proportion of ethanol-induced 1PN parthenogenones were found to exhibit an aneuploid chromosome constitution at metaphase of the first cleavage mitosis. The incidence of aneuploidy was directly related to the duration of ethanol exposure at HCG+ 17h. The number of chromosomes involved in each malsegregation event was also directly proportionate to the duration of ethanol exposure. This indicates that a dose dependent relationship exists between ethanol exposure and meiotic spindle dysfunction during the early stages of oocyte activation.

In this study, the specific chromosome constitution of both normal and aneuploid ethanol-induced 1PN parthenogenones has been determined from the karyotypic analyses of G-banded first cleavage metaphase chromosome preparations. The karyotypic analysis of those

preparations that exhibited a euploid chromosome constitution established that they were also genetically balanced. The analysis of both hyperhaploid and hypohaploid metaphase preparations has established that not all chromosomes were equally predisposed to undergo malsegregation. The fact that the meiotic chromosomes of the mouse were not equally involved in aneuploidy may relate more to their individual characteristics and their relationship to the meiotic spindle rather than reflect upon any specific property of the agent to which they were exposed. The majority of the smaller chromosomes of the mouse were more frequently involved in segregation errors than other members of the genome. However, the smallest member of the genome, chromosome 19, was underrepresented in malsegregation events. G-banded metaphase preparations that were nullisomic or disomic for chromosmes 5,6,7, were also rarely observed. Furthermore, the incidence of disomy 1 and 14 was greater than the complementary nullisomic conditions. The preferential involvement of chromosomes in segregation errors was not related to their higher constituitive heterochromatin content (Yoshida and Kodama, 1983) or restricted to those which possess nucleolar organising regions (Dev, Tantravahi, Miller and Miller, 1977). Centromeric associations between disomic pairs of chromosomes in hyperhaploid metaphase preparations of the ethanol-induced 1PN parthenogenones were not observed. This would indicate that ethanol exposure did not

interfere with the microtubule-independent process of centromeric separation (Figuerora and Vig, 1983).

The majority of hypohaploid chromosome spreads exhibited either 18 or 19 chromosomes. It is generally accepted that a small proportion of these preparations arise due to the physical loss of chromosomes during the fixation procedure. Metaphase preparations that exhibited less than this number were considered to arise, as determined from their physical appearance, by chromosome scattering incurred during the fixation procedure and were not analysed in this study. For most of the chromosomes that were karyotypically identified in aneuploid preparations, the incidence of hypohaploid and hyperhaploid events were found to be similar. However, several nullisomic conditions, notably nullosomy 1, 14 and 17 were infrequently observed. The majority of hyperhaploid preparations exhibited 21 chromosomes. However, when the duration of ethanol exposure exceeded 3 minutes, hyperhaploid preparations that exhibited 22 - 24 chromosomes were obtained. These observations indicate that three mechanisms the activity of each being dependent on the duration of ethanol exposure, may effect chromosome malsegregation. The hypohaploid and hyperhaploid conditions that occur when the duration of ethanol exposure is less than 3 minutes may be induced by nondisjunctional events (producing either hypohaploid or hyperhaploid parthenogenones) or by anaphase lagging.

This latter process is believed to be associated with a higher incidence of chromosome loss. These mechanisms may continue to function when the duration of ethanol exposure exceeds 3 minutes. The higher incidence of aneuploidy, and the induction of 1PN parthenogenones that exhibit several pairs of disomic chromosomes, may arise when ethanol significantly interferes with the structure of the meiotic spindle apparatus and its ability to effect chromosome movement. Such events would not be representative of either nondisjunction or anaphase lagging but would be due to the inhibition of chromosome movement.

The cytogenetic analysis of the 1PN parthenogenones details only the chromosome malsegregation events that occur in the activated oocytes that have successfully extruded a second polar body. Parthenogenones that have retained both genomic products of the second meiotic division most frequently develop as 2PN parthenogenones, possess two haploid sets of chromosomes and exhibit a total chromosome number of 40. Ethanol also has the potential to induce chromosome malsegregation during the completion of the second meiotic division in this class of parthenogenone. Parthenogenones in which malsegregation had occurred would develop two haploid pronuclei with complementary hypohaploid and hyperhaploid chromosome numbers. These malsegregation events and their influence on the chromosome constitution of both 1PN and

2PN parthenogenones are represented in Fig. 4.3. In these studies it was not possible to distinguish between those parthenogenones that exhibited metaphase chromosome spreads with complementary aneuploid chromosome sets as a result of ethanol-induced chromosome malsegregation and those in which chromosome displacement was induced during the fixation and spreading of the parthenogenone. The homologous metaphase chromosomes of each pronuclear set were not observed to exhibit differing degrees of chromosome contraction. In the metaphase chromosome spreads prepared from fertilised one-cell embryos (Chapter 7) the sperm-derived chromosomes can be easily recognised as they exhibit a lesser degree of contraction than the homologous oocyte-derived chromosomes. This observation indicates that the attenuated morphology of the sperm-derived chromosomes in fertilised one-cell embryos at metaphase of the first cleavage mitosis may be related more to their inherent molecular constitution than the differential activity of oocyte-derived cytoplasmic "factors".

The regulation of microtubule polymerisation has been found to be a Ca^{2+}-dependent process (Marcum, Dedman, Brinkley and Means, 1978) and localised micromolar alterations in the concentration of this ion regulate chromosome movement and segregation in both cultured PtK1 cells and sea urchin oocytes during the completion of the second meiotic division (Izant, 1983; Eisen and Reynolds,

1985). The localised alterations to the intracellular concentration of calcium ions is effected by their efflux primarily from the endoplasmic reticulum associated with the spindle apparatus (Kiehart, 1981). Compounds that have the ability to induce anaesthesia are believed to either exert their influence by the direct physical alteration of specific membrane bound receptor proteins (Franks and Lieb, 1982) or indirectly, by their ability to induce dysfunction of membrane bound proteins as a result of changes to the fluidity of plasma membranes (Miller, Firestone and Forman, 1987). These authors have found that exposed cells exhibit a net increase in the free intracellular concentration of calcium ions due to the efflux of this ion from cytoplasmic organelles (Hoek, 1987). However, analyses of the kinetics of calcium ion efflux from the sarcoplasmic reticulum of ethanol exposed rat muscle in vitro have found that the ability of these organelles to regulate the sequestration of calcium ions following ethanol exposure is disrupted. This was found to induce a net increase in the intracellular calcium concentration (Ohnishi, Flick and Rubin, 1984; Ohnishi, 1987).

The segregation of chromosomes in ethanol activated oocytes is observed several minutes after the brief exposure to this agent (Chapter 8; O'Neill and Kaufman, 1989a). It is unlikely that aneuploidy is induced by the direct interaction of this agent with the individual

microtubulues of the meiotic spindle apparatus. It has been proposed that one of the initial steps in the induction of segregation errors may be the displacement of chromosomes from the meiotic spindle (Ford & Roberts, 1983a). Cytogenetic analyses of human lymphocyte cultures has indicated that this phenomenon more frequently involved the smaller chromosomes (Ford & Lester, 1982) and that the incidence of these events increased when the pH (Ford & Roberts, 1983b) or the calcium concentration of the culture medium was altered prior to analysis (Ford & Roberts, 1984). Under normal conditions, the tendency towards displacement is reduced by the "stabilizing" activity of the microtubular components of the spindle. Ethanol-induced dysfunction of the processes that regulate the release and sequestration of calcium ions within the locality of the meiotic spindle apparatus may decrease the chances of the latter occurring and predispose a proportion of the activated oocytes to chromosome malsegregation.

CHAPTER 5

ETHANOL- AND AVERTIN-INDUCED PARTHENOGENETIC ACTIVATION in vivo

CONTENTS

5.1 Introduction

5.2 Methods

5.3 Results

5.3.1 The incidence of ethanol-induced parthenogenetic activation *in vivo*

5.3.2 The incidence of Avertin-induced parthenogenetic activation *in vivo*

5.3.3 Chromosome constitution of ethanol- and Avertin-induced parthenogenones at metaphase of the first cleavage mitosis.

5.4 Discussion

5.1 INTRODUCTION

A number of compounds, other than ethanol, that have the potential to induce anaesthesia, such as nitrous oxide, ether or Nembutal, also share the ability to induce parthenogenetic activation *in vivo* when female rodents are exposed to these agents several hours after ovulation has occurred (reviewed Kaufman, 1983c). The development of haploid parthenogenones to the egg cylinder stage has been achieved following the exposure of postovulatory aged mouse oocytes to Avertin anaesthesia in vivo (Kaufman, 1975a). Parthenogenesis has also been induced *in vivo* when postovulatory aged mouse oocytes were exposed to a dilute ethanol solution administered to female mice by intraperitoneal injection (Dyban and Khozhai, 1980; Kaufman, 1983c). The majority of *in vivo* ethanol and Avertin activated oocytes have been reported to develop as 1PN parthenogenones. The electrical stimulation of the oviduct *in situ* is the only other reliable stimulus that has the potential to induce parthenogenetic activation *in vivo* in mice (Tarkowski, Witkowska and Nowicka, 1970). This parthenogenetic stimulus inhibits the extrusion of the second polar body and the majority of activated oocytes develop as 2PN parthenogenones.

The preceeding cytogenetic study of *in vitro*

ethanol-induced parthenogenones has confirmed that a significant proportion exhibited an aneuploid chromosome constitution at metaphase of the first cleavage mitosis (Kaufman, 1982; O'Neill and Kaufman, 1989a; Chapter 4). Analysis of the chromosome constitution of fertilised one-cell embryos, isolated from female mice that received an intragastric injection of dilute ethanol shortly before or at the time of conception, has also found that a significant proportion exhibited numerical chromosomal anomalies (Kaufman, 1983b; Kaufman and Bain, 1984; O'Neill and Kaufman, 1987a). However, it has yet to be established whether ethanol-induced activation in vivo significantly increases the incidence of 1PN parthenogenones that possess an aneuploid chromosome constitution. Similarly, the influence of Avertin exposure in vivo on the proportionate incidence of the pathways of parthenogenesis and the capacity of this agent to interfere with chromosome segregation during the completion of the second meiotic division has still to be investigated.

Aims of investigation

The aims of this study are to examine the potential of both ethanol and Avertin to induce parthenogenetic activation in vivo when administered by intraperitoneal injection to female mice several hours after ovulation has occurred. The subsequent analysis of the chromosome constitution of the 1PN class of parthenogenone at

at metaphase of the first cleavage mitosis can determine if exposure to these parthenogenetic stimuli has the capacity to induce meiotic chromosome segregation errors.

Furthermore, the identification of a parthenogenetic stimulus that has the potential to induce both a high incidence of activation and aneuploidy in vivo would facilitate an analysis of the developmental fate of euploid and aneuploid parthenogenones without the need to perform oviduct transfers.

The exposure of human oocytes to ethanol normally occurs following the consumption of beers, wines or spirits. In this study, the capacity of an intragastric injection of a dilute ethanol solution to induce the parthenogenetic activation of mouse oocytes is also investigated. This can serve as a model to examine whether human alcohol consumption during the postovulatory period may have the potential to induce the parthenogenetic activation of human oocytes in vivo.

5.2 METHODS

Ethanol- and Avertin-induced parthenogenetic activation in vivo

F1 hybrid female mice were superovulated as described in Chapter 2.2. The female mice received either the standard (x1.0 Std.), x0.5 Std. or x1.5 Std. dose of Avertin anaesthetic by intraperitoneal injection at HCG+ 18h as described in Chapter 2.4.4. An additional group of F1 hybrid female mice were administered either 0.35ml or 0.5ml of a 25% solution of ethanol in Dulbecco's PBS by intraperitoneal injection at HCG+ 18h. In the control series, the F1 hybrid female mice received an intraperitoneal injection of an appropriate volume of Dulbecco's PBS at HCG+ 18h.

A third group of F1 hybrid female mice received an intragastric injection of 1ml of a 12.5% solution of ethanol in distilled water at either HCG+ 18 or +20h as decribed in Chapter 2.4.6.

Preparation of chromosome spreads at metaphase of the first cleavage mitosis

At 4h-6h after the administration of the parthenogenetic stimuli, the ethanol and Avertin exposed oocytes were isolated from the F1 hybrid female mice as described in Chapter 2.4.3 and 2.4.4. After a further 6h in culture,

the 1PN parthenogenones were transferred to M16 supplemented with 1ug/ml of Colcemid to arrest development at metaphase of the first cleavage mitosis. Metaphase chromosome spreads were prepared as described in Chapter 2.6.2

5.3 RESULTS

5.3.1 The incidence of ethanol-induced parthenogenetic activation in vivo

The incidence of parthenogenetic activation observed at 6 - 8h after the intraperitoneal injection of either 0.35ml (Group 1) or 0.5ml (Group 2) of a 25% solution of ethanol in Dulbecco's PBS to superovulated F1 hybrid female mice at HCG+ 18h is presented in Table 5.1. The higher frequency of activation (44.1%) was obtained following the administration of 0.5ml of the 25% ethanol solution. Although a lower frequency of activation (36.1%) was observed in Group 1, the proportionate incidence of the 1PN class of parthenogenone (95.5%) was higher than that observed in Group 2 (80.6%). This is represented in Fig. 5.1. However, the proportionate incidence of the 1PN class in these two groups was not found to differ significantly ($X^2 = 2.7$; $P = 0.1$). The intraperitoneal injection of 0.35ml of Dulbecco's PBS (control, Table 5.1) to superovulated F1 hybrid female mice at HCG+ 18h was not observed to induce parthenogenetic activation.

The intragastric injection of 1ml of a 12.5% (v/v) solution of ethanol to superovulated F1 hybrid females at HCG+ 18h or HCG +20h did not induce parthenogenetic activation.

TABLE 5·1

The proportionate incidence of the four classes of parthenogenone observed following the i.p. or i.g. injection of dilute ethanol to female F1 mice at either HCG +18h or 20h.

Group	Parthenogenetic Stimulus	Number of ovulated oocytes	Number of activated oocytes	Class of Parthenogenone				Activation frequency (%)
				1PN	2PN	IC	1PND	
1	0·35ml of 25% ethanol i.p.	310	112	107	5	0	0	36·1
2	0·50ml of 25% ethanol i.p.	152	67	54	9	4	0	44·1
3	0·35ml of PBS (control) i.p.	64	0	0	0	0	0	0
4	1·0ml of 12·5% ethanol i.g. at HCG+ 18h	48	0	0	0	0	0	0
5	1·0ml of 12·5% ethanol i.g. at HCG+ 20h	71	0	0	0	0	0	0

The activation frequency in Group 1 is not significantly different to that in Group 2 $X^2 = 2·72$, $P = 0·1$

The proportionate incidence of the 1PN class in Group 1 is significantly different to that in Group 2. $X^2 = 10·5$, $P < 0·01$.

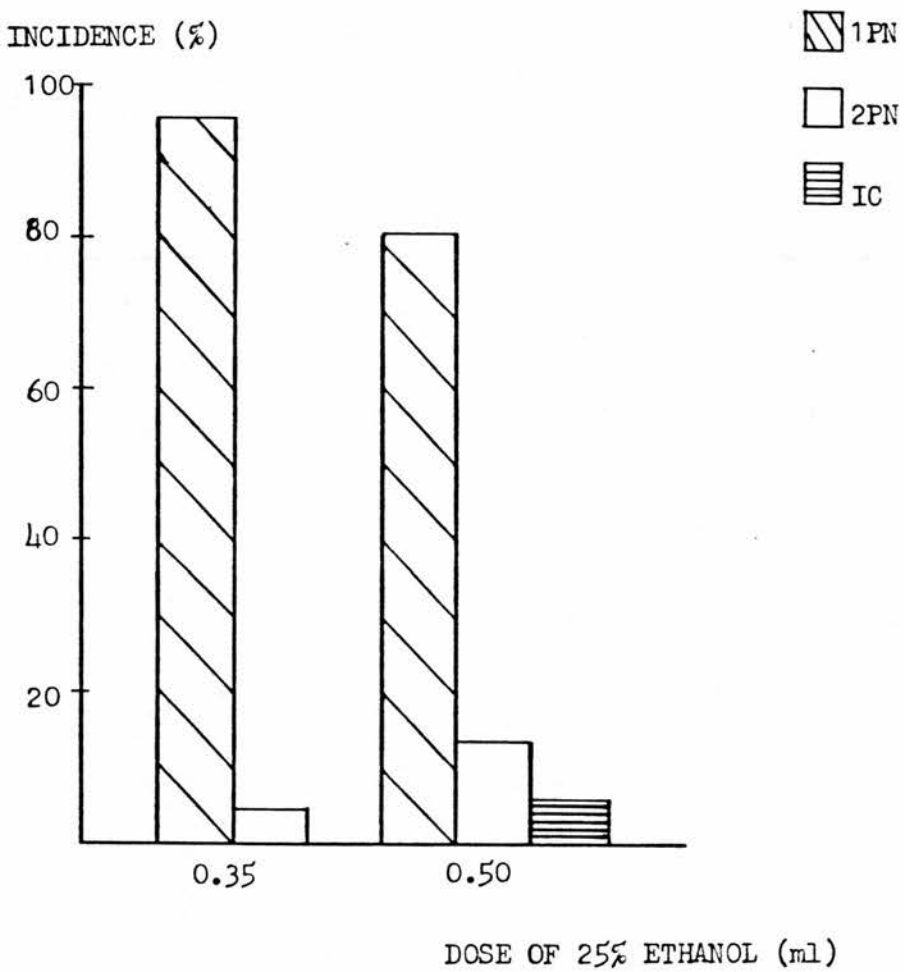


Fig. 5.1 The proportionte incidence of the four pathways of parthenogenetic development observed following the in vivo exposure of F1 hybrid oocytes to either 0.35ml or 0.50ml of a 25% ethanol solution at HCG+ 18h.

5.3.2 The incidence of Avertin-induced parthenogenetic activation in vivo

The frequency of parthenogenetic activation observed at 6 - 8h after the intraperitoneal injection of a standard (x1.0 Std.), x0.5 Std. or x1.5 Std. dose of Avertin to superovulated F1 hybrid female mice at HCG+ 18h is presented in Table 5.2. The injection of 0.4ml of Dulbecco's PBS (control) to F1 hybrid female mice at HCG+ 18h was not observed to induce parthenogenetic activation (Table 5.2). The highest frequency of activation was observed following the administration of a x0.5 Std. dose of Avertin (62.3%) and 88.1% of the activated oocytes developed as 1PN parthenogenones. The x1.0 Std dose of Avertin induced a lower frequency of activation (54.8%), decreased the proportionate incidence of the 2PN and IC pathways from 11.9% (Group 1) to 7.8% (see Fig. 5.2) and resulted in the necrotic degeneration of 18.8% of the exposed oocytes. The administration of the x1.5 dose of Avertin resulted in the degeneration of the majority of the exposed oocytes (72.0%) and exhibited the lowest frequency of activation (20.6%). All parthenogenones in this group developed as 1PN parthenogenones.

5.3.3 The chromosome constitution of ethanol- and Avertin-induced 1PN parthenogenones

Cytogenetic analysis of the 1PN parthenogenones that

TABLE 5·2

The proportionate incidence of the four classes of parthenogenone observed following the intraperitoneal injection of Avertin anaesthesia to F1 female mice at HCG+ 18h.

Group	Avertin Dose*	Ovulated oocytes	Degenerated oocytes	Nonactivated oocytes	Activated oocytes	Class of Parthenogenone				Activation frequency (%)
						1PN	2PN	IC	1PND	
1	X 0·5 Std	175	5	61	109	96	13	0	0	62·3
2	X 1·0 Std	303	57	80	166	153	11	2	0	54·8
3	X 1·5 Std	107	77	8	22	22	0	0	0	20·6
4	0·4ml PBS control	67	0	67	0	0	0	0	0	0

* The standard dose of Avertin anaesthesia is 0·02ml/g bwt. of a 1·2% solution of Avertin in PBS

The activation frequency in Group 1 is not significantly different to that observed in Group 2. $X^2 = 1·28$, $P = 0·3 - 0·2$.

The proportionate incidence of the 1PN class in Group 1 is not significantly different to that observed in Group 2. $X^2 = 2·6$, $P = 0·2 - 0·1$.

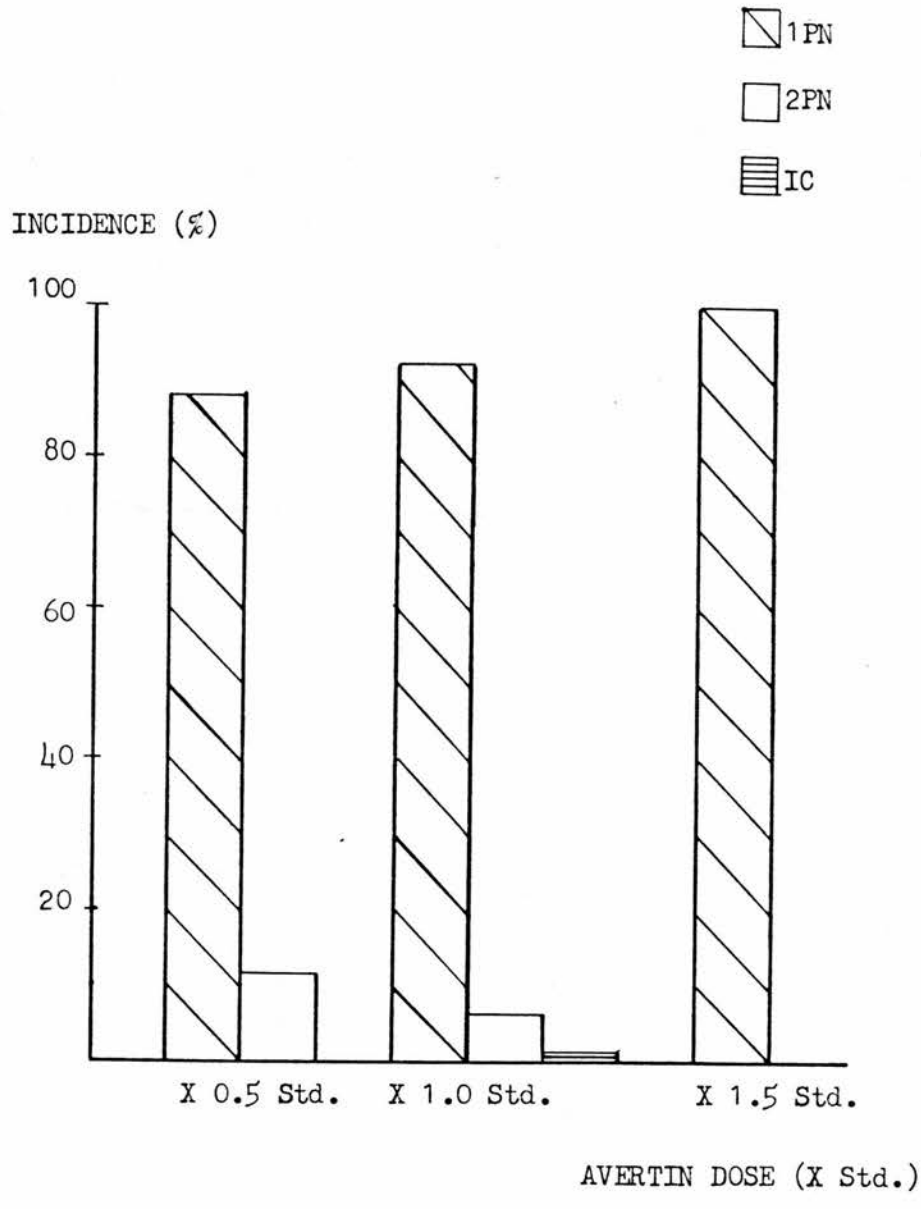


Fig. 5.2 The proportionate incidence of the four pathways of parthenogenetic development following the in vivo exposure of ovulated F1 hybrid oocytes to either a x0.5 Std., x1.0 Std. or x1.5 Std. dose of Avertin at HCG+ 18h.

TABLE 5·3

The chromosome constitution of 1PN parthenogenones at metaphase of the first cleavage mitosis following the exposure of F1 hybrid oocytes to either ethanol or Avertin at HCG+ 18h in vivo.

Group	Parthenogenetic stimulus	Number of chromosome preparations	Number of non-analysable preparations	Chromosome constitution 19	20	21	22	Aneuploid frequency (%)
1	0·35ml of 25% ethanol i.p.	84	7	3	72	2	0	6·4
2	0·50ml of 25% ethanol i.p.	45	6	2	37	0	0	5·1
3	X 0·5 Std. Avertin dose	70	12	1	56	0	1	3·4
4	X 1·0 Std. Avertin dose	133	12	2	117	2	0	3·3
5	X 1·5 Std. Avertin dose*	22	2	0	20	0	0	0·0

* See Table 5·2

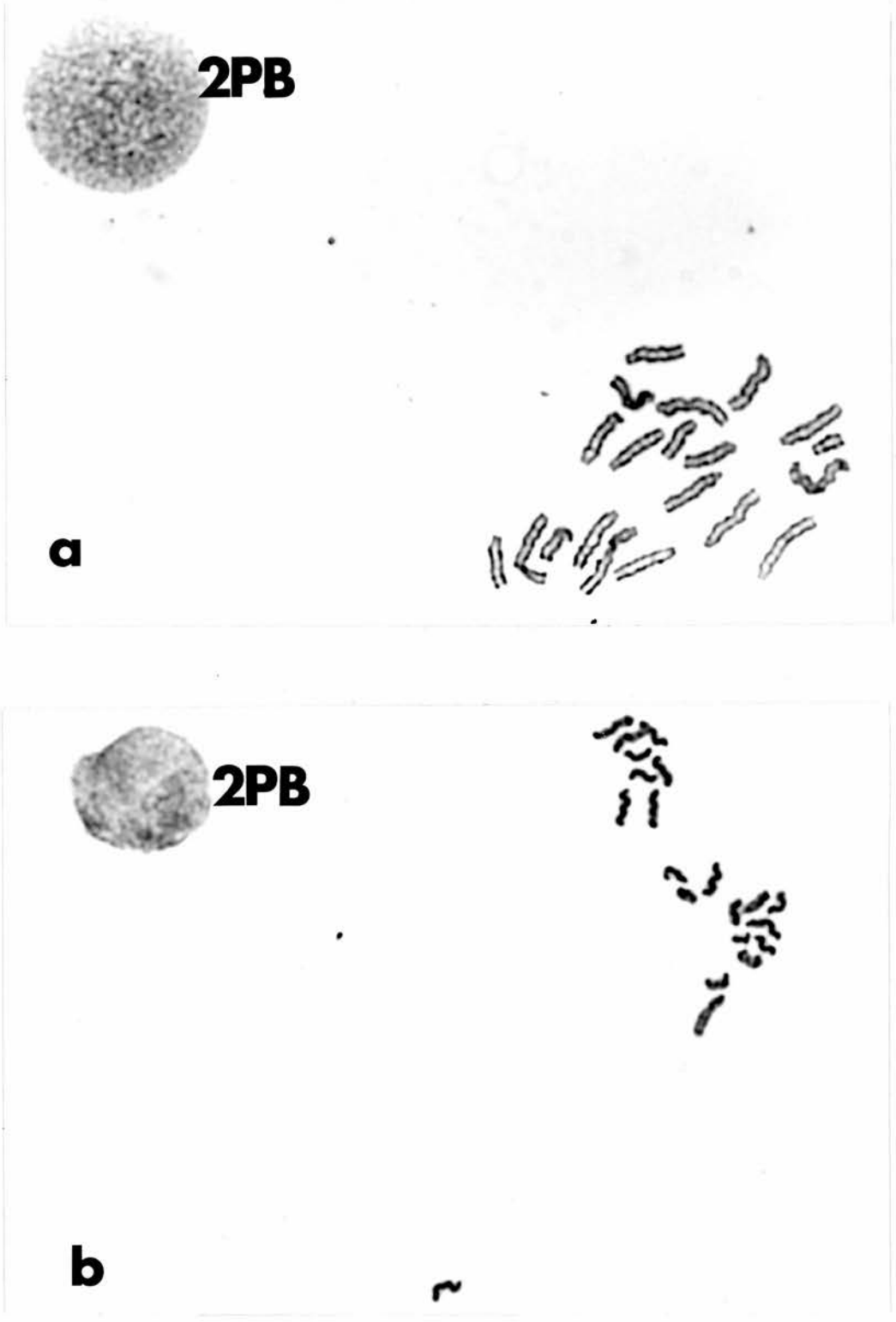


Fig. 5.3 Representative air-dried chromosome spreads of in vivo ethanol-induced 1PN parthenogenones at metaphase of the first cleavage mitosis that exhibit (a) 20 chromosomes and (b) 21 chromosomes. The nucleus of the second polar body (2PB) can be seen in both preparations. Giemsa stain.

developed following the intraperitoneal injection of 0.35ml of a 25% ethanol solution in Dulbecco's PBS to F1 hybrid females at HCG+ 18h has demonstrated that 6.5% exhibited an aneuploid chromosome constitution (Table 5.3). However, this value is not significantly different to the incidence of aneuploidy observed following the hyaluronidase-induced activation of oocytes at HCG+ 18h in vitro (Chapter 3.4.1), X^2 = 1.19, P = 0.3-0.2. The intraperitoneal injection of 0.5ml of the ethanol solution decreased the incidence of aneuploidy to 5.1% and both examples of aneuploidy were due to the loss of one chromosome. Representative air-dried euploid and hyperhaploid chromosomes spreads of in vivo ethanol-induced 1PN parthenogenones are illustrated in Fig. 5.3.

Analysis of the chromosome constitution of the 1PN parthenogenones that developed following the in vivo exposure of ovulated oocytes to x0.5 Std., x1.0 Std. and x1.5 Std. doses of Avertin at HCG+ 18h has revealed that the incidence of aneuploidy was 3.4%, 3.3% and 0.0% respectively (Table 5.3). The incidence of aneuploidy in Group 3 and 4 is not significantly different to that observed in the metaphase chromosome preparations of the hyaluronidase activated 1PN parthenogenones in Chapter 3 (X^2 = 0.08, P = 0.9-0.8).

5.4 Discussion

The incidence of ethanol-induced activation *in vivo* in this study was comparable to that achieved by Dyban and Khozhai (1980). It is, however, considerably lower than the incidence of parthenogenetic activation observed when ovulated oocytes are exposed to a dilute solution of ethanol *in vitro* (Kaufman, 1982; O'Neill and Kaufman, 1989a, Chapter 4.). In this study, the incidence of parthenogenetic activation induced following the intraperitoneal injection of either a standard or x0.5 standard dose of Avertin anaesthetic was at least twice that previously reported by Kaufman (1975a).

The highest frequency of parthenogenetic activation in this study was observed following the administration of a x0.5 standard dose of Avertin. However, a lower frequency of activation as well as the degeneration of a significant proportion of the exposed oocytes and a decrease in the proportionate incidence of the 2PN class of parthenogenone was induced following the administration of the standard dose of Avertin to female mice. The administration of a x1.5 Std. dose killed the majority of the exposed ovulated oocytes, but all activated oocytes developed as 1PN parthenogenones. This study found has found that the optimum conditions for Avertin-induced parthenogenetic activation required, at

most, the administration of a x0.5 standard dose of this agent. The overall incidence of ethanol-induced parthenogenesis was lower than that achieved following Avertin exposure. The majority of ethanol activated oocytes also developed as 1PN parthenogenones but the proportionate incidence of the 2PN and IC pathways of development was increased when the administered dose of ethanol was raised from 0.35ml to 0.5ml. These studies have clearly indicated that the administered doses of ethanol and the maximum dose of Avertin were toxic to a large proportion of the exposed oocytes. The present study demonstrates that only the x0.5 Std. dose of Avertin was a reliable in vivo parthenogenetic stimulus Although both ethanol and Avertin induce anaesthesia and parthenogenetic activation, the evidence from this study indicates that it is not valid to compare the activity of these two agents in vivo as the developmental response of the exposed oocytes has been observed to differ considerably.

In this study, the intragastric injection of a 12.5% ethanol solution to female mice at either HCG+ 18h or HCG+ 20h failed to induce parthenogenetic activation. Kaufman (1983c) has reported that the oral administration of 1ml of a 10% ethanol solution to F1 hybrid female mice several hours after ovulation had occurred induced a low (10.9%) incidence of parthenogenesis. Dyban and Baranov (1987) have also observed a comparable incidence of

parthenogenesis following the intragastric injection of ethanol to F1 hybrid female mice (14.3%), but these levels of activation were only achieved when the administered dose of ethanol (0.5ml/25%) raised the blood ethanol level approximate with the LD_{50} value for the mouse. The results from both this study and those cited above indicate that parthenogenesis cannot be induced effectively by the intragastric administration of ethanol. This strongly indicates that human alcohol consumption during the postovulatory period is unlikely to induce the parthenogenetic development of human oocytes. However, several cytogenetic studies of early human development have encountered parthenogenetically activated oocytes at a low frequency (Angell, et al., 1979; Plachot, et al., 1987, 1988). It is believed that the exposure of human ovulated oocytes to inhalational anaesthetics, as occurred during laparoscopy in the studies cited above, was the factor most responsible for the initiation of human parthenogenesis (see, Kaufman, 1983a).

The cytogenetic analysis of the in vivo ethanol-induced 1PN parthenogenones at metaphase of the first cleavage mitosis has found that the proportion that exhibited numerical chromosome anomalies did not differ significantly to that observed in the metaphase chromosome preparations of hyaluronidase-induced 1PN parthenogenones. Similarly, the proportion of

Avertin-induced 1PN parthenogenones that exhibited an aneuploid chromosome constitution was also not significantly different from that observed following hyaluronidase-induced activation (O'Neill and Kaufman, 1988, Chapter 3).

These findings initially appear to conflict with those of the previous study in which it was observed that a significant proportion of ethanol-induced 1PN parthenogenones exhibited aneuploidy (O'Neill and Kaufman, 1989a, Chapter 4). However, the high proportion of degenerated and non-activated oocytes observed following the exposure to these agents strongly indicates that they are toxic to the oocyte under the conditions employed. The incidence of aneuploidy is, therfore, only a reflection of the chromosome segregation errors that occurred in oocytes that retained the ability to initiate parthenogenesis and survived exposure to these noxious conditions. This study has clearly demonstrated that ethanol- and Averin-induced parthenogenetic activation does not provide a model system by which euploid and aneuploid haploid embryos could be produced <u>in vivo</u>.

CHAPTER 6

THE OVULATION AND FERTILISATION OF PRIMARY AND SECONDARY OOCYTES IN LT/Sv MICE.

CONTENTS

6.1 Introduction

6.2 Methods

6.3 Results

6.3.1 Cytogenetic analysis of ovulated oocytes isolated from superovulated LT/Sv mice

6.3.2 Cytogenetic analysis of LT/Sv fertilised one-cell embryos

6.3.3 Analysis of the litter size of the LT/Sv breeding colony

6.3.4 Analysis of the spontaneous Robertsonian translocation (6.13) of LT/Sv mice

6.4 Discussion

6.1 INTRODUCTION

The spontaneous parthenogenetic activation of ovulated oocytes *in vivo* is an extremely rare phenomenon in mammals, occurring only in the LT/Sv strain of the mouse with any degree of regularity (see Stevens & Varnum, 1974). Pre- and early post-implantation stages of parthenogenetic development have been recognised both within the ovary, and within the reproductive tract. A proportion of the ovulated LT/Sv oocytes may also activate spontaneously following their incubation in tissue culture medium. Several investigators have indicated that the spontaneous activation of ovarian oocytes in mature females is causally related to the high incidence of ovarian teratomas that have been observed in this strain. (Stevens and Varnum, 1974; Stevens, 1975; Eppig, 1978; reviewed by Kaufman, 1983c).

The majority of spontaneously activated oocytes develop a single pronucleus following the extrusion of a polar body (Kaufman, 1983c). Although this pathway is usually associated with the development of single-pronuclear haploid parthenogenones, the chromosome spreads prepared from activated oocytes that had progressed to metaphase of the first cleavage mitosis, indicated that both haploid and diploid parthenogenones had developed. The latter observation was particularly surprising, as the presence of a polar body is almost invariably associated

with haploid development. Furthermore, the diploid chromosome preparations exhibited evidence of "homologous chromosome pairing". In some of the chromosome preparations, clear evidence of terminal end-to-end associations were still visible at the centromere regions.

A subsequent cytogenetic and nucleardensitometric study (Kaufman and Howlett, 1986) revealed that LT/Sv females ovulated both primary and secondary oocytes. Two populations of single-pronuclear activated oocytes could be readily distinguished on account of the significant difference between their nuclear diameters. Those with a large pronuclear volume were invariably found to be diploid (Fig. 2.2), while those parthenogenones in which the pronuclear volume was significantly smaller were invariably found to be haploid (Fig 2.1). The activation of primary oocytes resulted in the separation of bivalents, the extrusion of a polar body and the subsequent formation of a single diploid pronucleus. The centromeric separation of meiotic chromosomes does not occur until anaphase of the second meiotic division. The "homologous pairing" of chromosomes observed at metaphase of the first cleavage mitosis is due to the absence of the centromeric division of chromosomes prior to the formation of the diploid pronucleus.

Aims of investigation

The purpose of this study is to determine whether the ovulated primary and secondary oocytes of the LT/Sv strain exhibit an equal competence to undergo monospermic fertilisation. The fertilisation of primary oocytes would result in the development of a unique class of digynic triploid embryo that exhibits a single female diploid pronucleus and a haploid male-derived pronucleus during the first cell cycle. This study also identifies some of the factors that regulate the ovulation of primary oocytes in this strain of the mouse.

6.2 METHODS

Isolation and cytogenetic analysis of the ovulated oocytes and fertilised one cell embryos of LT/Sv mice.

Eight- to ten-week-old female LT/Sv mice were superovulated as described in Chapter 2.2. After the HCG injection the females were individually caged with F1 hybrid males. Successful matings were identified early the next morning by the presence of a vaginal plug. An additional group of young mature naturally cycling female LT/Sv female mice were caged with F1 males for 1-3 days and were checked early each morning for the presence of a vaginal plug. This was taken as evidence that mating had occurred following natural ovulation. The fertilised oocytes from both groups of female LT/Sv mice were isolated and transferred to microdrops of M16 medium equilibrated with 5% CO_2 in air at 37°C as decribed in Chapter 2.5.

Fertilised one-cell embryos were recognised by the presence of a polar body and two pronuclei. A small number of oocytes had activated spontaneously and exhibited a polar body and a single pronucleus. Oocytes that were neither fertilised nor activated did not exhibit pronuclei. The unfertilised oocytes were isolated from culture, and chromosome spreads were prepared by the

air-drying technique described in Chapter 2.6.

The superovulated female mice that did not mate with F1 males were killed by cervical dislocation at HCG+ 16h. The ovulated oocytes were isolated and air-dried chromosome spreads were prepared as described above.

At 27-32h after the HCG injection, the fertilised eggs and spontaneous parthenogenones were observed to enter metaphase of the first cleavage mitosis. This was signalled by the breakdown of the pronuclear membranes. These embryos were harvested, and metaphase chromosome spreads were prepared as described above. The chromosome preparations were processed for G-band analysis as described in Chapter 2.6.1.

The LT/Sv females that mated with F1 hybrid males after natural ovulation were killed 6-8h after the expected time of fertilisation. The fertilised one-cell embryos and spontaneous parthenogenones were isolated and incubated in M16 medium as previously descibed (Chapter 2.5). At 12h after the expected time of fertilisation the oocytes were transferred to M16 medium supplemented with 1ug/ml Colcemid and air-dried metaphase chromosome spreads were prepared early the next morning as described in Chapter 2.6.2.

6.3 RESULTS

6.3.1 Cytogenetic analysis of ovulated oocytes from superovulated LT/Sv females

The analysis of the chromosome constitution of the ovulated oocytes, isolated at HCG+ 16h from superovulated female LT/Sv mice, has clearly demonstrated that both primary and secondary oocytes, arrested at metaphase of the first meiotic and second meiotic divisions, respectively, were ovulated by females of this strain (see Table 6.1). Chromosome preparations of primary oocytes exhibited the characteristic "tetrad" chromosome configuration (Fig 6.1), while secondary oocytes exhibited the "dyad" chromosome configuration usually observed in recently ovulated oocytes. An analysis of the findings presented in Table 6.1 reveals that of the total number of ovulated oocytes recovered from the oviducts of the LT/Sv females, 33% had been ovulated as primary oocytes.

6.3.2. Cytogenetic analysis of fertilised LT/Sv one-cell embryos

The results of the analysis of the chromosome constitution of the fertilised one-cell embryos isolated from superovulated LT/Sv females that had mated to F1

Fig. 6.1 Representative air-dried chromosome spread of an LT/Sv ovulated primary oocyte at metaphase of the first meiotic division. Giemsa stain.

TABLE 6·1

Chromosome constitution of ovulated oocytes isolated from superovulated LT/Sv female mice as determined from air-dried chromosome preparations.

Total number of ovulated oocytes	Number of oocytes ovulated at M I*	Number of oocytes ovulated at M II*	Number of spontaneous parthenogenones	Number of non-analysable preparations
24	8	16	0	0

* M I : metaphase of the first meiotic division
M II : metaphase of the second meiotic division

hybrid males are presented in Table 6.2, Group 1. These results demonstrate that the incidence of fertilisation was 76.8% and that 31.6% of these exhibited a triploid chromosome constitution. A further 12.1% of the ovulated oocytes developed as spontaneous parthenogenones.

The analysis of the chromosome constitution of the fertilised one-cell embryos isolated after natural matings between F1 hybrid males and LT/Sv females are also presented in Table 6.2, Group 2. These results demonstrate that the incidence of fertilisation, in this study, was 85.1%, but that a smaller proportion (14.2%) of the one-cell embryos exhibited a triploid chromosome constitution. One triploid one-cell embryo was found to possess three distinct pronuclear sets of chromosomes. This was an example of either the dispermic or digynic class of triploid embryo usually observed following the fertilisation of postovulatory aged oocytes.

Representative air-dried chromosome preparations of triploid and diploid fertilised one-cell embryos at metaphase of the first cleavage mitosis are presented in Fig. 6.2 (natural mating) and Fig. 6.3 (superovulation). Under normal circumstances, when a secondary oocyte is fertilised, and incubated overnight in the presence of colcemid, two distinct haploid chromosome sets may be observed at the first cleavage mitosis, one being of paternal and the other of maternal origin. In the

TABLE 6·2

Chromosome constitution of fertilised and unfertilised oocytes isolated from LT/Sv female mice mated to F1 hybrid males.

Group	Total number of oocytes	Number of unfertilised oocytes at		Number of fertilised one-cell embryos that exhibit a chromosome constitution of		Number of spontaneous parthenogenones		Incidence of triploidy (%)
		M I*	M II*	(20♀/20♂)	(40♀/20♂)	1PN	1DPN	
1	99	1	10	52	24	8	4	31·6
2	141	2	8	102	18**	5	6	14·2

* see Table 6·1

** one embryo was a dygynic triploid that developed following the failure to extrude the second polar body. (see Fig. 6·5 pathway iia)

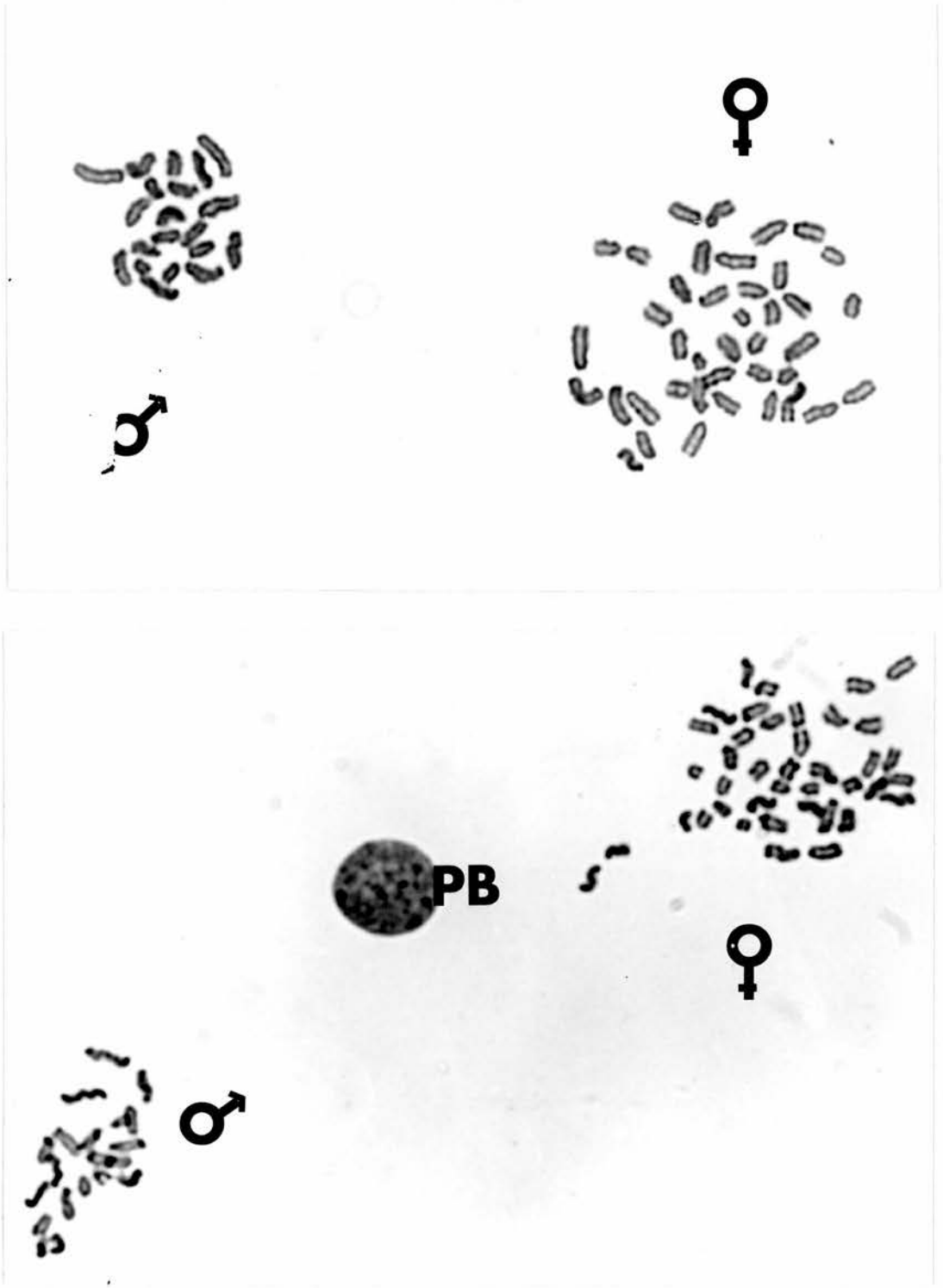


Fig 6.2 Representative air-dried chromosome spreads of two triploid (LT/Sv X F1) fertilised one-cell embryos at metaphase of the first cleavage mitosis. The F1 hybrid male pronuclear set of chromosomes is haploid (N = 20), whereas the LT/Sv oocyte-derived pronuclear set of chromosomes is diploid (N = 40) and exhibits centromeric associations between homologous chromosomes. The polar body (PB) has the appearance similar to that of a normal second polar body. Giemsa stain

Fig. 6.3 (A) Giemsa-banded, air-dried chromosome spread of a diploid fertilised one-cell (LT/Sv x F1) embryo at metaphase of the first cleavage mitosis. (B) Karyotypic analysis illustrates that this embryo is genetically balanced but is heterozygous for an oocyte-derived Robertsonian translocation between chromosomes 6 and 13.

metaphase preparations of triploid one-cell embryos (Fig. 6.2) the male-derived set is haploid but the female chromosome complement, as it is derived from a single diploid pronucleus, is represented by a distinct single group of 40 chromosomes. The homologous female chromosomes are clearly observed to be paired at their centromeres.

Another unusual feature observed in air-dried preparations of the triploid embryos was the morphological appearance of the polar body chromatin. The latter was invariably found to be present in the form of a single discrete nuclear mass (see Fig. 6.2), very closely resembling the nuclear morphology characteristic of the second polar body in Giemsa stained preparations of 1PN parthenogenones, fertilised diploid and dispermic triploid one-cell embryos. Under normal circumstances, the chromosomes of the first polar body tend to remain as a compact group, and invariably degenerate rather than progress to form a recognisable nuclear mass (Rodman, 1971; Abramczuk & Sawicki, 1975). In the present situation, the appearance of the chromatin mass has all the features of that seen when air-dried preparations are made of the second polar body. However, this polar body appears after the first meiotic division and is therefore, by definition, a first polar body.

6.3.3 Analysis of the litter size of the LT/Sv breeding colony

The mean number of oocytes ovulated following superovulation and natural ovulation was 9.7 ±3.0 (n=10) and 8.3 ±1.28 (n=16), respectively. The mean number of offspring (269 ♂ : 214 ♀, 1.0:0.7) was 5.03 ± 1.92 (n=96). The results of this study indicate that the major factor that contributes to the disparity between the number of ovulated oocytes and the litter size of the LT/Sv strain is the prenatal loss of triploid embryos (14.2%) and the limited developmental potential of spontaneous parthenogenones (7.0%).

6.3.4 Analysis of the spontaneous Robertsonian Translocation (6.13) of LT/Sv mice

A spontaneous Robertsonian translocation between chromosomes 6 and 13 was isolated from the LT/Sv breeding colony. Several mice that possessed this novel chromosomal rearrangement were used in this and the following study before the parental source was identified. The karyotype of a fertilised one-cell embryo that exhibited this chromosomal rearrangement is represented in Fig 6.3. Chromosomal rearrangements have been reported to increase the incidence of spontaneous meiotic nondisjunction (see, Ford, 1975; Dyban and

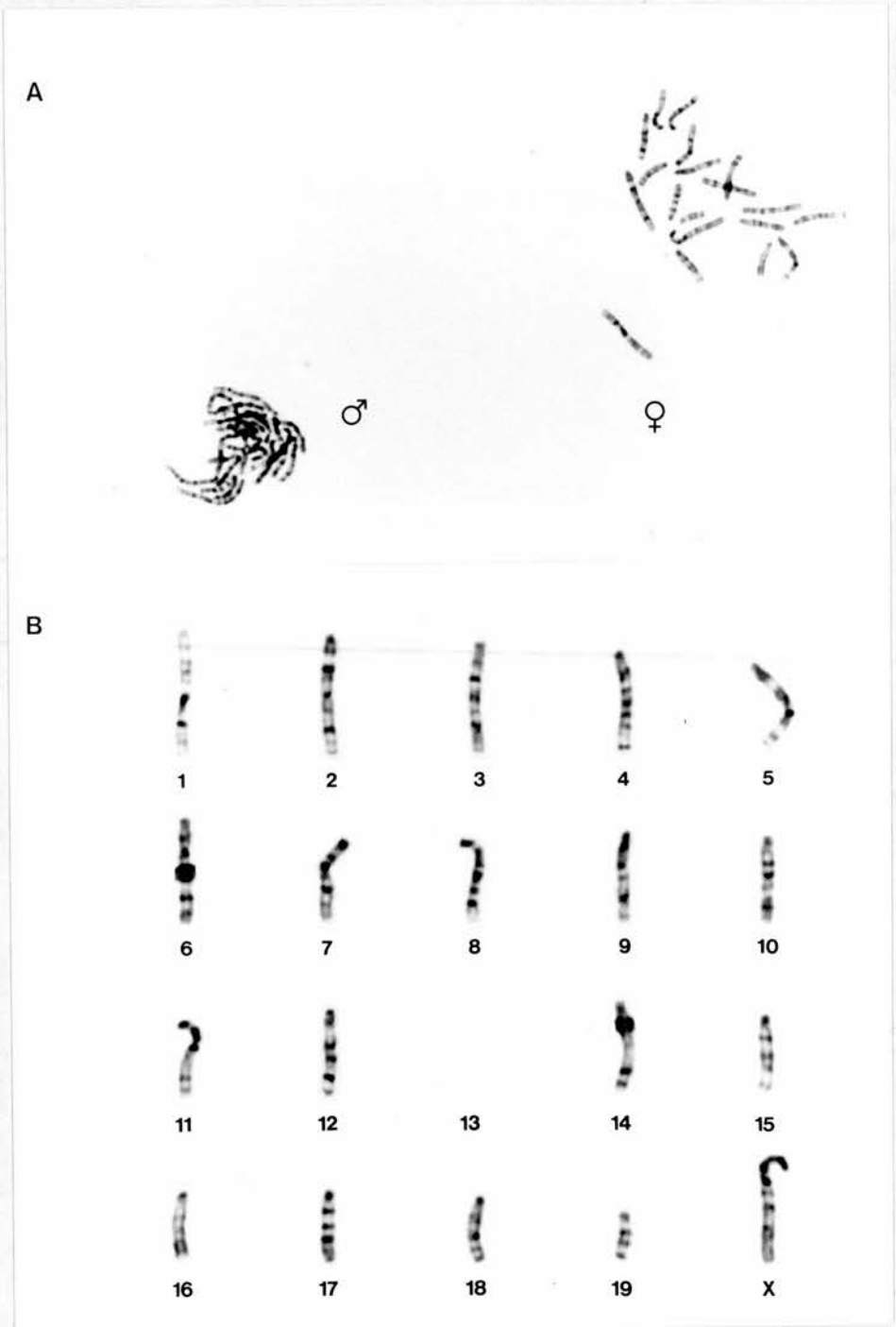


Fig 6.4 Giemsa-banded, air-dried chromosme spread of a diploid fertilised one-cell (LT/Sv x F1) embryo at metaphase of the first cleavage mitosis. The chromosome spread also illustrates that the male- and female-derived chromosome sets exhibit differential degrees of contraction at metaphase of the first cleavage mitosis.. (B) Karyotypic analysis demonstrates that the oocyte-derived pronuclear set of chromosomes is nullosomic for chromosome 13.

Baranov, 1987) and the monosomy 13 chromosome constitution of the fertilised one-cell embryo in Fig. 6.4 may be the product of nondisjunction in a Rb(6.13)/++ oocyte. The mice that possesed the (6.13) translocation also exhibited hyperactive behaviour. This is also the first identification of a Robertsonian translocation that has involved chromosomes 6 and 13 (Searle, 1981).

6.4 DISCUSSION

Analysis of the chromosome constitution of unfertilised ovulated oocytes has confirmed the previous observation that LT/Sv female mice ovulate both primary and secondary oocytes (Kaufman and Howlett, 1986). Ovulated oocytes are normally arrested at metaphase of the second meiotic division and the chromosomes exhibit the characteristic "dyad" configuration. However in this study, one third of the ovulated oocytes exhibited the "tetrad" chromosome configuration usually only observed when chromosome spreads are prepared from ovarian oocytes that have matured to diakinesis/metaphase of the first meiotic division.

Furthermore, examination of the chromosome constitution of fertilised one-cell embryos at metaphase of the first cleavage mitosis revealed that both naturally or induced ovulated primary and secondary oocytes were capable of monospermic penetration and pronuclear development. This study has revealed that 31.6% of the fertilised oocytes obtained by the administration of exogenous hormones exhibited a triploid chromosome constitution and developed following the fertilisation of primary oocytes. In contrast, a lower incidence of triploidy was observed when spontaneously ovulated oocytes were fertilised. The

administration of exogenous hormones did not significantly increase the number of ovulated oocytes but increased the incidence of this particular meiotic anomaly. The comparison of the litter size and the number of spontaneously ovulated oocytes per mouse in this strain has demonstrated that the limited developmental potential of the LT/Sv triploids is one of the significant factors that reduce the number of live born offspring in this strain. Recent cytogenetic analysis of a larger number of fertilised one-cell embryos isolated from LT/Sv females following either exogenous hormone stimulation or natural mating has confirmed these initial observations (Speirs and Kaufman, 1988)

The fertilised triploid embryos isolated from the oviducts of LT/Sv mice are of an unusual type, and developmentally different from the other classes of digynic triploid embryos that have been observed at a low frequency (1 - 2%) in previous analyses of the chromosome constitution of one-cell embryos at metaphase of the first cleavage mitosis (Donahue, 1972; Kaufman, 1973b; Nijhoff and de Boer, 1981; Dyban and Baranov, 1987). Digynic triploid one-cell embryos isolated from other strains of mice generally exhibit three haploid pronuclei and develop following the monospermic fertilisation of a secondary oocyte in which second polar body extrusion has failed to occur. In the latter group, the embryo generally contains one male-derived pronucleus and two

female-derived haploid pronuclei. Failure to extrude the second polar body and polyspermy are facilitated by postovulatory ageing of the oocyte within the oviduct prior to fertilisation (Marston and Chang, 1964; Vickers, 1969; Austin, 1970; Szollosi, 1971).

The triploid one-cell embryos of the LT/Sv strain developed from fertilised primary oocytes and consequently exhibited only two pronuclei. The male-derived pronucleus was haploid, whereas the female pronucleus, since it was formed after the extrusion of a first rather than a second polar body, was invariably diploid. Triploidy in the LT/Sv strain is unrelated to the various changes that occur due to post-ovulatory ageing of the oocyte. The origin and developmental pathways of the various classes of triploid embryos described above, are represented diagrammatically in Figure 6.5.

A third feature that differentiates LT/Sv triploid embryos from the other classes of triploidy induced following the fertilisation of postovulatory aged secondary oocytes, is the presence of "homologous chromosome pairing" which may be observed at metaphase of the first cleavage mitosis. In "spontaneous" digynic triploids, the maternally- derived first cleavage chromosomes normally originate from two distinct pronuclei formed after the centromeric division of dyads

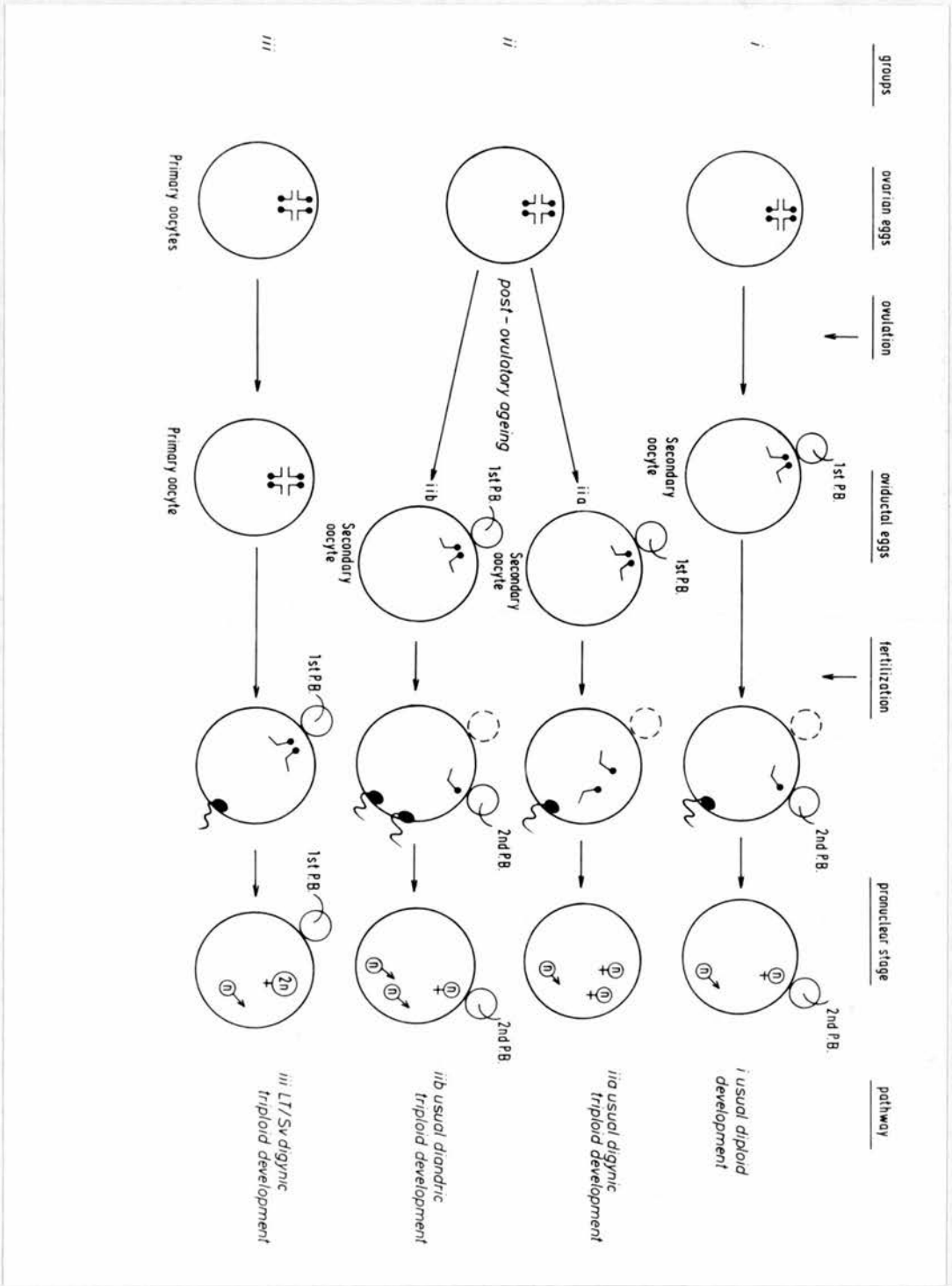


Fig. 6.5 Usual pathways of development taken following the fertilisation of (i) normal secondary and (ii) ovulated LT/Sv primary oocytes. The figure also demonstrates two pathways of triploid development that occur following the fertilisation of postovulatory aged secondary oocytes (iia, iib). The LT/Sv triploid embryos encountered in this study develop along the pathway illustrated in (iii).

in secondary oocytes. The diploid female-derived pronucleus in the LT/Sv triploids is, however, formed after the segregation of bivalents, in which centromeric division does not occur, and the extrusion of a first rather than a second polar body. The chromosomes have not separated at their centromeres and maintain the capacity to retain the morphological features characteristic of "homologous chromosome pairing" when they enter metaphase of the first cleavage mitosis.

Previous attempts to fertilise primary oocytes (of ovarian origin) of rodents <u>in vitro</u> has been singularly unsuccessful in that only a small proportion were capable of progressing as far as metaphase of the first cleavage mitosis (Iwamatsu and Chang, 1971, 1972; Niwa and Chang, 1975; Niwa, Miyake, Iritani and Nishikawa, 1976). Oocytes penetrated by sperm prior to anaphase were frequently unable to support sperm head decondensation or polar body extrusion and development was limited to the pronuclear stage of the first cell cycle. These authors have proposed that the primary oocyte, prior to ovulation, needs to undergo a period of "cytoplasmic maturation" necessary for normal fertilisation to occur. However, the results of this study indicate that ovulated primary oocytes from LT/Sv strain mice have necessarily undergone some form of "cytoplasmic maturation" within the ovary prior to ovulation, and that this phenomenon has occurred without the need for the oocyte to complete its first

meiotic division. Furthermore, these results have clearly demonstrated that ovulated LT/Sv primary oocytes are capable of progressing to metaphase of the first cleavage division with a frequency equal to that of normal secondary oocytes. Subsequent analysis of the development potential of LT/Sv triploids in vivo has found that this class of triploid embryo can regularily develop to the forelimb bud stage with about 20 - 25 somites present (Kaufman and Speirs, 1987).

In three mammalian species, namely the dog (Van der Stricht, 1923), the fox (Pearson and Enders, 1943) and the horse (Hamilton and Day, 1945), the oocyte is reported to be ovulated as a primary oocyte. These early investigations found that meiotic maturation continued in the oviduct as sperm penetration did not occur until after the oocyte had matured to metaphase of the second meiotic division. Van der Stricht (1923) reported that sperm occasionally entered the oocyte before meiotic maturation had been completed, but that sperm head decondensation was only observed to occur in secondary oocytes. These observations indicate that the final stages of "cytoplasmic maturation" are probably completed when the oocytes of these three species are within the oviduct and that the primary oocytes are not normally competent to initiate 'premature' embryonic development. More recent cytological and cytogenetic analyses of horse oocytes, isolated from mature

preovulatory follicles after HCG treatment, have clearly demonstrated that the majority exhibited a first polar body and the chromosome constitution of secondary oocytes (King, et al., 1987). This more recent analysis indicates that meiotic maturation in the dog and fox should be examined again and suggests that all mammalian oocytes may be ovulated as secondary oocytes.

The ovulation of primary oocytes occurs at a very low frequency (< 0.5%) in the majority of other mouse strains (Hansmann and El-Nahaas, 1979). The ovulated primary oocytes of the LT/Sv strain have the chromosomal constitution of normal ovarian oocytes but have the same initial developmental capacity as secondary oocytes. The only other mice that regularly ovulate primary oocytes are the NMRI/Han strain, which bear the inheritable trait "diploidy" (Beermann, Francke and Hansmann, 1986). In this strain up to 2-4% of the oocytes may be ovulated as primary oocytes following exogenous gonadotrophin stimulation. It has also been established that the "Diploidy" trait of the NMRI strain is maternally inherited through the ooplasm. The most recent studies indicate that this trait is due to a genetic mutation in the mitochindrial genome (Beermann, Hummler, Francke and Hansmann, 1988)

Eppig (1978) has drawn attention to the fact that a proportion of the ovarian follicles in LT/Sv females are

morphologically unusual in that they have a deficiency in their granulosa cell population. These granulosa-cell-deficient (or G.C.D.) follicles have previously been shown to be the location of spontaneous parthenogenetic activation of ovarian eggs and teratoma formation (Stevens and Varnum, 1974). However, the G.C.D. follicles are probably not capable (for purely mechanical reasons) of ovulating an oocyte. A third class of "unstable", but morphologically normal, follicles may exist that develop an abberrant relationship with the developing oocyte contained within them. The oocytes ovulated from these "unstable" follicles would be those that are ovulated as primary oocytes, but have the initial developmental potential of normal secondary oocytes. The influence of the ooplasm ie, mitochondrial genome function, on the incidence of "diploidy" in the LT/Sv strain has still to be analysed. However, the higher incidence of spontaneous primary oocyte ovulation observed in the LT/Sv strain and the capacity of the ovulated primary and secondary oocytes of this strain to initiate spontaneous parthenogenesis indicate that the meiotic anomalies expressed by the LT/Sv and NMRI strains are different. No information is available, as yet, on the endocrinological status of the LT/Sv strain. However, the high incidence of spontaneous primary oocyte ovulation, and its significant increase following the administration of exogenous hormones, indicates that a distinct population of follicles are sensitive to normal

levels of endogenous hormones rather than the female mice having an unusual endocrinological status.

CHAPTER 7

ETHANOL-INDUCED PARTHENOGENETIC ACTIVATION OF LT/Sv OOCYTES in vitro

CONTENTS

7.1 Introduction

7.2 Methods

7.3 Results

7.3.1 The incidence of ethanol- and hyaluronidase-induced parthenogenesis

7.3.2 Cytogenetic analysis of LT/Sv parthenogenones

7.4 Discussion

7.1 INTRODUCTION

A proportion of the ovarian and ovulated oocytes of LT/Sv strain mice have the potential to initiate spontaneous parthenogenetic development both *in vivo* and *in vitro* (Stevens and Varnum, 1974; Stevens, 1975). A higher incidence of parthenogenetic activation can be achieved when the ovulated oocytes of this strain are exposed *in vitro* to recognised parthenogenetic stimuli such as ethanol (Kaufman and Howlett, 1986). Several studies, (see, O'Neill and Kaufman, 1987b, Chapter 6) have now confirmed that a proportion of the ovulated oocytes in this strain have the chromosome constitution of primary oocytes. The primary and secondary oocytes exhibit the same capacity to initiate either parthenogenetic or sperm-activated embryonic development.

Aims of study

In the previous chapter, the ovulated primary and secondary oocytes were found to possess an equal ability to initiate embryonic development. A comparative examination of the potential of both ethanol and hyaluronidase to induce the parthenogenetic activation of LT/Sv ovulated oocytes *in vitro*, can determine not only the potential of each agent to act as a parthenogenetic stimulus, but can also identify whether the ovulated

primary and secondary oocytes of this strain exhibit the same ability to initiate parthenogenesis. The analysis of the proportionate incidence of the numerous pathways of parthenogenesis, observed several hours after the activation of primary and secondary LT/Sv oocytes, also permits an examination of the influence of both postovulatory ageing and ethanol exposure on the ability of both classes of oocyte to maintain the ability to extrude a polar body.

The cytogenetic analysis of the ethanol- and hyaluronidase-induced LT/Sv parthenogenones can also be used to investigate the ability of these agents to interfere with the segregation of meiotic chromosomes in the activated oocytes of a second strain of the mouse. Furthermore, the ovulation of primary oocytes, that possess the initial developmental potential of normal secondary oocytes provides the unique opportunity to examine, in vitro, the interaction between ethanol and the segregation of chromosomes in a primary oocyte.

7.2 METHODS

Ethanol-induced parthenogenetic activation

Eight- to 12 week old LT/Sv female mice were superovulated as described in Chapter 2.2. The female mice were killed by cervical dislocation at HCG+ 17h and their oviducts were removed. The cumulus masses, containing the ovulated oocytes, were isolated and briefly incubated in a 7% solution of ethanol *in vitro* for either 1min. or 5min., to induce parthenogenetic activation as described in Chapter 2.4.2.

Hyaluronidase-induced parthenogenetic activation

An additional group of young mature female LT/Sv mice were superovulated as described in Chapter 2.2. Their ovulated oocytes were exposed to M16 culture medium supplemented with 3mg/ml of hyaluronidase at HCG+ 20h for 10-15min, as described in Chapter 2.4.1, to induce parthenogenetic activation.

Six hours after the exposure of LT/Sv ovulated oocytes to either ethanol or hyaluronidase, the majority of the activated oocytes exhibited a single pronucleus and a polar body. All activated oocytes were transferred to fresh microdrops of equilibriated M16 cuture medium as described in Chapter 2.4.

Preparation of chromosome spreads from LT/Sv parthenogenones.

Chromosome preparations of LT/Sv ethanol- and hyaluronidase-induced parthenogenones were prepared during metaphase of the first cleavage mitosis by the method described in Chapter 2.6.1.

7.3 RESULTS

7.3.1 The incidence of ethanol- and hyaluronidase-induced parthenogenesis

The proportionate incidence of the numerous pathways of ethanol- and hyaluronidase-induced parthenogenesis are presented in Table 7.1 (Fig. 7.1 & 7.2). The highest frequency of parthenogenetic activation (73.1%) was observed following the exposure of ovulated LT/Sv oocytes to a 7% solution of ethanol in Dulbecco's PBS for 1min. at HCG+ 17h (Group 1) This value was higher than the frequency of parthenogenetic activation observed following the exposure of ovulated oocytes to hyaluronidase at HCG+ 20h (Group 3). The frequency of ethanol-induced parthenogenesis was significantly reduced to 54.1% when the duration of ethanol exposure was increased from 1min. to 5min (Group 2). The reduction in the activation frequency was not restricted to the inhibition of parthenogenesis in one of the two populations of ovulated LT/Sv oocytes. In each group, the proportion of primary oocytes (Group 1, 39.1%; Group 2, 37.4%; Group 3, 33.3%), and thus secondary oocytes, that initiated parthenogenonesis did not differ significantly .

The majority of hyaluronidase and ethanol activated

TABLE 7·1

The proportionate incidence of the four classes of parthenogenone observed following either the ethanol- or hyaluronidase-induced parthenogenetic activation of ovulated primary and secondary oocytes of LT/Sv mice.

Group	Activation stimulus	Number of ovulated oocytes	Number of activated oocytes	Class of Parthenogenone						Frequency of activation (%)
				1PN	2PN	IC	1PND	1DPN	2DPN	
1	7% ethanol, 1min. at HCG+ 17h.	119	87	45	6	3	0	33	1	73·1
2	7% ethanol, 5min. at HCG+ 17h.	183	99	34	23	5	0	35	2	54·1
3	3mg/ml hyaluronidase at HCG+ 20h.	165	102	52	13	2	1	34	0	61·8

The activation frequency in Group 1 is significantly greater than that observed in Group 2. X^2 = 10·9. P 0·01.

The proportionate incidence of the 1PN class in Group 1 is significantly greater than that observed in Group 2. X^2 = 10·4, $P < 0·01$.

The frequency of primary oocyte activation in Group 1 does not differ significantly to that observed in Group 3. X^2 = 0·67, $P = 0·8$.

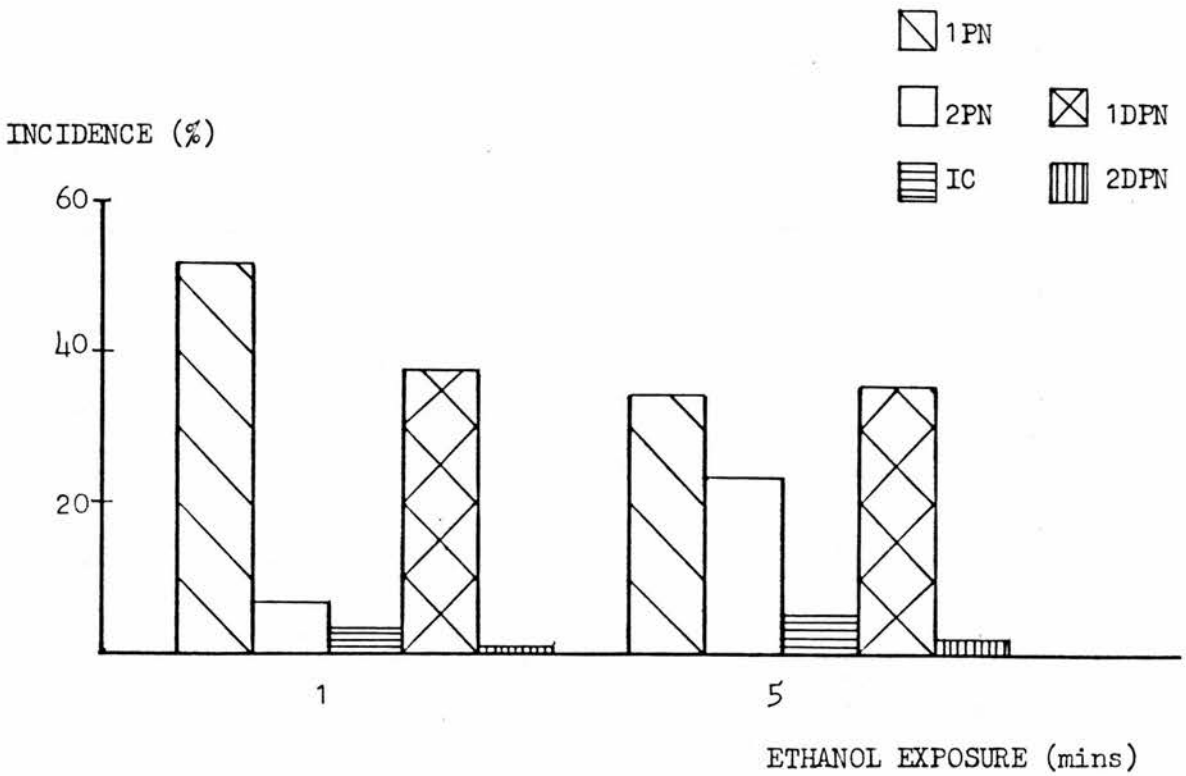


Fig. 7.1 The proportionate incidence of the pathways of parthenogenetic development observed following the ethanol-induced activation of the ovulated secondary and primary oocytes of LT/Sv mice at HCG+ 17h.

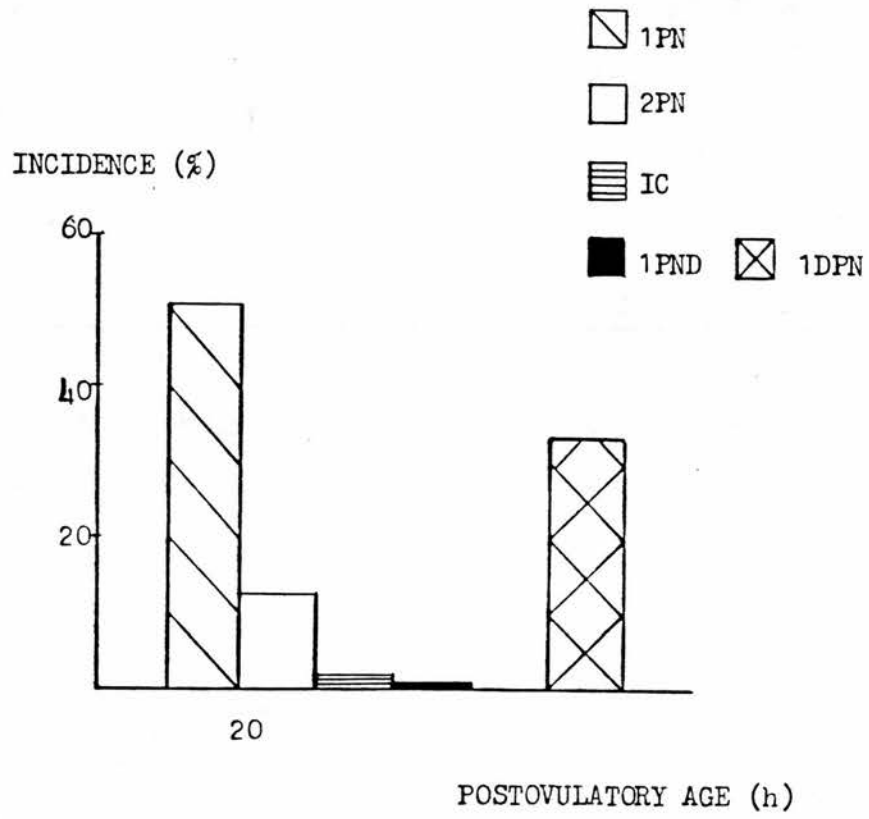


Fig. 7.2 The proportionate incidence of the pathways of parthenogenetic development observed following the brief exposure of ovulated primary and secondary LT/Sv oocytes to M16 medium supplemented with 3mg/ml of hyaluronidase at HCG+ 20h

secondary oocytes extruded the second polar body and developed as 1PN parthenogenones (Table 7.1). The proportion of secondary oocytes that developed as 1PN parthenogenones following exposure to hyaluronidase at HCG+ 20h (51.0%), or to ethanol for 1min. at HCG+ 17h (51.7%) did not differ significantly. However, when the duration of ethanol exposure was increased from 1min. to 5min. the proportionate incidence of 1PN parthenogenones was significantly reduced to 34.3% as an increased proportion of ethanol exposed oocytes failed to extrude the second polar body and developed as 2PN, IC or 1PND parthenogenones.

All hyaluronidase activated primary oocytes developed a single diploid pronucleus (1DPN) following the extrusion of a polar body (Table 7.1, Group 3). The majority of ethanol activated primary oocytes also developed as 1DPN parthenogenones but a small proportion (Group 1, 2.9%; Group 2, 5.4%) failed to extrude a polar body and subsequently developed two diploid pronuclei (2DPN). This unique tetraploid class is only observed following the activation of LT/Sv primary oocytes.

7.3.2 Chromosome constitution of LT/Sv parthenogenones

The chromosome constitution of all activated oocytes was analysed in this study. The analysis of the chromosome constitution of hyaluronidase activated 1PN and 1DPN parthenogenones at metaphase of the first cleavage

mitosis has found that 3.2% of the 1PN parthenogenones exhibited aneuploidy and that all 1DPN parthenogenones had a normal chromosome constitution (Table 7.2).

Cytogenetic analysis of the ethanol-induced 1DPN parthengenones has revealed that the majority exhibited a normal chromosome constitution at metaphase of the first cleavage mitosis (Fig. 7.3). In both Group 1 and 2, 6.2% of this class of parthenogenone exhibited an aneuploid chromosome constitution. However, karyotypic analysis of the chromosome spreads of these two aneuploid parthenogenones in Group 2 revealed that they were heterozygous for Rb(6.13) and the chromosome segregation errors that involved chromosome 6 and 13, respectively, may have been the result of unbalanced segregation due to the chromosomal rearrangement and not the product of ethanol-induced spindle disorder (Fig. 7.4). The ethanol-induced hyperdiploid 1PN parthenogenone in Group 1 was found to be due to the malsegregation of chromosome 8 (Fig. 7.5)

The cytogenetic analysis of the ethanol-induced 1PN parthenogenones at metaphase of the first cleavage mitosis (Table 7.2, Groups 1,2) has demonstrated that a significant proportion of this class exhibited a high incidence of aneuploidy following exposure to this agent for either 1min. (14.0%) or 5min. (52.0%) at HCG+ 17h. The incidence of ethanol-induced malsegregation events

TABLE 7·2

The chromosome constitution of ethanol- and hyaluronidase-induced 1PN and 1DPN LT/Sv parthenogenones at metaphase of the first cleavage mitosis.

Group	Activation stimulus	Number of chromosome preparations	Number of nonanalysable preparations	Chromosome Constitution									Aneuploidy (%)	
				38	40	42	19	20	21	22	23	27	1PN	1DPN
1	7% ethanol, 1min. at HCG+ 17h.	82	7	1	30[a]	1	1	37[+++]	3	2	0	0	14·0	6·2
2	7% ethanol, 5min. at HCG+ 17h.	75	20	0	32[b]	2*	3	12[++]	3	5	1	1	52·0	6·2
3	3mg /ml hyaluronidase at HCG+ 20h.	87	19	0	36	0	0	31	1	0	0	0	0·0	3·2

* Two 1DPN parthenogenones that carried the Rb(6·13) translocation were hyperdiploid for chromosome 13 and 6, respectively.

+++ and ++ 12 and 3 1PN parthenogenones exhibited the Rb(6·13) translocation but did not exhibit an aneuploid chromosome constitution

a., 15 and b.,7 1DPN parthenogenones possessed the Rb (6·13) but did not exhibit an aneuploid chromosome constitution

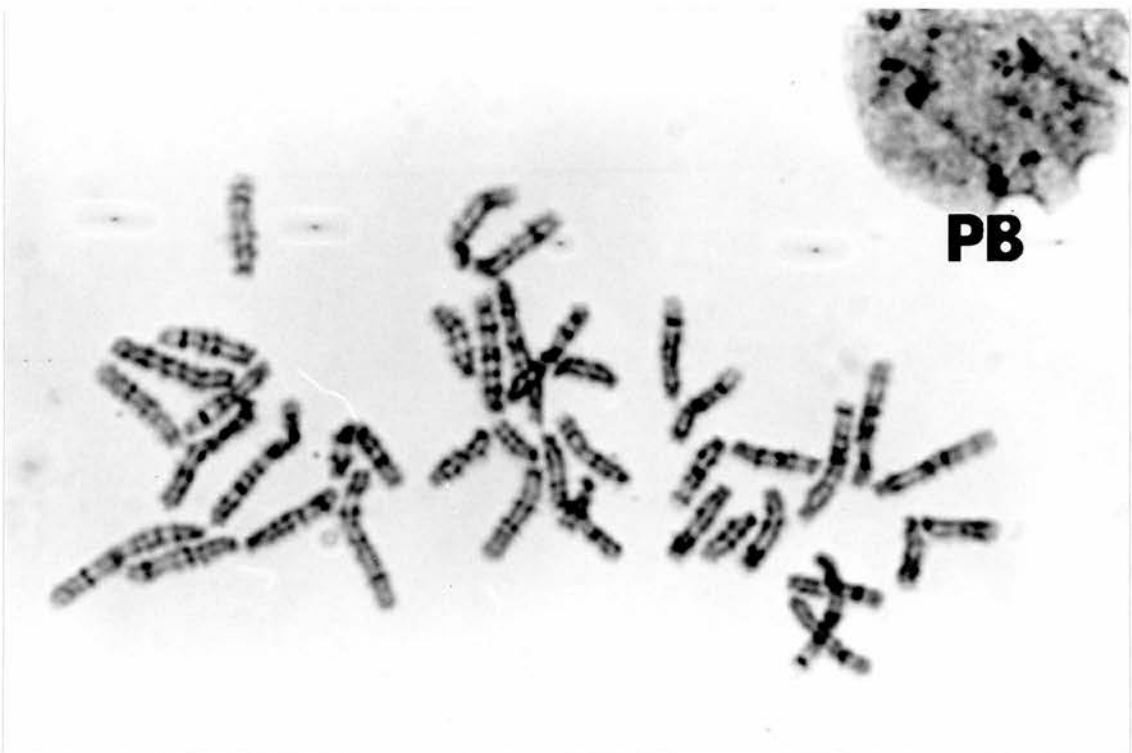


Fig 7.3 Giemsa-banded air-dried chromosome spread of an ethanol-induced LT/Sv diploid 1DPN parthenogenone at metaphase of the first cleavage mitosis (N = 40). The majority of the homologous chromosomes exhibit centromeric associations.

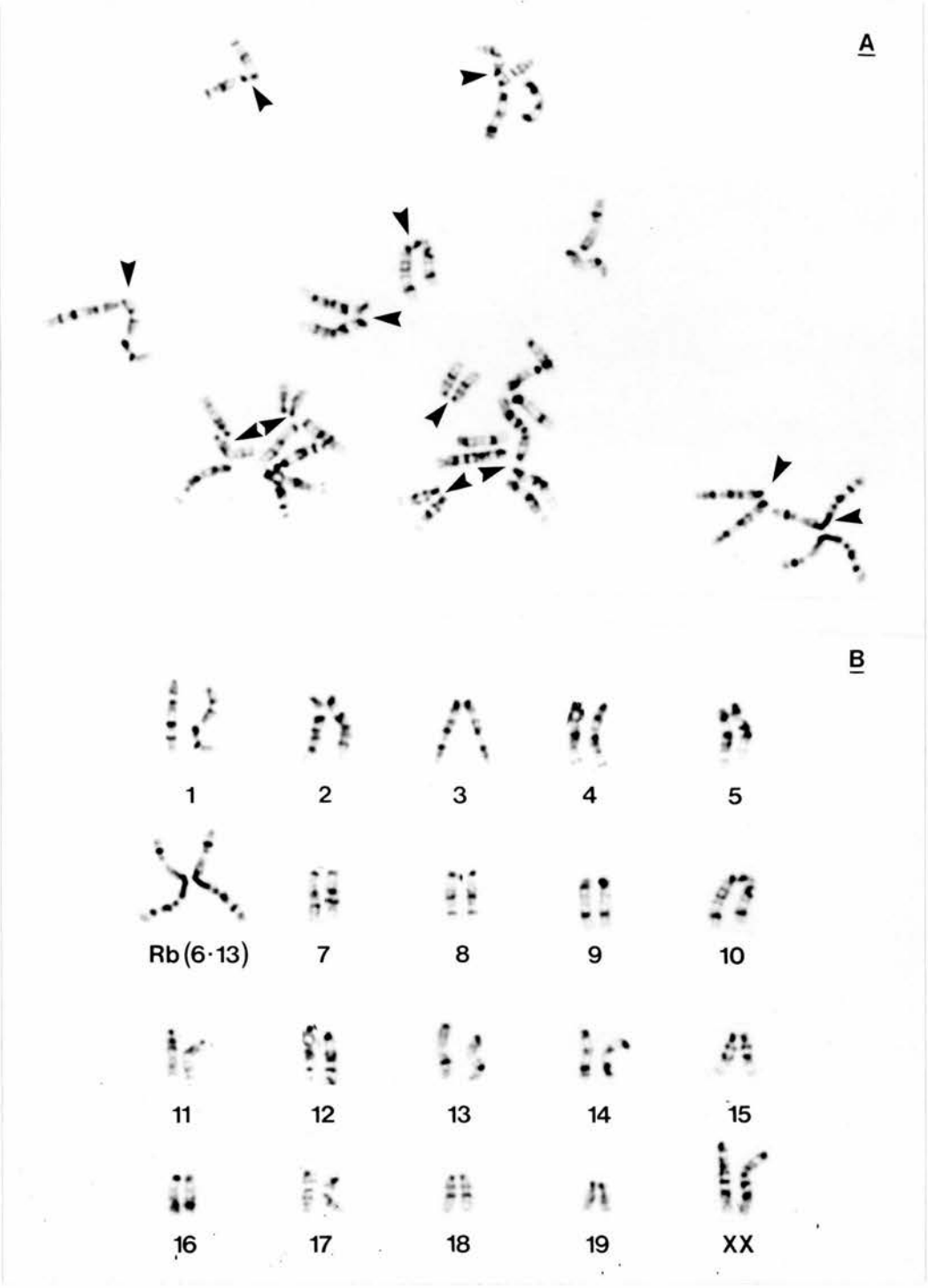


Fig 7.4 (A) Giemsa-banded, air-dried chrompsome spread of an ethanol-induced hyperdiploid (N.F. = 42) LT/Sv 1DPN parthenogenone at metaphase of the first cleavage mitosis. (B) Karyotypic analysis demonstrates that this preparation is heterozygous for the Robertsonian translocation (6.13) and tetrasomic for chromosome 13. The majority of the homologous chromosomes exhibit centromeric associations (arrows).

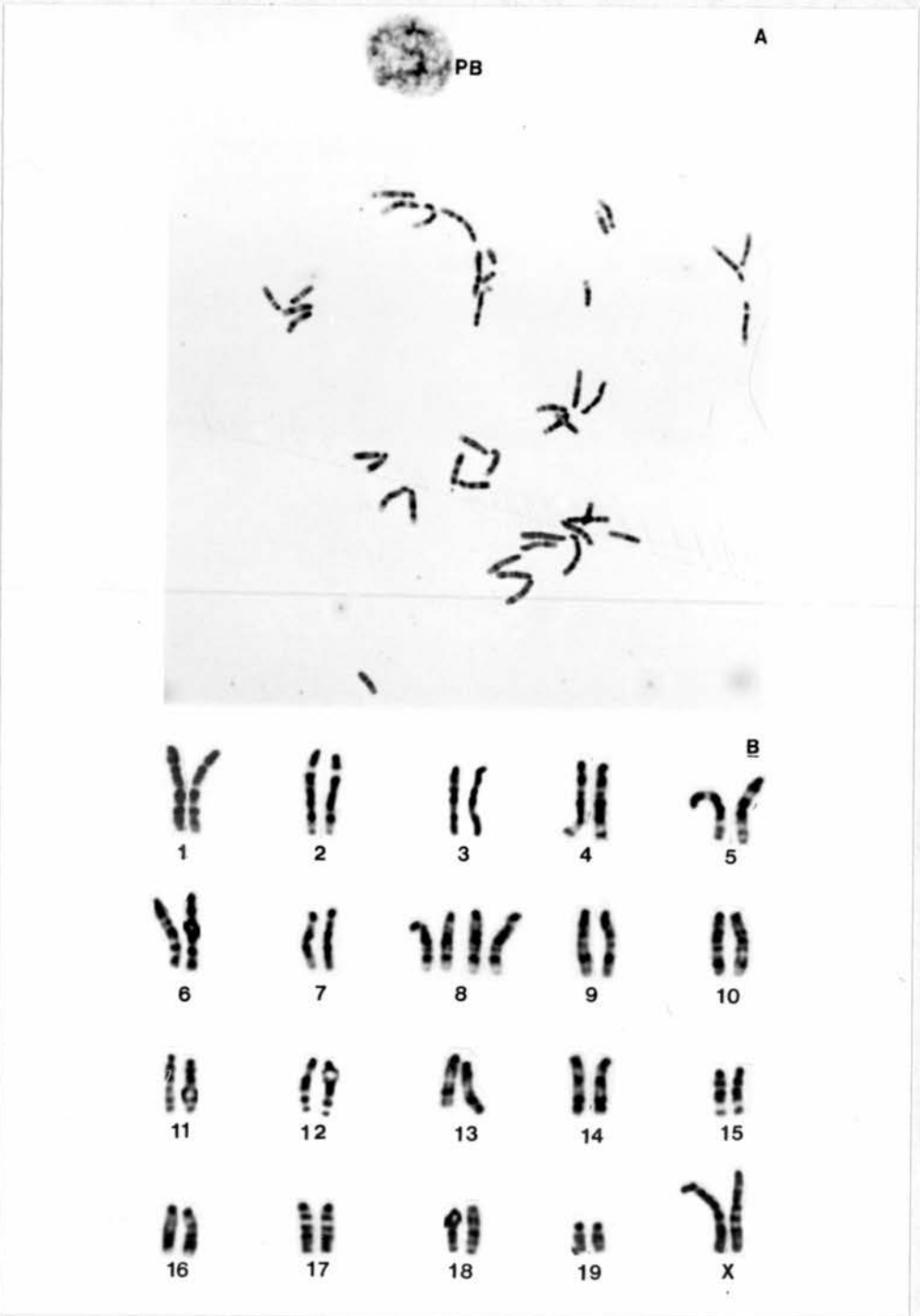


Fig 7.5 (A) Giemsa-banded, air-dried chromosome preparation of an ethanol-induced hyperdiploid (N = 42) LT/Sv 1DPN parthenogenone at metaphase of the first cleavage mitosis. (B) Karyotypic analysis demonstrates that this preparation is tetrasomic for chromosome 8.

that involved more than one chromosome increased from 33.3% to 53.8% when the duration of ethanol exposure increased from 1min. to 5min. The majority of chromosome preparations from ethanol-induced aneuploid 1PN parthenogenones failed to exhibit distinct G-band patterns. Two metaphase preparations from Group 2 (5min exposure) that exhibited 22 chromosomes were hyperhaploid for chromosomes 10 & 6 and 14, 17, 18 & X, respectively . The latter preparation was also hypohaploid for chromosomes 3, 12. Two hypohaploid preparations were nullosomic for chromosome 3 (Group 1) and 18 (Group 2) respectively. Ten of the activated oocytes in Group 1, and a further three in Group 2, possessed the Rb(6.13) translocation but none of these parthenogenones exhibited numerical chromosomal anomalies.

Karotypic analysis of G-banded metaphase spreads prepared from ten 1PN (Fig. 7.6), eight 1DPN and two 2PN (Fig. 7.7) parthenogenones that exhibited the euploid number confirmed that they were also genetically balanced.

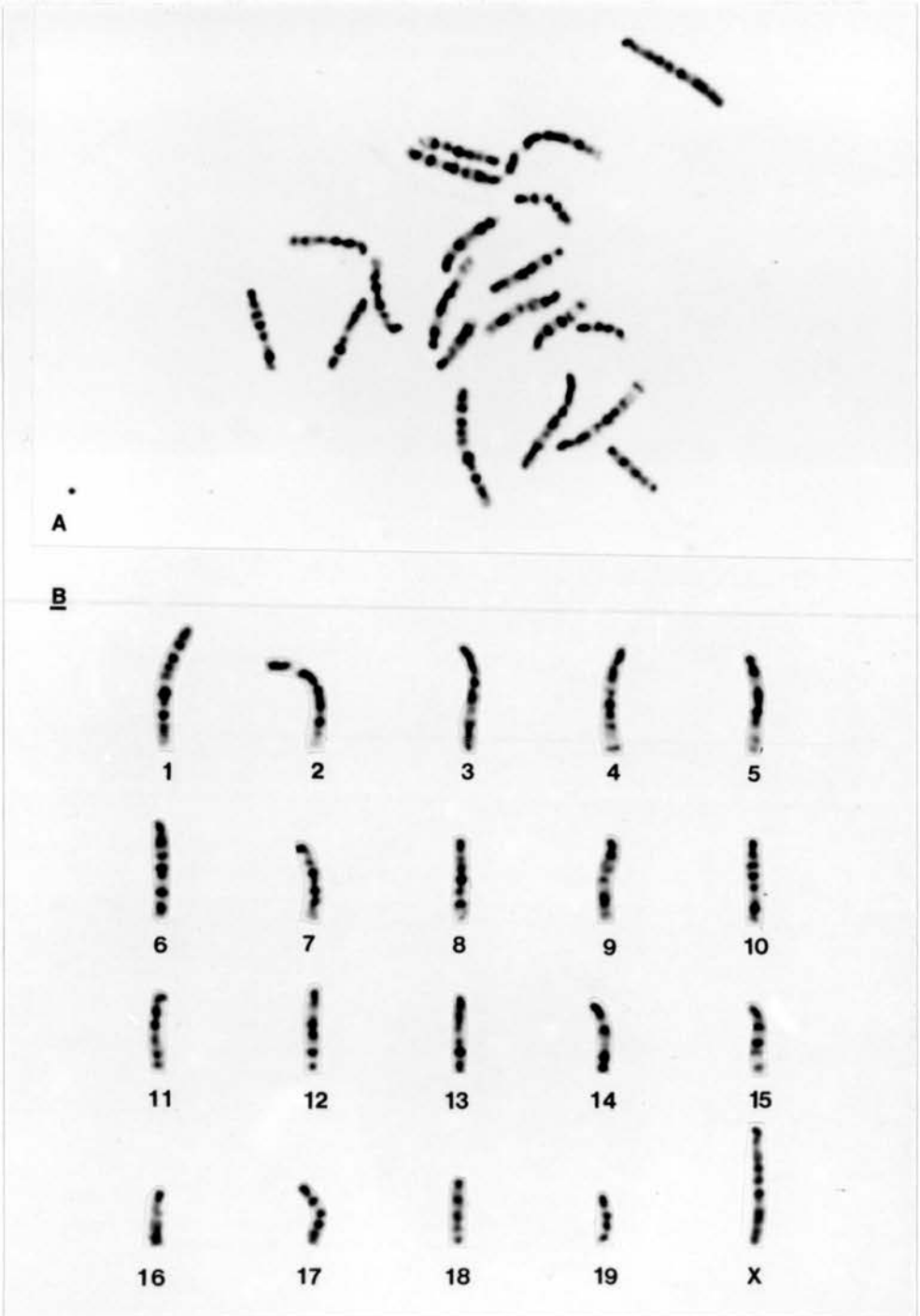


Fig. 7.6 (A) Giemsa-banded, air-dried chromosome spread of an ethanol-induced LT/Sv haploid 1PN parthenogenone at metaphase of the first cleavage mitosis. (B) Karyotypic analysis illustrate that this preparation is euploid and genetically balanced.

Fig. 7.7 (A) Giemsa-banded, air-dried chromosome spread of an ethanol-induced LT/Sv diploid 2PN parthenogenone at metaphase of the first cleavage mitosis. (B) Karyotypic analysis illustrates tha this parthenogenone is euploid and genetically balanced.

DISCUSSION

In this study it was observed that a high proportion of the primary and secondary LT/Sv oocytes initiated parthenogenetic development following their brief exposure to either ethanol or hyaluronidase in vitro. However, the incidence of ethanol-induced parthenogenesis was significantly reduced when the duration of exposure to this agent was increased from 1min. to 5min. When the secondary oocytes of F1 hybrid mice were exposed to this parthenogenetic stimulus, the duration of exposure was not found to significantly alter the frequency of activation (O'Neill and Kaufman, 1989a, Chapter 4). These finding indicate that the oocytes of these two mouse strains do not exhibit similar levels of ethanol tolerance. The reduced frequency of parthenogenesis observed following an increase in the duration of ethanol exposure was found to inhibit the activation of equal proportions of primary and secondary oocytes.

All the ovulated LT/Sv primary oocytes that initiated hyaluronidase-induced parthenogenesis were found to exhibit a diploid pronucleus following the extrusion of a polar body (1DPN). The majority of hyaluronidase activated secondary oocytes extruded the second polar body and developed as 1PN parthenogenones. However, a

significant proportion of the activated secondary oocytes failed to extrude the second polar body and developed as 2PN, IC or 1PND parthenogenones. The proportionate incidence of the activated secondary oocytes that retain both genomic complements of the second meiotic division increases when the loss of the close alignment between the meiotic spindle apparatus and the plasma membrane in postovulatory aged secondary oocytes interferes with the ability of the oocyte to extrude the second polar body after activation has occurred (Webb, et al., 1986). Although the primary and secondary oocytes were activated at the same time, this study has found postovulatory ageing did not adversely influence the events associated with polar body extrusion in the activated primary oocytes.

Furthermore, the majority of ethanol activated primary oocytes also developed as 1DPN parthenogenones. Only a very small proportion of the ethanol activated primary oocytes failed to extrude a polar body. These parthenogenones exhibited two diploid pronuclei (2DPN). Metaphase chromosome preparations of this parthenogenetic class at metaphase of the first cleavage mitosis exhibited two distinct groups of forty chromosomes. Homologous chromosome pairs were observed to be associated at their centromeric ends. These observations clearly indicate that ethanol exposure, even for 5 minutes, cannot significantly reduce the proportion of

activated primary oocytes that develop as 1DPN parthenogenones. These findings indicate that both ethanol exposure and postovulatory ageing do not interfere with the organisation of the cytoskeletal elements of activated primary oocytes.

The majority of ethanol activated primary and secondary oocytes developed as 1DPN and 1PN parthenogenones, respectively. This study has found that the proportion of secondary oocytes that developed as 1PN parthenogenones following either ethanol exposure for 1min. at HCG+ 17h or hyaluronidase activation at HCG+ 20h was equivalent even though the postovulatory age of the two groups of oocytes differed by 3h. The increased duration of ethanol exposure to 5min. further reduced the ability of activated LT/Sv secondary oocytes to extrude the second polar body and a greater proportion of the oocytes developed as 2PN, IC, or 1PND parthenogenones. This strongly indicates that ethanol can inhibit the extrusion of the second polar body in a signifiant proportion of the exposed oocytes. The duration of ethanol exposure has also been previously observed to regulate the proportionate incidence of the four main classes of parthenogenones derived from F1 ovulated oocytes (O'Neill and Kaufman, 1989a, Chapter 4). However the exposure of LT/Sv primary oocytes to ethanol did not significantly reduce the incidence of polar body extrusion. These findings indicate that both postovulatory ageing and

ethanol exposure do not interfere with either the alignment of the meiotic spindle apparatus with the plasma membrane of the primary LT/Sv oocyte or the subsequent extrusion of the polar body.

The cytogenetic analyses of hyaluronidase and ethanol activated primary oocytes have found that numerical chromosome anomalies were very rarely observed in 1DPN parthenogenones. In the chromosome preparations of these parthenogenones the twenty homologous chromosomes pairs of the mouse genome were frequently observed to be paired at their centromeric ends as previously reported by Kaufman and Howlett (1986). This phenomenon was also observed in the female chromosome complement of the metaphase spreads prepared from LT/Sv one-cell triploid embryos (O'Neill and Kaufman, 1987b, Chapter 6). One ethanol-induced 1DPN parthenogenone was hyperdiploid for chromosome 8. The other two ethanol-induced hyperdiploid parthenogenones were derived from oocytes that were heterozygous for Rb(6.13) and it is more likely that aneuploidy resulted from chromosome segregation anomalies due to the presence of this chromosome translocation.

The hyaluronidase activated 1PN parthenogenones were found to exhibit a low incidence of aneuploidy and this did not differ from previously observed in the chromosome spreads of hyaluronidase activated F1 hybrid oocytes (O'Neill and Kaufman, 1988, Chapter 3). However, the

ethanol-induced LT/Sv 1PN parthenogenones exhibited a very high incidence of aneuploidy. It was observed that a majority of the 1PN parthenogenones exhibited an aneuploid chromosome constitution when oocytes were exposed to this agent for 5min.. This was greater than the incidence of aneuploidy observed in ethanol activated F1 hybrid oocytes and indicates that ethanol exposure induces a greater degree of disorder in the meiotic spindle and cytoskeletal organisation of LT/Sv secondary oocytes.

These studies have clearly shown that the two populations of LT/Sv oocytes initiate parthenogenesis, whether the stimulus is hyaluronidase or ethanol, at a high frequency. The majority of the ethanol activated primary oocytes developed as 1DPN parthenogenones and did not exhibit a range of distinct parthenogenetic classes. Factors, such as ethanol and postovulatory age, that have been observed to reduce the incidence of polar body formation in activated secondary oocytes did not influence the proportionate incidence of the 1DPN parthenogenones. Ethanol-induced activation, recognised to increase the incidence of chromosome segregation errors during the completion of the second meiotic division, was clearly unable to induce spindle anomalies in primary oocytes, as the 1DPN parthenogenones did not exhibit a high incidence of aneuploidy. These studies strongly indicate that the integrity of the meiotic

spindle apparatus and associated cytoskeletal elements of the ovulated primary oocyte is maintained following both postovulatory ageing and ethanol exposure, and that the cytoskeletal organisation of this class of oocyte is more stable than that of secondary oocytes.

CHAPTER 8

ULTRASTRUCTURAL ANALYSIS OF THE SECOND MEIOTIC DIVISION IN HYALURONIDASE AND ETHANOL ACTIVATED F1 HYBRID OOCYTES

CONTENTS

8.1 Introduction

8.2 Methods

8.3 Results

8.3.1 The incidence of hyaluronidase and ethanol-induced parthenogenetic activation

8.3.2 Ultrastructural observations of the second meiotic division in hyaluronidase- and ethanol-induced parthenogenones.

8.4 Discussion

8.1 INTRODUCTION

The parthenogenetic activation of ovulated mouse oocytes has been observed to occur at a high frequency following their brief exposure to either hyaluronidase (O'Neill and Kaufman, 1988, Chapter 3) or a dilute solution of ethanol *in vitro* (Kaufman, 1982; O'Neill and Kaufman, 1989a, Chapter 4). The cytogenetic analysis of ethanol-induced 1PN parthenogenones at metaphase of the first cleavage mitosis has consistently shown that this class of activated oocyte exhibits a high incidence of aneuploidy. In contrast, both hyaluronidase activation and postovulatory ageing were found not to significantly increase the incidence of aneuploid 1PN parthenogenones.

It has been reported that one of the first electophysiological events to occur in fertilised oocytes is a series of localised and transient increases in the intracellular concentration of free Ca^{2+} ions (Cuthbertson, *et al.*, 1981; Igusa and Miyazaki, 1986). The physical and chemical stimuli that induce parthenogenesis are believed to activate the completion of the second meiotic division as their interaction with ovulated oocytes subsequently induces an increase in the intracellular concentration of free Ca^{2+} ions that is homologous to the events observed in fertilised oocytes (Siracusa, Whittingham, Codonesu and De Felici, 1978;

Eusebi and Siracusa, 1983). Similarly, Surani and Kaufman (1977), demonstrated, although indirectly, that the disruption of the intracellular concentation of calcium ions following the the incubation of ovulated mouse oocytes in Ca^{2+} -free M16 culture medium induced parthenogenetic activation at a high frequency. It has been proposed in Chapter 4 and by O'Neill and Kaufman (1989a) that ethanol-induced parthenogenesis disrupts the regulation of the intracellular concentration of Ca^{2+} ions in oocytes exposed to this agent. In activated oocytes, this probably also interferes with the Ca^{2+}-mediated regulation of microtubule dynamics in the second meiotic spindle apparatus. The subsequent development, in a proportion of activated oocytes, of meiotic spindle dysfunction or structural anomaly may predispose chromosomes to malsegregation.

Aims of investigation

The cytogenetic analysis of ethanol-induced 1PN parthenogenones has been able to demonstrate that the brief exposure to this agent *in vitro* significantly increased the incidence of chromosome segregation errors during the completion of the second meiotic division. This form of analysis is, however, unable to reveal directly how exposure to this agent interferes with the mechanism of chromosome segregation.

Immunofluorescent and immunocytochemical localisation

techniques facilitate the examination of the complete meiotic spindle in whole oocytes and have been used sucessfully to detect the presence of gross abnormalities in the meiotic spindle morphology of 10% - 12% of postovulatory aged mouse oocytes (Eichenlaub-Ritter, et al., 1986). However, the incidence of the anomalies observed by Eichenlaub-Ritter et al. (1986) was several times greater than the incidence of aneuploidy detected in the first cleavage metaphase chromosome preparations of hyaluronidase-induced parthenogenones (O'Neill and Kaufman, 1988, Chapter 3). This disparity between these two studies may indicate that a significant proportion of the structural anomalies observed in the former study do not result in chromosome segregation errors. In addition, a proportion of the meiotic spindle anomalies reported by Eichenlaub-Ritter, et al.(1986) may be responsible for the 15% -20% of hyaluronidase or ethanol exposed oocytes that consistantly fail to initiate parthenogenesis. Under both conditions, it is unlikely that the gross anomalies observed in the meiotic spindles of non-activated postovulatory aged oocytes are directly related to those events associated with ethanol-induced aneuploidy. The majority of ethanol-induced malsegregation events are restricted to only the loss or gain of one chromosome. The spindle anomalies associated with these events may be beyond the power of resolution of of immunocytochemical techniques used in conjunction with light microscopy..

An ultrastructural analysis of activated oocytes at specific times after hyaluronidase or ethanol exposure permits an examination of the sequence of cytological events that occur during the completion of the second meiotic division. However, in contrast to previous studies that have analysed spindle anomalies using light microscopy, transmission electron microscopy facilitates an examination of both gross anomalies and discrete alterations in the structure of the meiotic spindle. This form of analysis can identify whether chromosome malsegregation occurs as a result of alterations to the morphology ,and thus function, of individual spindle elements, or whether aneuploidy is the product of disorganised microtubule dynamics during the brief period of chromosome segregation that occurs several minutes after activation. This analysis can also identify if ethanol exposure alters the morphology or location of other cytoplasmic organelles.

8.2 METHODS

Ethanol-induced parthenogenetic activation

F1 hybrid female mice were superovulated as described in Chap. 2.2. At HCG+ 17h the female mice were killed by cervical dislocation and the cumulus masses, containing the ovulated oocytes, were isolated and exposed to a solution of 7% ethanol in Dulbecco's PBS for 3min, as described in Chap. 2.4.2, to induce parthenogenetic activation. This duration of ethanol exposure was chosen as as it has been demonstrated to induce both a high incidence of parthenogenesis and aneuploidy (O'Neill and Kaufman, 1989, Chapter 4). The exposed oocytes were incubated for either 15, 30, 60 or 75 min. in microdrops of M16 medium prior to fixation and processing for electron microscopy.

Hyaluronidase-induced parthenogenetic activation

An additional group of young mature female F1 hybrid mice were superovulated, as describded above, and were killed by cervical dislocation at HCG+ 20h. Hyaluronidase activation at this postovulatory age has been found to induce parthenogenesis at a frequency equivalent to that observed following ethanol-induced activation at HCG+ 17h (Chapter 4). Their cumulus masses, containing the ovulated oocytes, were isolated and transferred to M16 culture medium supplemented with 3mg/ml of hyaluronidase

for 10 - 15 min., as described in Chapter 2.4.1, to induce parthenogenesis. The exposed oocytes were then incubated for either 30 min. or 75min. in microdrops of M16 medium prior to fixation and processing for electron microscopy.

Non-activated ovulated oocytes

F1 hybrid female mice were superovulated as described above. The cumulus masses were isolated at HCG+ 17h and washed briefly in M16 culture medium supplemented with 0.5mg/ml of hyaluronidase for 2 - 3min. to remove the adhering cumulus cells. The cumulus cell-free oocytes were then fixed immediately and processed for transmission electron microscopy.

Transmission electron microscopy

The details of the method used to fix and subsequently process the activated and nonactivated oocytes for transmission electron microscopy are presented in Chapter 2.7.

8.3 RESULTS

8.3.1 The incidence of hyaluronidase- and ethanol-induced parthenogenetic activation

The frequency of ethanol- and hyaluronidase-induced parthenogenetic activation was determined from the proportion of sectioned oocytes that exhibited evidence of chromosome disjunction, spindle rotation and second polar body formation. These criteria have confirmed that the non-activated ovulated oocytes fixed at HCG+ 17h were arrested at metaphase of the second meiotic division. Complete chromatid separation was not observed in all of the sections from individual oocytes that had been retained in culture for 15min. following ethanol exposure, and therefore it was not possible to unequivocally establish the proportion of oocytes in which activation had occurred. It was possible, however, to identify the early events associated with the resumption of meiosis in the activated oocytes that had been retained in culture for periods of 30 - 75min. (Table 8.1). These initial changes associated with activation were observed in 56.5% of the ethanol exposed oocytes (X of groups 2-4) and 65.7% of the hyaluronidase-activated oocytes (X of Groups 5-6).

Table 8.1

The incidence of ethanol- and hyaluronidase-induced parthenogenetic activation as determined from semi-thin and ultra-thin sections of (C57BL x CBA)F1 hybrid oocytes

Group	Period of culture (min)	Total number of oocytes analysed	Number of activated oocytes	Overall activation frequency (%)
1 [a]	15	(9)*		
2	30	36	21	)
3	60	10	5	) 56.5
4	75	16	9	)
5 [b]	30	17	10	) 65.7
6	75	18	13	)
7 [c]	0	19	0	

* At this developmental stage it was not possible to determine unequivocally whether ethanol exposure had induced activation or not.

a Groups (1-4): Oocytes were exposed to a 7% ethanol solution for 3 mins at HCG + 17h

b Groups (5-6): Oocytes were exposed to 3mg/ml of hyaluronidase for 10-15 min at HCG + 20h

c Group 7: Ovulated oocytes were fixed at HCG + 17h.

8.3.2 Ultrastructural observations of the completion of the second meiotic division in hyaluronidase- and ethanol-induced parthenogenones

Ovulated non-activated oocytes

The meiotic spindle apparatus of 19 non-activated oocytes isolated at HCG+ 17h was consistently found closely aligned with the avillous zone of the plasma membrane (Fig. 8.1) and the polar axes of the spindle were orientated in a tangential plane with respect to the plasma membrane. Electron dense material was observed to be located at the spindle poles in this group. These have recently been identified as foci of microtubule polymerisation (Maro, et al., 1985) and termed the pericentriolar material (PCM). Microtubules extend from the spindle poles to the chromosomes located at the equatorial region of the spindle apparatus. The majority of mitochondria, smooth endoplasmic reticulum (sER) and lipid vesicles were positioned in the cytoplasm subjacent to the spindle apparatus.

Ethanol exposure +15min. incubation in culture medium

The meiotic spindle apparatus of the oocytes in this group were also observed to lie subjacent to the plasma membrane. The chromosomes were still retained at the equator of the meiotic spindle apparatus. It was not possible to clearly identify morphological differences in the arrangement of the microtubules or chromatin in

Key to Figures 8.1 - 8.17:

AVZ:	Avillous zone of the plasma membrane.
PM:	Villous region of the plasma membrane.
CG:	Cortical granules
Chr:	Chromosomes.
Chr A:	Chromatin body.
GC:	Golgi complex.
LV:	Lipid vesicles.
MB:	Midbodies.
Mt:	Microtubules.
Mc:	Mitochondria.
PCM:	Pericentriolar material.
sER:	Smooth endoplasmic reticulum.
YP:	Yolk platelets.
ZP:	Zona pellucida.

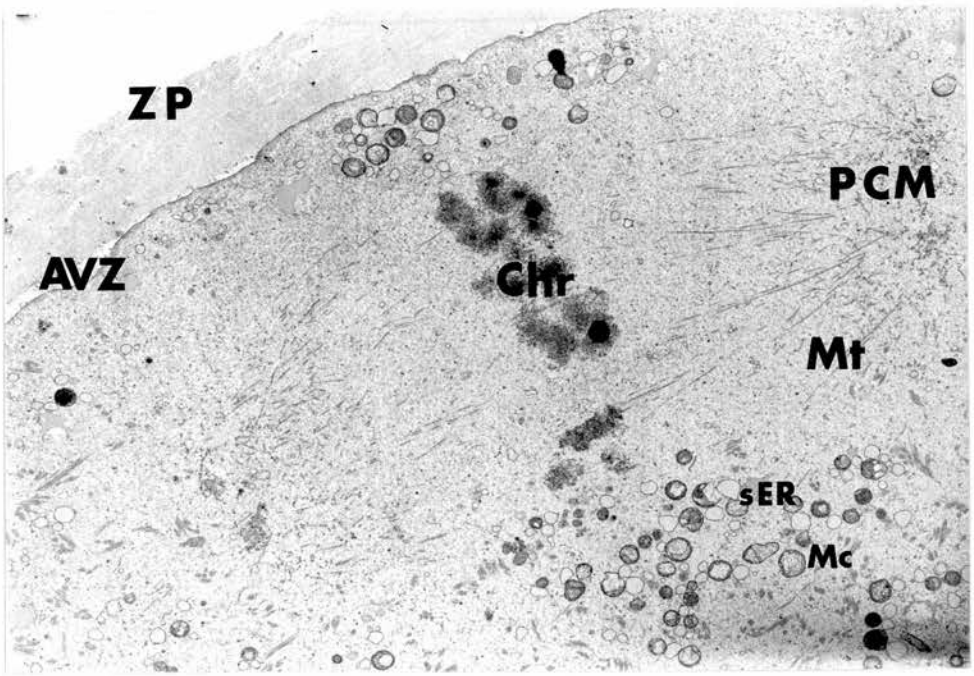
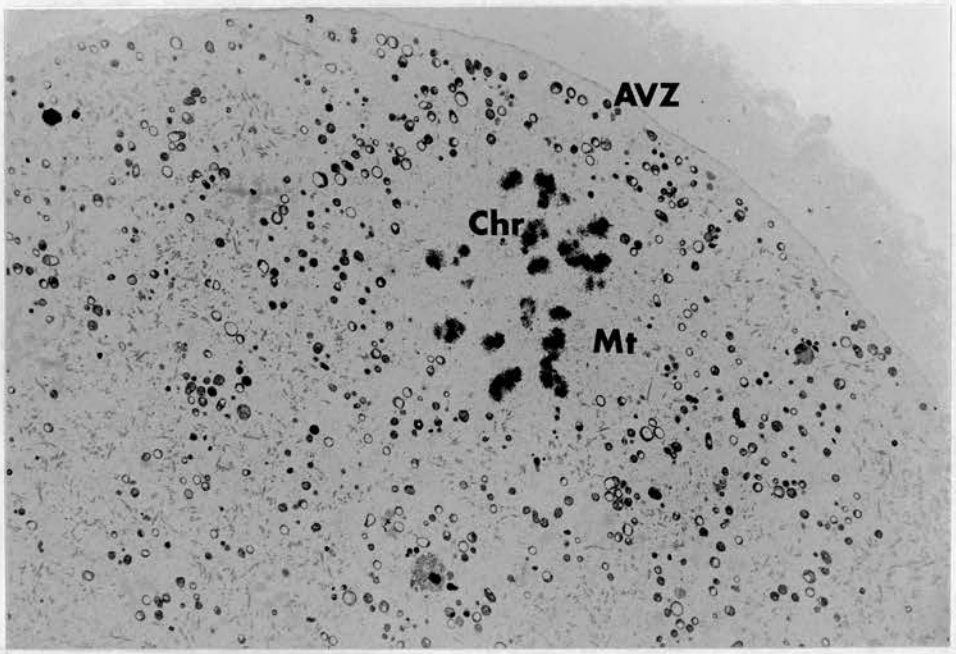


Fig. 8.1. Mag. X 4,300. The spindle apparatus of an nonactivated ovulated oocyte, arrested at metaphase of the second meiotic division and fixed at HCG+ 17h. The spindle apparatus lies in a tangential plane with respect to the avillous zone of the plasma membrane. Several foci of pericentriolar material are situated at the the spindle poles.



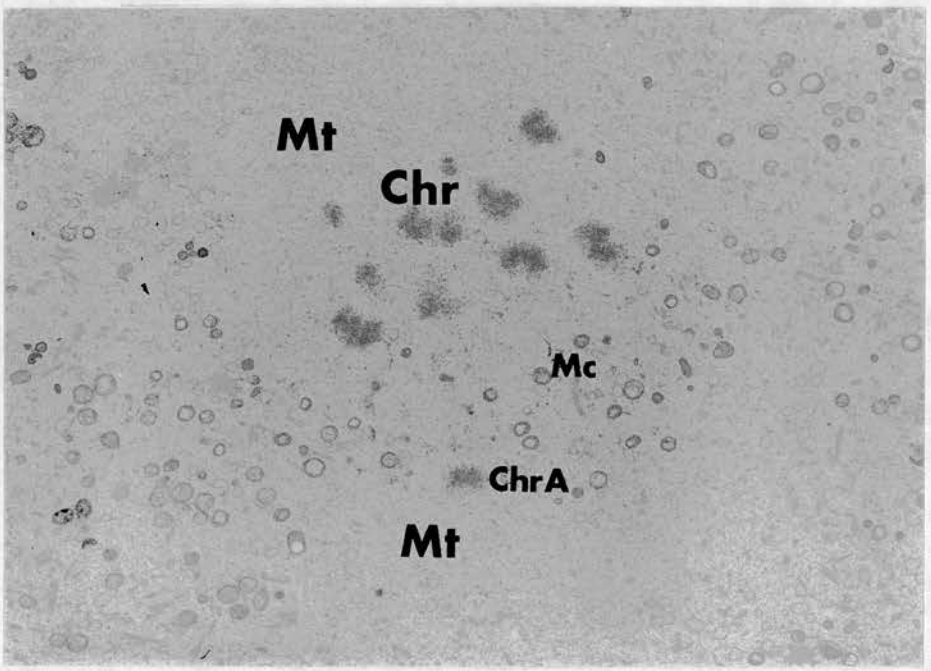


Fig. 8.2 & 8.3. Mag. X 1,800 & X 3,600 respectively. The meiotic spindle apparatus of two ethanol exposed oocytes that were incubated in culture medium for 15 mins. In both oocytes the meiotic spindle apparatus is aligned with the plasma membrane and sectioned in the transverse plane. In Fig. 8.3 one chromosome is displaced from the main body of the spindle apparatus.

activated or non-activated oocytes as a significant degree of centromeric separation or chromosome segregation could not be recognised. In two oocytes sectioned in the transverse plane it was observed that the chromosomes were not restricted to the periphery of the meiotic spindle apparatus (Fig. 8.2). The second of these oocytes exhibited a chromosome that lay outwith the main body of the meiotic spindle and cytoplasmic organelles were observed in the zone between this chromosome and the meiotic spindle apparatus (Fig. 8.3). In these oocytes the lipid vesicles had a stellate morphology and cortical granules were observed to lie subjacent to the villous region of the plasma membrane.

Hyaluronidase activation +30min. incubation in culture medium

The activated oocytes in this group were observed at late anaphase of the second meiotic division (Fig. 8.4). The avillous zone of the plasma membrane overlying the spindle apparatus protruded into the perivitelline space and a close relationship between this region and the meiotic spindle was maintained. Electron dense midbody material was present in the central region of the spindle, and groups of microtubules were organized into distinct arrays at this location. Numerous mitochondria and sER were associated with the spindle pole that was destined to be retained within the oocyte. The site of polar body formation and extrusion was restricted to the

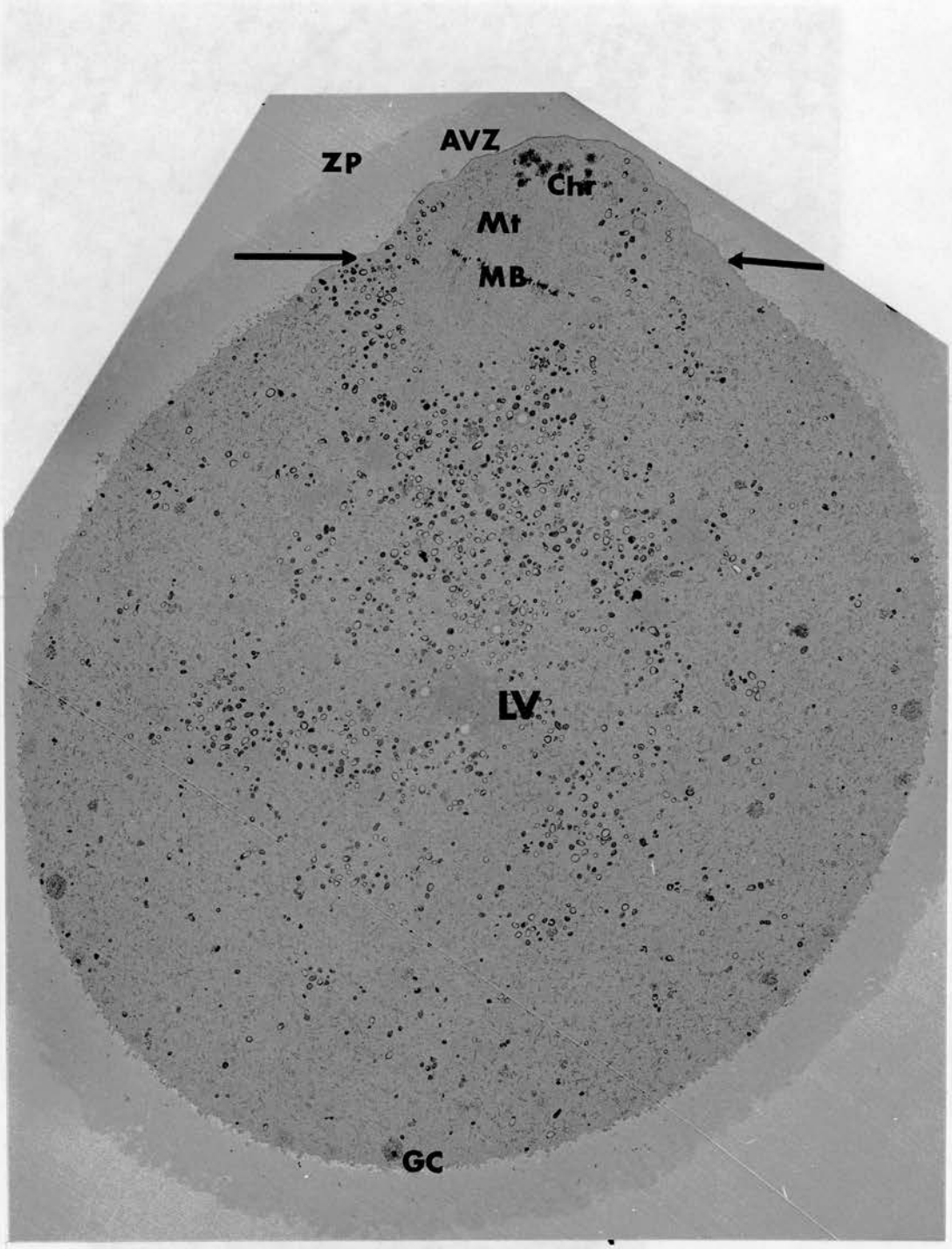
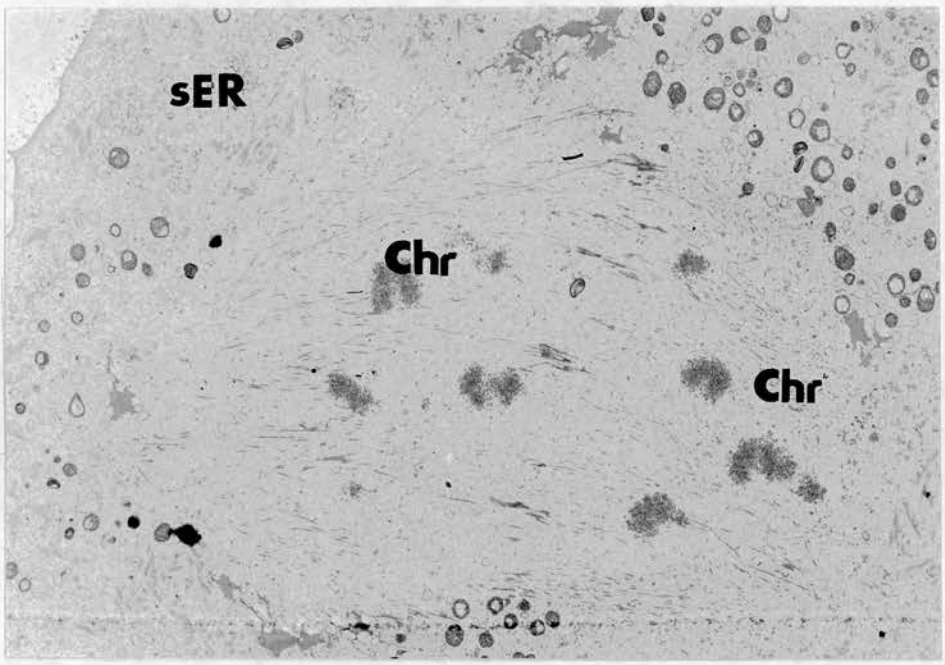


Fig. 8.4. Mag. X 980. Hyaluronidase activated oocyte at late anaphase of the second meiotic division following 30 min. incubation in culture medium. The avillous zone (between arrows) is destined to form the second polar body. The complete spindle apparatus has rotated into a perpindicular alignment with respect to the plasma membrane.

avillous zone of the plasma membrane. Cortical granules, located subjacent to the plasma membrane, were also observed in oocytes at this stage of development. In this control group, only one of the 10 oocytes analysed exhibited an abnormal spindle morphology (Fig. 8.5 & 8.6). In the latter, disordered arrays of microtubules deviated from the periphery of each half of the spindle apparatus but did not appear to be attached to laggard chromosomes.

Ethanol activation +30min. incubation in culture medium

Ethanol activated oocytes at this stage of development were observed at both the early (Fig. 8.7 & 8.8) and late anaphase (Fig. 8.9) of the second meiotic division. Abnormalities in the morphology of the meiotic spindle apparatus were observed in several of the oocytes examined. Three of the 21 activated oocytes exhibited multipolar spindles (Fig. 8.10 & 8.11). Two additional oocytes at late anaphase possessed a lagging chromosome within the midbody region (Fig. 8.12), while in two others the segregation of chromatids to the poles of the spindle appeared to be disordered (Fig. 8.13). The lipid vesicles that surround the spindle apparatus were more electron dense and stellate in appearance than those observed in both the non-activated oocytes and hyaluronidase-induced parthenogenones. Cortical granules were also observed in these activated oocytes(Fig. 8.14 & 8.15)



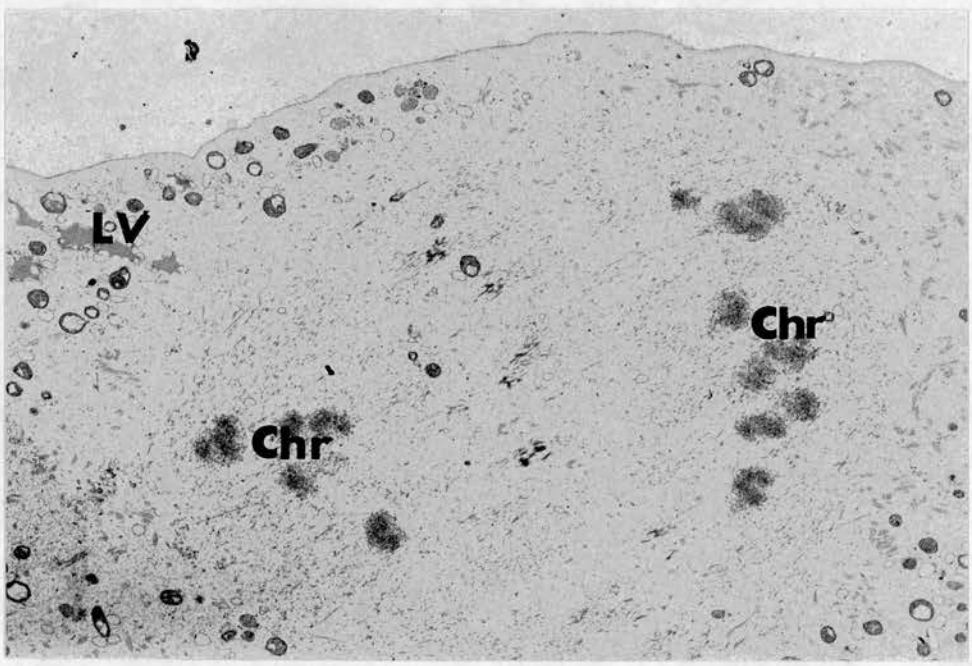


Fig. 8.7 & 8.8. Mag. X 4,300. Meiotic spindle apparatus of two ethanol activated oocytes at anaphase of the second meiotic division following 30 min. incubation in culture medium. The lipid vesicles exhibit a stellate morphology. The mitochondria and vesicles of smooth endoplasmic reticulum are located subjacent to the chromosomes that are to be retained within the oocyte following the completion of the second meiotic division.

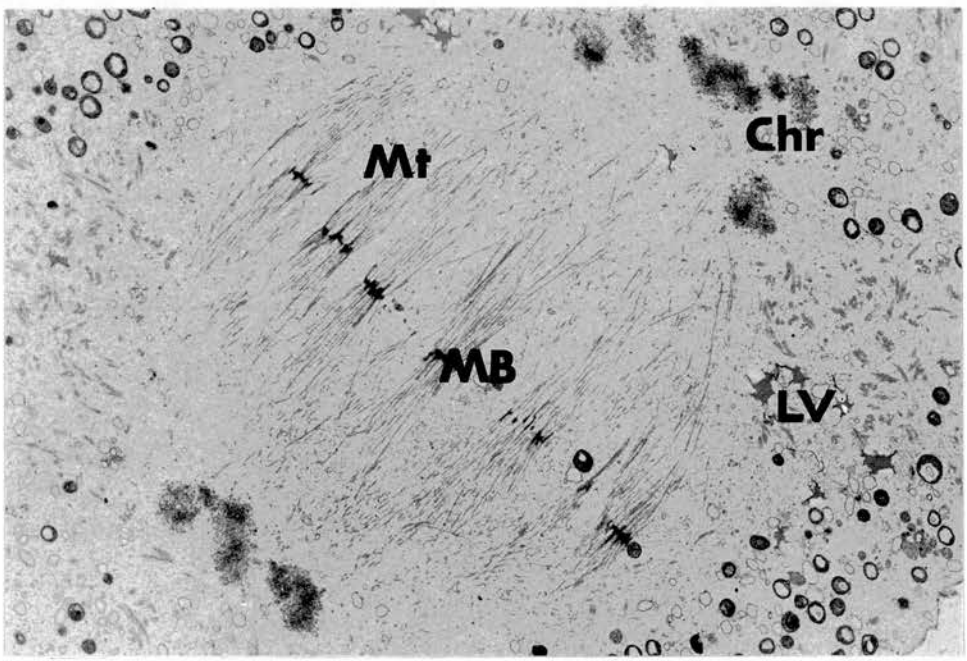
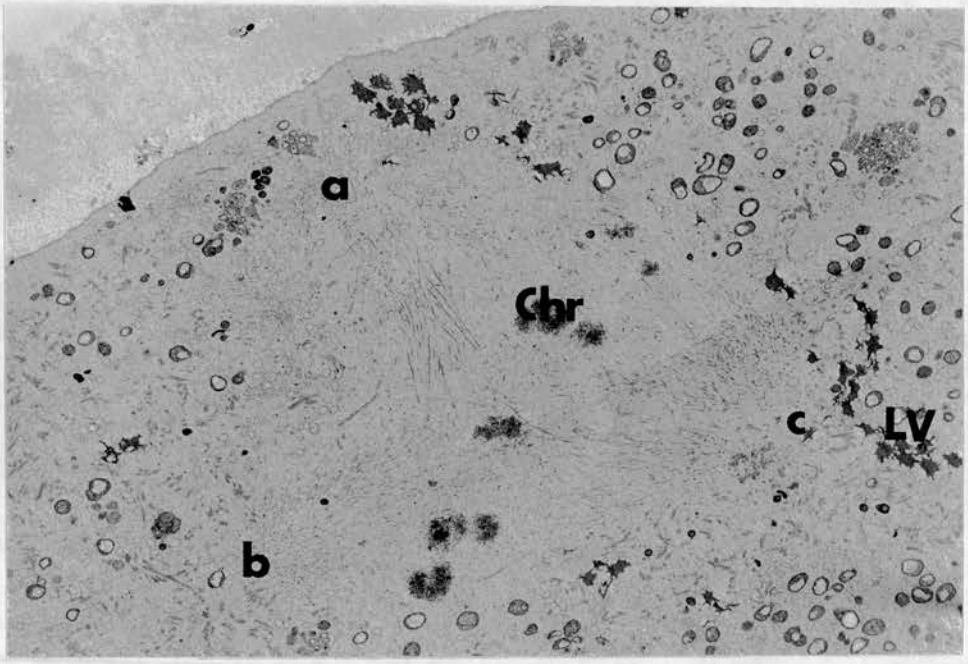


Fig. 8.9. Mag. X 4,300. Meiotic spindle apparatus of an ethanol activated oocyte at late anaphase of the second division following 30 min. incubation in culture medium. Numerous stellate lipid vesicles are present.



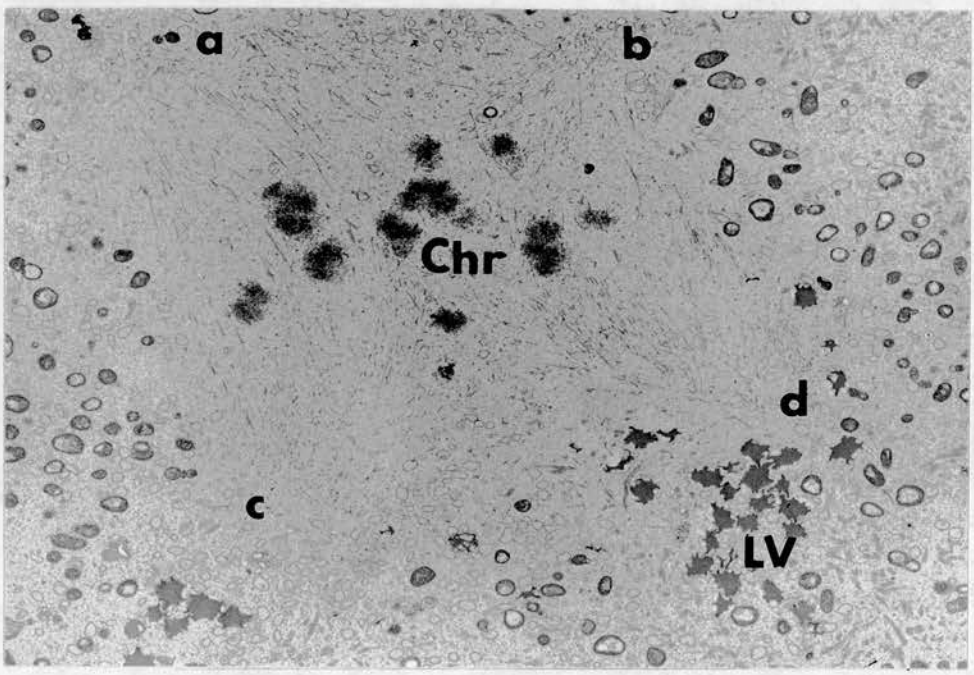


Fig. 8.10 & 8.11 Mag. X 4,300. Meiotic spindle apparatus of two ethanol activated oocytes at anaphase of the second meiotic division following 30 min. incubation in culture medium. The alignment of the chromosomes is disorganised and arrays of microtubules extend to the chromosomes from several distinct spindle poles (a - d). Stellate lipid vesicles are also present.

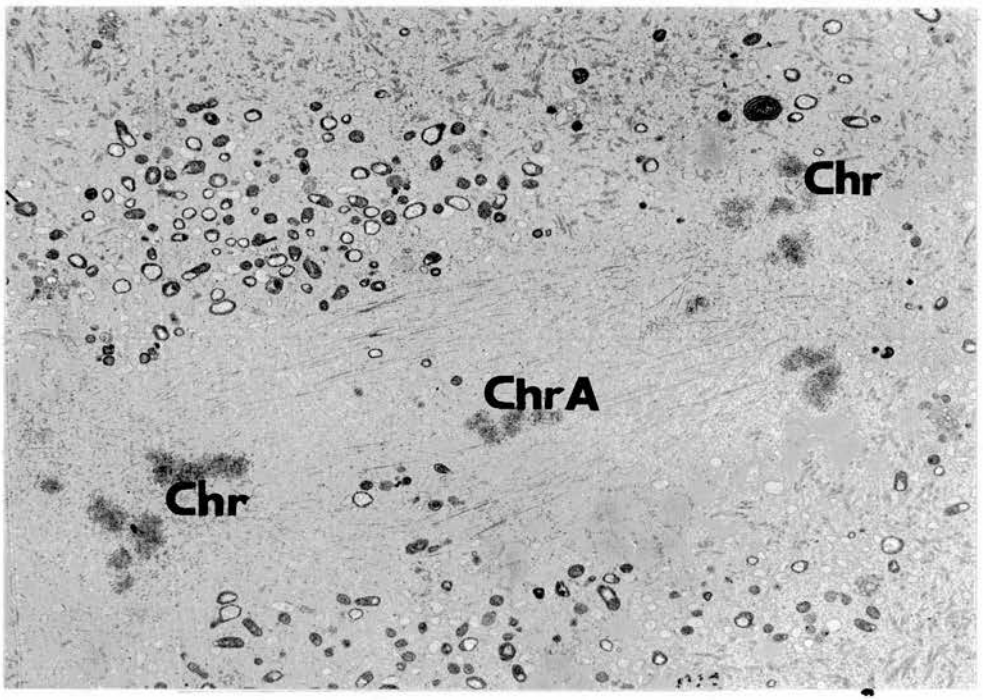


Fig. 8.12. Mag. X 3,600. Meiotic spindle apparatus of an ethanol activated oocyte at late anaphase of the second meiotic division following 30 min. incubation in culture medium. One chromatin body appears to be lagging behind and is located at the equator of the meiotic spindle apparatus.

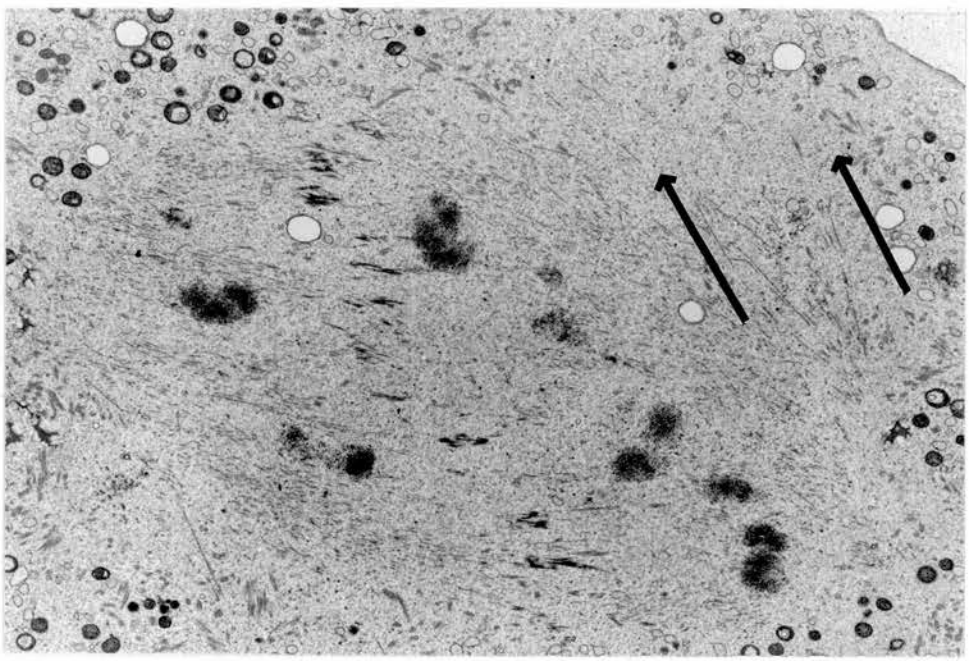
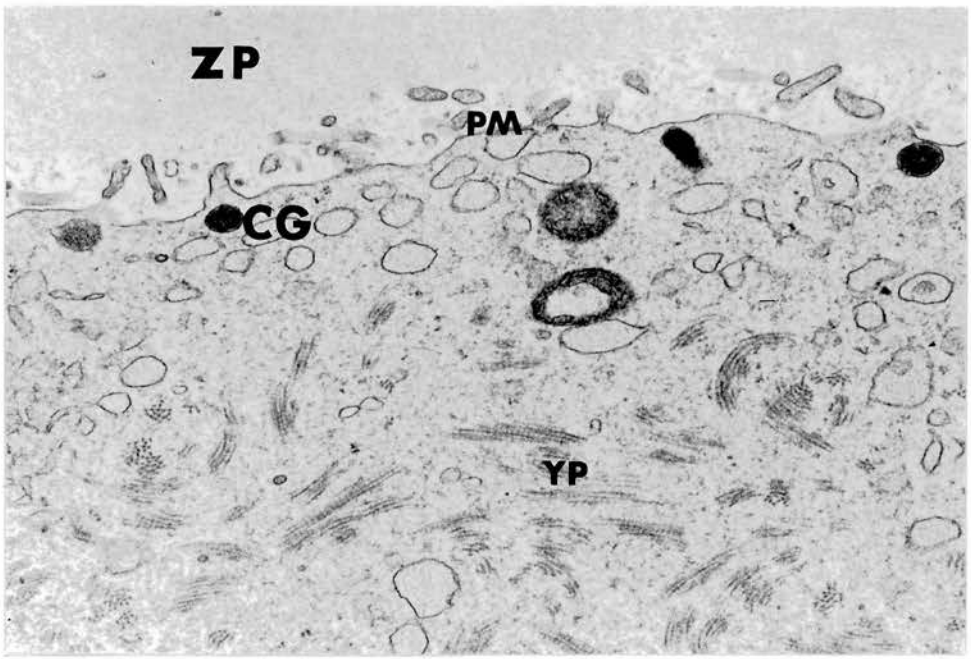


Fig. 8.13. Mag. X 4,300. Meiotic spindle apparatus of an ethanol activated oocyte at anaphase of the second meiotic division after 30 min. incubation in culture medium. The location of the midbodies is irregular and the pole - midbody - pole length is not uniform. Arrays of microtubules also extend outwith the main body of the spindle apparatus (arrows).



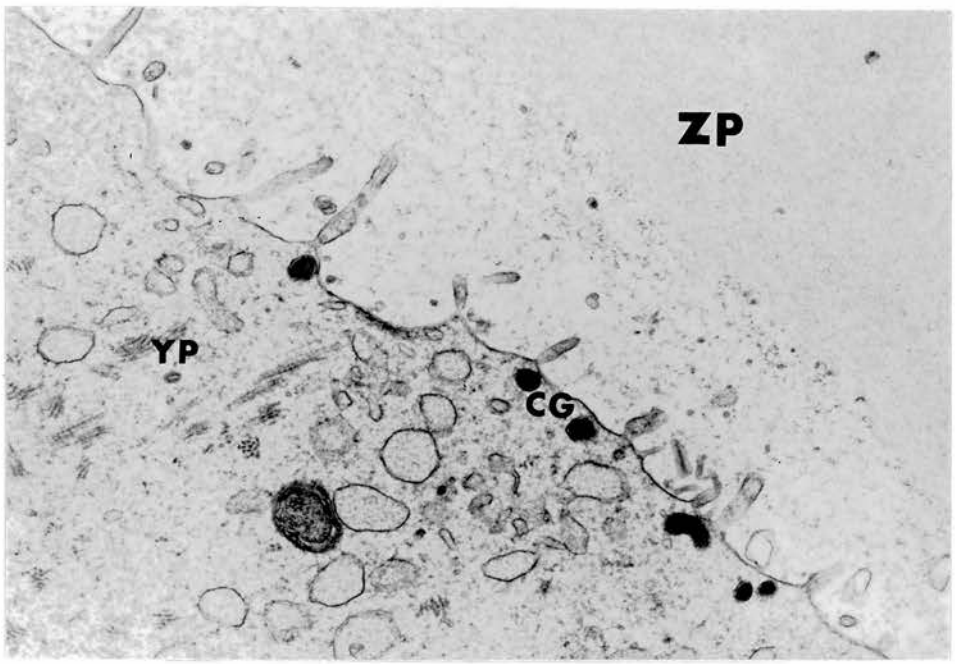


Fig. 8.14 & 8.15. Mag. x 18,000. The villous region of plasma membrane of two ethanol activated oocytes that were incubated in culture medium for 30 min. Numerous cortical granules are located in close proximity with the plasma membrane of both parthenogenones.

Hyaluronidase activation +75min. incubation in culture medium.

The activated oocytes in this group were at telophase of the second meiotic division and all oocytes exhibited a pronounced extrusion of the avillous zone of the plasma membrane that would later form the second polar body (Fig. 8.16) The main cytoplasmic constituents of the polar body were microtubules and chromatin. The latter was located at the pole furthest from the oocyte while the midbody region was situated at the narrow junction between the oocyte and the polar body. Spindle abnormalities were not observed in any of the 13 activated oocytes in this group.

Ethanol activation +60min. and +75min. incubation in culture medium.

The activated oocytes in these two experimental groups were at the same developmental stage as the oocytes isolated 75min. after hyaluronidase activation (Fig. 8.10). Spindle abnormalities were not observed in the 5 oocytes fixed after 60 min incubation in culture. However abnormal polar body formation was observed in one of the activated oocytes fixed after 60min and in two of the 9 oocytes that had been fixed after 75 min. incubation in culture medium. In these specimens the protruding region of the plasma membrane was larger than normal, included numerous cytoplasmic organelles and the spindle apparatus

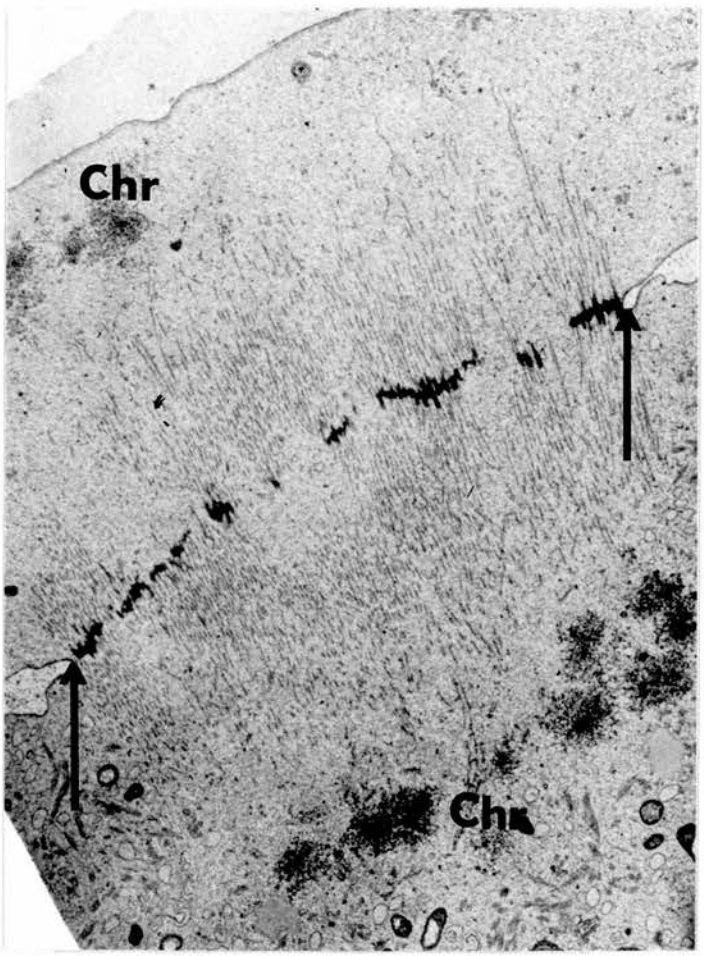


Fig. 8.16. Mag. X 5,900. Meiotic spindle apparatus of a hyaluronidase activated oocyte at telophase of the second meiotic division following 75 min. incubation in culture medium. One set of chromatids is retained within the oocyte. The other is located subjacent to the avillous zone of the plasma membrane and will be extruded in the second polar body. The midbodies are positioned at the site of excision between the oocyte and the developing polar body (arrows).

was not aligned with the site of cytokinesis. (Fig. 8.17).

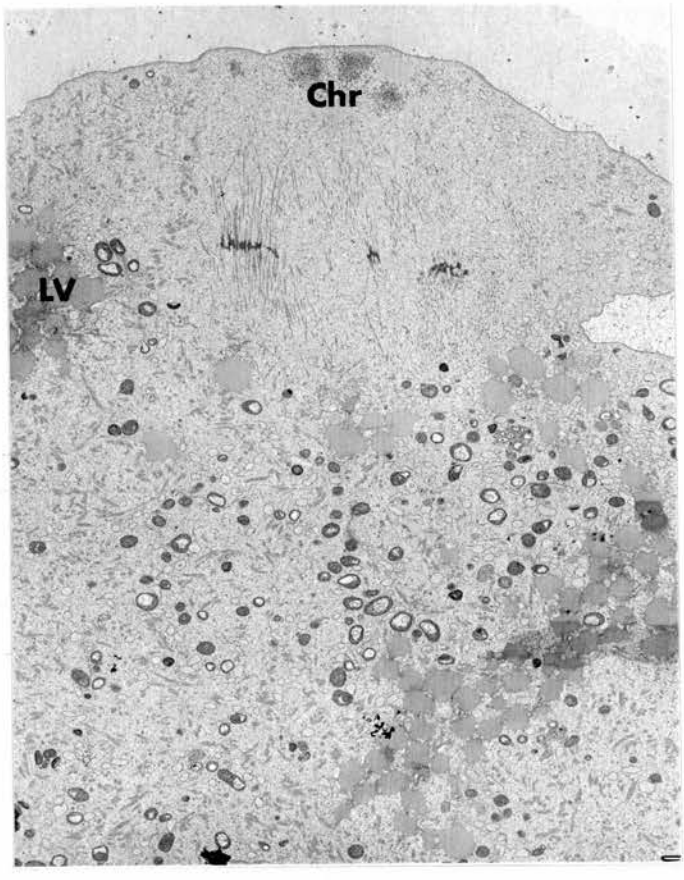


Fig. 8.17. Mag. X 3,600. Meiotic spindle apparatus of an ethanol activated oocyte at telophase of the second meiotic deivision following 75 min. incubation in culture medium. The chromosomes to be expelled with the polar body are seen in this micrograph. The avillous zone of the plasma memebrane in this oocyte is abnormally extensive. The numerous vesicles that are present within this region appear to have displaced the spindle apparatus towards the the periphery of the oocyte from its normal location at the junction between the oocyte and the developing second polar body. The lipid vesicles do not exhibit a pronounced stellate morphology.

8.4 DISCUSSION

The temporal and spatial alterations to the organisation and location of the meiotic spindle apparatus, cytoskeletal elements and cytoplasmic organelles in murine oocytes from the resumption of meiosis at germinal vesicle breakdown (GVBD) to ovulation (Zamboni, 1970; Wasserman and Fujiwara, 1978; Longo and Chen, 1985; Maro, et al., 1985; Van Blerkom and Bell, 1984, 1986) and subsequent fertilisation (Maro, et al., 1984, 1986) or postovulatory ageing and degeneration (Szöllősi, 1975; Webb, et al., 1986; Eichenlaub-Ritter et al., 1986) have been examined by transmission and scanning electron microscopy, phase contrast optics and both immunofluorescent and immunocytochemical localisation techniques. These studies have consistently found that within 2-4h of GVBD the dictyate stage chromosomes progress to metaphase of the first meiotic division, promote the polymerisation of microtubules and become aligned at the equator of a meiotic spindle. The complete meiotic spindle apparatus is then translocated from the central region of the oocyte to lie subjacent to the plasma membrane. The first meiotic division occurs 6-8h after GVBD and the completion of the second meiotic division follows shortly after fertilisation or parthenogenetic activation. The female genome maintains a peripheral location until the female pronucleus of either fertilised one-cell embryos or parthenogenones migrate

towards a more central position within the oocyte towards the completion of the first cell cycle. The cortical zone of cytoplasm between the meiotic spindle apparatus and the overlying plasma membrane has been found to be rich in actin filaments and to exhibit a lesser number of cytoplasmic organelles than the remainder of the oocyte. The region of the plasma membrane that overlies this zone is devoid of microvilli and the extrusion of the first and second polar bodies is normally restricted to this avillous zone (AVZ) of the oocyte (Maro, et al., 1984; 1986). The formation of the AVZ is reported to be induced by the subcortical presence of meiotic chromosomes. Furthermore, individual meiotic chromosomes, displaced from the main body of the meiotic spindle apparatus, retain the ability to promote their own meiotic spindle, to induce an overlying AVZ and to extrude a polar body. However, the nature of the mechanisms by which meiotic chromosomes actively regulate cytoplasmic differentiation have yet to fully established (Van Blerkom and Bell, 1986). Although the mechanism of second polar body formation in fertilised one-cell embryos has been analysed using immunofluorescent and immunocytochemical techniques, the present study represents the first ultrastructural analysis of the cytokinetic and karyokinetic events in hyaluronidase and ethanol activated oocytes.

The ultrastructural organisation of the nonactivated

oocytes isolated at HCG+ 17h was not observed to differ from that previously described by the authors cited above. The cytoplasm between the meiotic spindle and the plasma membrane was found to be relatively free of organelles. Mitochondria were located within this region, but only in association with the chromosomes at the equatorial zone of the spindle. In these oocytes, the mitochondria were ovoid in appearance and possessed a large vesicle that displaced the cristae to the periphery of these organelles. This unusual mitochondrial structure has been observed in the oocytes of several species (Thibault, Szöllösi and Gérard, 1987). Szöllösi (1971, 1975) has reported that the meiotic spindle of unfertilised oocytes migrates from its initial position subjacent to the plasma membrane as the postovulatory age of the oocyte exceeds HCG+ 18h. However, in this study, evidence of this phenomenon was not observed in the non-activated ovulated oocytes isolated at HCG+ 17h.

Numerous studies have demonstrated that the frequency of parthenogenetic activation induced by ethanol (Kaufman, 1982; O'Neill and Kaufman, 1989a, Chapter 4) or hyaluronidase (Kaufman, 1973a; O'Neill and Kaufman, 1988, Chapter 3) is usually in the range of 70-90%, and that the majority of the activated oocytes develop as 1PN parthenogenones. A slightly lower frequency of oocyte activation was observed in this study, possibly because an assessment of the activation frequency was made from

observations of the changes in the ultrastructure of the exposed oocyte within a limited period of time after exposure to the activating stimuli. In other parthenogenetic studies the activation frequency has normally been determined from the proportion of activated oocytes that develop pronuclei.

Activated oocytes were identified as those that exhibited evidence of chromosome segregation, spindle rotation and extrusion of the avillous region of the plasma membrane. The first of these changes in the activated spindle was not clearly observed until at least 30 minutes after exposure to either ethanol or hyaluronidase. The overall sequence of alterations that were observed in the ultrastructural organisation of the cytoskeleton and meiotic spindle apparatus in the majority of ethanol and hyaluronidase activated oocytes did not differ from that previously reported to occur in fertilised oocytes examined by phase contrast optics and immunofluorescent localisation techniques (Sato and Blandau, 1979; Maro, et al., 1984, 1986; Howlett, Webb, Maro and Johnson, 1985).

The fusion of the cortical granules with the plasma membrane, followed by the release of their proteolytic enzyme contents into the perivitelline space, occurs during fertilisation and is believed to prevent polyspermy (Szőllősi, 1967). However, in these studies, the cortical granules were retained within the ethanol

and hyaluronidase activated oocytes. This demonstrates that parthenogenetic activation fails to induce complete cortical granule exocytosis and that, complete cortical granule exocytosis is probably not required for subsequent parthenogenetic development. Incomplete cortical granule exocytosis has also been reported to occur in the ovulated oocytes of several other rodent species exposed to parthenogenetic stimuli (Kaufman, 1983c). The persistence of cortical granules within the cytoplasm of parthenogenones is a further indication that the sequence of electrophysiological events that stimulate the resumption of the second meiotic division are not totally homologous with those that occur following fertilisation.

The hyaluronidase activated oocytes that were incubated in culture medium for 30 minutes before being processed for electron microscopy were found to be at late anaphase of the second meiotic division. Ethanol activated oocytes that were retained in cuture medium for the same period of time were observed at both early and late meiotic anaphase. The lower proportion of ethanol activated oocytes that had progressed to late anaphase after 30 minutes incubation may indicate that the rate of the second meiotic division is briefly retarded in a proportion of these oocytes. The small number of activated oocytes examined in the present investigation prevents a more quantitative anaysis of this phenomenon

to be made. In addition, it is possible that interference with the rate of anaphase chromosome movement in ethanol activated oocytes is a factor that serves to predispose chromosomes to malsegregation. These differences may also be due, in part, to the activation procedures used. The postovulatory age of the oocytes exposed to hyaluronidase was greater than those in the ethanol activation group and this may have increased the ability of these oocytes to initiate parthenogenesis.

The arrangement of the microtubules and midbodies in the meiotic spindles of hyaluronidase and ethanol activated oocytes at anaphase and telophase did not differ considerably. However, abnormalities in the alignment of the spindle fibers and/or chromosome segregation at anaphase were almost invariably confined to the ethanol-activated oocytes. Multipolar spindles, disorganised anaphase chromosome movement and the persistence of chromatin in the central region of late anaphase spindles, were the principal forms of spindle anomaly observed. It is significant that similar spindle disorders have also been induced following the exposure of both meiotic and mitotic spindles to low doses of Colcemid (La Fontaine, 1985; Selletto and Kuriyama, 1988) or colchicine (Sugawara and Mikamo, 1980). However, there is no evidence to indicate that ethanol binds directly to tubulin. The findings of this ultrastructural analysis indicate that ethanol-induced dysfunction of microtubule

dynamics may be one of the initial events in the sequence that leads to the development of aneuploid parthenogenones. Ethanol exposure did not induce a direct structural change in the morphology of individual microtubules. The occurrence of spindle abnormalities in the present study was higher than the incidence of aneuploidy commonly encountered in ethanol-induced parthenogenones, and this indicates that all spindle "abnormalities" do not invariably induce chromosome segregation errors (Ford and Roberts, 1983a). The ethanol-induced spindle abnormalities were not obvious until at least 30min. after activation. This would seem to suggest that a minimum period must elapse before a change in the normal ultrastructural morphology of the spindle apparatus is apparent and as discussed in Chapter 4, it would appear that aneuploidy is induced by the ability of this agent to disrupt the Ca^{2+}-mediated regulation of chromosome segregation and not by a direct interaction with the spindle elements.

The ethanol and hyaluronidase activated oocytes developed to telophase of the second meiotic division after 60 - 75 minutes incubation in culture medium. However, the morphology of the extruded avillous zone of the plasma membrane in several of the ethanol activated oocytes isolated at these stages of development differed to that observed in the hyaluronidase activated oocytes. In the abnormal ethanol-induced parthenogenones the extruded

zone of the plasma membrane was not totally restricted to the avillous zone, the spindle apparatus was not closly aligned with the site of cytokinetic constriction and numerous organelles had become included in the region destined to form the second polar body. The regulation of actin filament dynamics, necessary for the process of cytokinesis (Karasiewicz, 1981, Karasiewicz and Slotynska, 1986) is also a calcium dependent process (Schliwa, 1981; Mooseker, Coleman and Conzelman, 1986), and the finding that ethanol activated oocytes also exhibited anomalies of polar body formation indicates that this process is also sensitive to the influence of ethanol. However, it is not known if these anomalies could induce aneuploidy, but would, if polar body extrusion fails to occur, predispose the oocyte to retain both genomic products of the second meiotic division. The incidence of second polar body extrusion is significantly reduced when the duration of ethanol exposure exceeds 5min. (Kaufman, 1982; O'Neill and Kaufman, 1989a, Chapter 4).

The deformities in the ultrastructural morphology of the spindle structure may induce chromosome malsegregation if microtubular disorder persists throughout the completion of the second meiotic division. This report therefore represents the first direct ultrastructural analysis of induced abnormalities in the spindle apparatus of oocytes stimulated to complete the second meiotic division, and

strongly suggests that exposure to ethanol and other spindle-active agents in vivo may lead to the production of aneuploid gametes. If similar events occur in human oocytes, this may give rise to genetically unbalanced conceptuses, many of which are destined to be spontaneously aborted during the first trimester of pregnancy.

CHAPTER 9

THE INFLUENCE OF AN INTRAGASTRIC INJECTION OF A DILUTE ETHANOL SOLUTION ON THE SEGREGATION OF MEIOTIC CHROMOSOMES IN F1 HYBRID FEMALE MICE

CONTENTS

9.1 Introduction

9.2 Methods

9.3 Results

9.3.1 The influence of an intragastric injection of a dilute ethanol solution to female mice during the first meiotic division on the frequency of parthenogenetic activation *in vitro*

9.3.2 The influence of an intragastric injection of a dilute ethanol solution to female mice during the first meiotic division on the chromosome constitution of hyaluronidase activated 1PN parthenogenones

9.3.3 The karyotypic analysis of hyaluronidase activated oocytes that were exposed to ethanol during the first meiotic division

9.3.4 The influence of an intragastric injection of a dilute ethanol solution to female mice during the first meiotic division on the frequency of

hyaluronidase activated oocytes induced to develop as 2PN parthenogenones *in vitro*

9.3.5 The influence of an intragastric injection of a dilute ethanol solution to female mice during the first meiotic division on the chromosome constitution of hyaluronidase activated 2PN parthenogenones

9.3.6 The influence of an intraperitoneal injection of a standard dose of Avertin to female mice during the first meiotic division on the frequency of parthenogenetic activation *in vitro*

9.3.7 The influence of an intraperitoneal injection of a standard dose of Avertin to female mice during the first meiotic division on the chromosome constitution of hyaluronidase activated 1PN parthenogenones.

9.4 Discussion

9.1 INTRODUCTION

The preceding studies have clearly demonstrated that the ovulated mouse oocytes of F1 hybrid and LT/Sv strain mice exhibit a high frequency of parthenogenetic activation when exposed briefly to a dilute solution of ethanol *in vitro*. The majority of activated oocytes were observed to develop as 1PN parthenogenones and cytogenetic analyses of this parthenogenetic class have found that a significant proportion exhibited an aneuploid chromosome constitution (Kaufman, 1982). O'Neill and Kaufman (1989a) have proposed that under these *in vitro* conditions the chromosome malsegregation errors were induced by the ability of this agent to disorganise the Ca^{2+}-dependent regulation of microtubule dynamics.

In Chapter 5, parthenogenetic activation *in vivo* was not observed following the intragastric administration of 1ml of a 12.5% ethanol solution to superovulated female mice at either HCG+ 18h or HCG+ 20h. Activation presumably failed to occur as a much higher concentration of blood ethanol, equivalent to the ethanol LD_{50} for the mouse, is normally required to induce even a low incidence of parthenogenetic activation *in vivo* (Dyban and Baranov, 1987). Several attempts to induce a high incidence of ethanol-induced parthenogenesis *in vivo* by exposing ovulated oocytes to ethanol by intraperitoneal injection

have been, overall, unsuccessful as ethanol was found to be toxic to the ovulated oocytes under these conditions (Dyban and Khozhai, 1980; Chapter. 5).

A dilute solution of ethanol, administered by intragastric injection to female mice, may be unable to induce parthenogenesis in vivo but has been found to maintain the ability to interfere with the segregation and movement of meiotic chromosomes. Several cytogenetic studies (Kaufman, 1983b; Kaufman and Bain, 1984; O'Neill and Kaufman, 1987a) have demonstrated that the intragastric injection of a dilute solution of ethanol to female mice during the first meiotic division or at the sperm-activated completion of the second meiotic division can significantly increase the frequency of fertilised one-cell mouse embryos that exhibit numerical chromosome anomalies in the female-derived genome at metaphase of the first cleavage mitosis. The intragastric administration of ethanol to female mice during the first or second meiotic division was not observed to significantly increase the incidence of triploidy. It has been proposed that under these conditions ethanol interferes with the ability of the microtubular components of the meiotic spindle apparatus to effect the proper segregation of chromosomes (Kaufman, 1985; Kaufman and O'Neill, 1988).

The intragastric injection of the dilute solution of

ethanol to female mice has been found to induce anaesthesia for 1 - 1.5h. The segregation of chromosomes at the metaphase-anaphase transition of the second meiotic division proceeds rapidly after oocyte activation and is complete after a period of approximately 15 minutes (Chapter 8, O'Neill, McDougall and Kaufman, 1989b). The mechanism by which ethanol exposure in vivo disrupts chromosome segregation at conception is believed to be comparable to the events that have been encountered during ethanol-induced parthenogenesis (Chapters, 4 and 8).

In contrast, primary oocytes were exposed to high blood concentrations of ethanol and its metabolites for several hours when female received an intragastric injection of dilute ethanol. Under these conditions, the mechanism by which ethanol interferes with chromosome segregation during this stage of meiosis may differ to that which occurs at fertilisation. The exposure of ovulated oocytes to ethanol at the time of conception interferes with chromosome segregation and movement during the completion of the second meiotic division. The administration of ethanol to female mice at specific periods during the first meiotic division interfers with both metaphase chromosome alignment on the meiotic spindle apparatus and their subsequent separation at anaphase I. Furthermore, chromosome segregation at this division involves the separation of the bivalent chromosome arrangement and is

not dependent on the centromeric separation of sister chromatids.

Aim of Investigation

In previous studies (Kaufman, 1983b; Kaufman and Bain, 1984; O'Neill and Kaufman, 1987a) the ability of an intragastric injection of a dilute ethanol solution to induce chromosome segregation errors during the first meiotic division or at the sperm-activated completion of the second meiotic division has been examined by an analysis of the chromosome constitution of the resultant fertilised one-cell embryos at metaphase of the first cleavage mitosis.

In the following series of experiments, the trisomogenic potential of ethanol is examined by a cytogenetic analysis of the ethanol exposed oocytes that subsequently develop as hyaluronidase activated 1PN and 2PN parthenogenones. This approach has several advantages over previous studies in which meiotic chromosome segregation errors were detected as numerical chomosomal anomalies in the metaphase spreads of fertilised one-cell embryos. The incidence of mating was considerably reduced when female mice were administered an intragastric injection of ethanol at HCG+ 2h and especially at HCG+ 4h. Matings did not occur if ethanol was administered to the female mice at HCG+ 6h or HCG+ 8h. This reduced the opportunities to analyse the relationship between ethanol

ethanol exposure and chromosome segregation errors in those studies that depended on the isolation and cytogenetic analysis of fertilised one-cell embryos. This was especially the case in those experiments where ethanol was administered during the later stages of the first meiotic division. Ethanol has been considered as a contributary factor in the aetiology of human aneuploidy (Kaufman, 1985, Kaufman and O'Neill, 1988). As the majority of the chromosome segregation errors in human oocytes occur during the first meiotic division it is a fundamental necessity of this mouse model to examine the trisomogenic potential of ethanol during the first meiotic division. In the following study, ovarian oocytes are exposed to ethanol at HCG+ 4h to HCG+ 8h as both metaphase chromosome alignment and separation occur during this period (see, Edwards and Gates, 1959). The present model also permits the activation and subsequent cytogenetic analysis of those mouse oocytes that have otherwise remained unfertilised in previous studies.

A preliminary karyotypic analysis of the ethanol-induced aneuploid metaphase chromosome preparations of fertilised one-cell mouse embryos did not identify the specific chromosomes involved in the majority of malsegregation events (O'Neill and Kaufman, 1987a). The problem was largely a technical one, since the presence of residual cytoplasm adjacent to either one or both of the parental genomes inhibited the development of uniform G-band

patterns. The karyotypic analysis was also made more difficult as the incidence of aneuploidy observed in the above study was lower than that previously observed by Kaufman (1983b) and Kaufman and Bain (1984). A higher proportion of the metaphase chromosome preparations from 1PN parthenogenones have been observed to exhibit good quality G-band patterns (O'Neill and Kaufman, 1989a, Chapter 4). This appears to be simply due to the higher probability of producing G-bands in the metaphase chromosomes of only one maternally-derived genome instead of two, often widely separated, parental genomes.

The following parthenogenetic study has the potential to examine if in vivo ethanol exposure during the first meiotic division influences postovulatory age-related changes in ovulated oocytes and whether this interferes with the initiation of parthenogenetic activation. This study also aims to provide an analysis of the mechanisms by which ethanol-induced meiotic chromosome segregation errors arise when primary oocytes are exposed to this agent. The cytogenetic analysis of both the 1PN and 2PN classes of parthenogenone also has the potential to identify the specific chromosomes involved in malsegregation events in a higher proportion of in vivo ethanol exposed oocytes than previously observed. The ability of Avertin anaesthesia to induce chromosome segregation errors when oocytes are exposed to these agents in vivo is also examined.

9.2 METHODS

Hyaluronidase-induced parthenogenetic activation

Ten- to 12-week old F1 hybrid female mice were induced to superovulate as described in Chapter 2.2. At specific times after the HCG injection the mice were administered either a dilute solution of ethanol or Avertin as described below. The cumulus masses, containing the ovulated oocytes, were isolated from these superovulated mice at either HCG+ 20h, 22h, or 24h. They were incubated in microdrops of M16 culture medium supplemented with 3mg/ml of hyaluronidase for 10-15min., as described in Chapter 2.4.1, to induce parthenogenetic activation. The oocytes were then transferred to microdrops of equilibriated M16 culture medium for 6h. At the end of this period the activation frequency was determined and the parthenogenones were transferred to fresh microdrops of M16 medium.

Ethanol administration

A 1ml solution of 12.5% (v/v) ethanol or 1ml of distilled water (control) was injected via a fine plastic tube into the stomach of superovulated F1 female mice as described in Chapter 2.5.1 at HCG+ 4h, 6h, or 8h.

Avertin administration

A standard dose of Avertin anaesthesia (Chapter 2.4.4) or

0.4ml of Dulbecco's PBS (control) was administered to superovulated female F1 hybrid mice by intraperitoneal injection at HCG+6h.

Cytochalasin D-induced hyaluronidase activated 2PN parthenogenones

An additional group of superovulated mice were administered either a 1ml 12.5% (v/v) ethanol solution or 1ml of distilled water (control) at HCG+ 4h or 6h. The oocytes were incubated in equilibriated microdrops of M16 medium supplemented with 3mg/ml of hyaluronidase at HCG+ 22h to induce parthenogenetic activation (Chapter 2.4.1). The oocytes were then transferred initially to microdrops of M16 medium supplemented with 1ug/ml of cytochalasin D for 4h to inhibit second polar body extrusion (Chapter 2.4.5). At the end of this period the cytochalasin-D exposed oocytes were transferred to fresh microdrops of M16 culture medium. The majority of activated oocytes developed as 2PN parthenogenones and were transferred to fresh microdrops of M16 culture medium after the activation frequency was determined.

Preparation of chromosome spreads from 1PN and 2PN parthenogenones at metaphase of the first cleavage mitosis

The majority of the metaphase chromosome spreads prepared from hyaluronidase activated oocytes that were exposed to ethanol in vivo during the first meiotic division were

prepared following the Colcemid-induced arrest of parthenogenones at metaphase of the first cleavage mitosis as described in Chapter 2.6.2. The metaphase chromosome spreads of oocytes exposed to ethanol in vivo at HCG+ 4h, 6h and 8h and induced to initiate parthenogenesis at HCG+ 20h (9.3.2.; Table 9.5) were prepared during metaphase of the first cleavage mitosis as described in Chapter 2.6.1. The metaphase chromosome spreads of the oocytes that were exposed to ethanol in vivo at HCG+ 4h and then induced to initiate parthenogenesis at HCG+ 22h were also prepared by this method. All chromosome spreads of hyaluronidase activated oocytes exposed to cytochalasin D for 4h were prepared following the Colcemid-induced arrest of the 2PN parthenogenones at metaphase of the first cleavage mitosis. The chromosome spreads of the hyaluronidase activated oocytes that were exposed to Avertin in vivo during the first meiotic division were also prepared following the Colcemid-induced metaphase arrest.

9.3 RESULTS

9.3.1 The influence of an intragastric injection of a dilute ethanol solution to female mice during the first meiotic division on the frequency of parthenogenetic activation in vitro

The mean number of oocytes isolated from superovulated F1 hybrid female mice was 27.3± 1.6 (n=47) per mouse. When female mice received an intragastric injection of 1ml 12.5% (v/v) of ethanol at either HCG+ 4h, 6h or 8h the mean number of ovulated oocytes per superovulated mouse was reduced to 22.0± 2.17 (n=19), 23.0± 1.14 (n=58) and 22.1± 1.7 (n=18), respectively.

A very high incidence of parthenogenetic activation was observed (81.1% - 97.1%) when ovulated oocytes, isolated from F1 hybrid mice that received an intragastric injection of 1ml of distilled water at either HCG+ 4h, 6h, or 8h, were briefly incubated in M16 medium supplemented with 3mg/ml of hyaluronidase at either HCG+ 20h, 22h, 24h (Table 9.1 Groups 1-3).

A reduced incidence of hyaluronidase-induced parthenogenetic activation at HCG+ 20h, 22h and 24h was consistently observed when the activated ovulated oocytes were isolated from female mice administered an

TABLE 9·1

The influence of an intragastric injection of distilled water to F1 hybrid female mice during the first meiotic division on the frequency of hyaluronidase-induced parthenogenetic activation.

Group	Administration of distilled water	Postovulatory age of oocytes at activation(h)	Number of ovulated oocytes	Number of activated oocytes	Class of Parthenogenone				Frequency of activation (%)
					1PN	2PN	IC	1PND	
1	1ml dist. water at	20	107	88	70	17	1	0	82·2
	HCG+ 4h, i.g.	22	25	22	17	5	0	0	81·1
		24	95	92	61	26	5	0	96·8
2	1ml dist. water at	20	29	27	25	2	0	0	93·1
	HCG+ 6h, i.g.	22	34	33	29	3	1	0	97·1
		24	113	97	37	56	4	0	85·8
3	1ml dist. water at	20	169	148	111	34	3	0	87·6
	HCG+ 8h, i.g.	22	103	84	67	16	1	0	81·6
		24	134	123	55	51	13	4	91·8

The proportionate incidence of the 1PN class at HCG+ 20h is significantly greater than that observed at HCG+ 24h. $X^2 = 11·98$, $P < 0·01$.

TABLE 9·2

The influence of an intragastric injection of a dilute ethanol solution to F1 hybrid female mice during the first meiotic division on the frequency of hyaluronidase-induced parthenogenetic activation.

Group	Administration of ethanol solutions	Postovulatory age of oocytes at activation (h)	Number of ovulated oocytes	Number of activated oocytes	Class of Parthenogenone				Frequency of activation (%)
					1PN	2PN	IC	1PND	
1	1ml 12·5% ethanol	20	79	32	32	0	0	0	40·5
	at HCG+ 4h, i.g.	22	377	165	144	18	2	1	43·8
		24	59	54	43	7	4	0	91·5
2	1ml 12·5% ethanol	20	307	144	109	4	0	1	37·1
	at HCG+ 6h, i.g.	22	370	194	172	22	0	0	52·4
		24	166	87	80	5	2	0	52·4
3	1ml 12·5% ethanol	20	190	47	47	0	0	0	24·7
	at HCG+ 8h, i.g.	22	88	56	50	6	0	0	63·6
		24	284	191	165	25	0	1	67·3

The proportionate incidence of the 1PN class at HCG+ 20h is not significantly different to that observed following activation at HCG+ 24h. $X^2 = 0{\cdot}78$, $P = 0{\cdot}3 - 0{\cdot}5$.

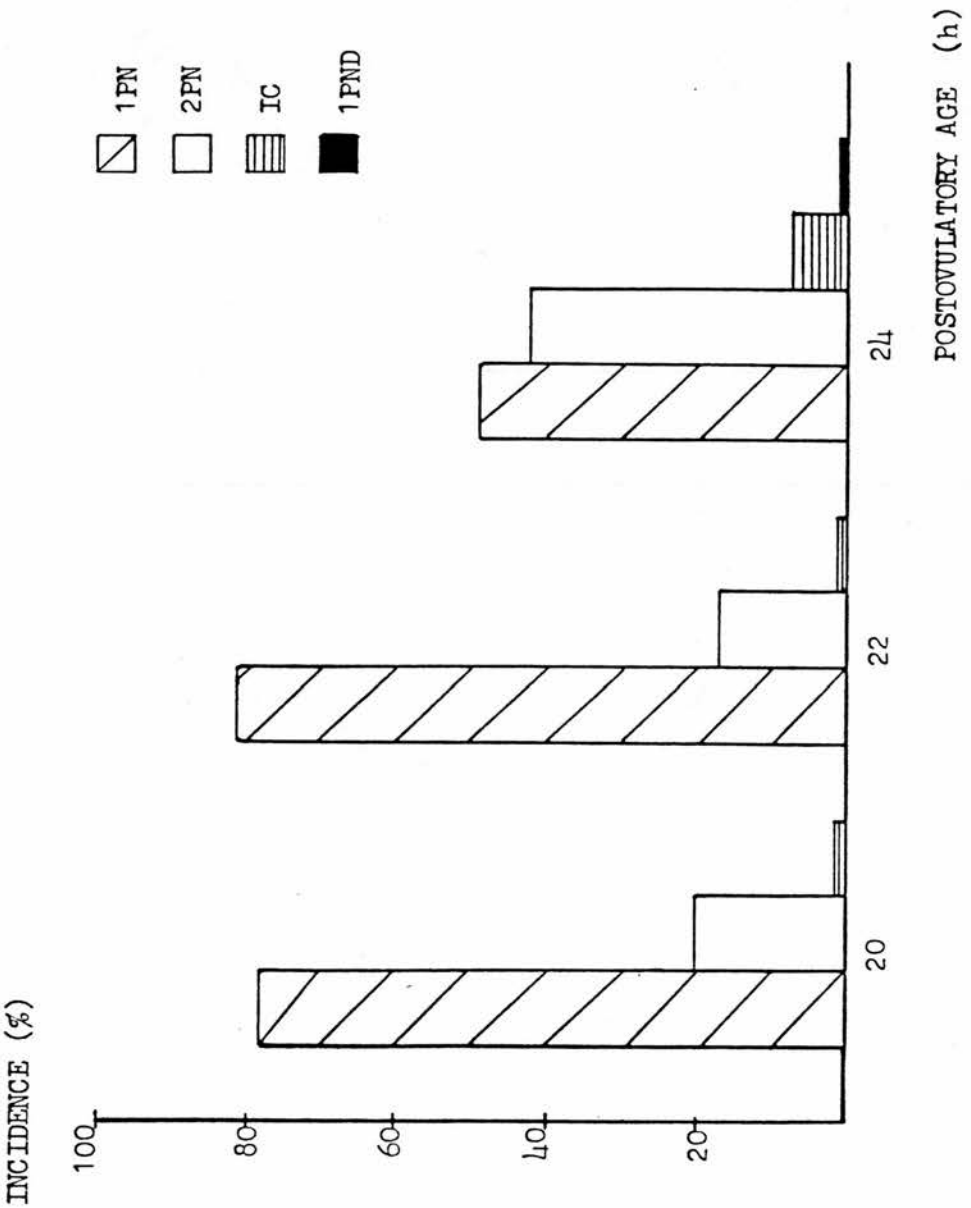


Fig. 9.1 The proportionate incidence of the four pathways of parthenogenetic development observed following the exposure of F1 hybid oocytes to hyaluronidase at HCG+ 20h - 24h in vitro. The ovulated oocytes were recovered from female mice that had received an i.g. injection of 1ml distilled water at either HCG+ 4h 6h or 8h.

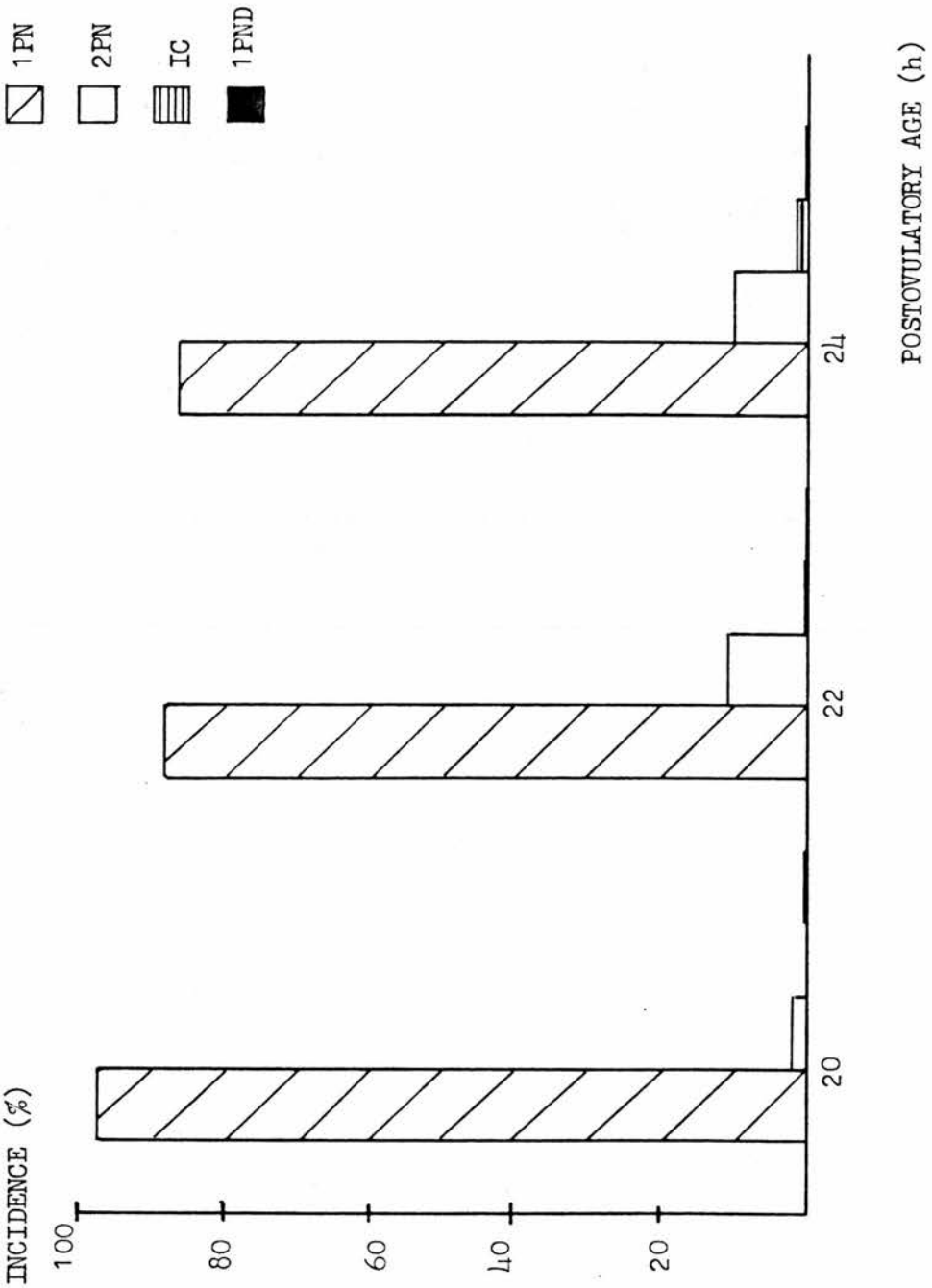


Fig. 9.2 The proportionate incidence of the four pathways of parthenogenetic development observed following the exposure of F1 hybrid oocytes to hyaluronidase in vitro. The ovulated oocytes were recovered from female mice that received an i.g. injection of 1ml 12.5% ethanol at either HCG+ 4h, 6h or 8h.

intragastric injection of a 1ml 12.5% (v/v) solution of ethanol at either HCG+ 4h, 6h, or 8h (Table 9.2). In Group 1, the incidence of parthenogenetic activation increased from 40.5% to 91.5% when the postovulatory age of the activated oocytes, previously exposed to ethanol at HCG+ 4h, increased from HCG+ 20h to HCG+ 24h. In this group, the time interval between in vivo ethanol administration and hyaluronidase activation in vitro was 16h, 18h and 20h respectively. The incidence of parthenogenetic activation in Group 2 increased from 37.3% to 52.4% when the postovulatory age of the activated oocytes, previously exposed to ethanol at HCG+ 6h, was increased from HCG+ 20h to HCG+ 24h. In this group the time interval between ethanol exposure in vivo and activation in vitro was 14h, 16h and 18h, respectively. The incidence of parthenogenetic activation in Group 3 increased from 24.7% to 67.3% when the postovulatory age of activated oocytes, previously exposed to a dilute solution of ethanol in vivo at HCG+ 8h, increased from HCG+ 20h to HCG+ 24h. The relationship between the incidence of parthenogenetic activation, the time interval between ethanol exposure in vivo and the postovulatory age of the activated oocyte is presented in Table 9.3.

The proportionate incidence of hyaluronidase activated oocytes that developed as 1 PN parthenogenones (control) was significantly reduced from 78.3% to 49.0% as the

TABLE 9·3

The influence of the time of ethanol administration and the postovulatory age of hyaluronidase exposed oocytes on the frequency of parthenogenetic activation.

Postovulatory Age (h)	Time interval between ethanol administration and parthenogenetic activation (h).				
	12	14	16	18	20
20	24·7	37·1	40·5	-	-
22	-	63·6	52·4	43·8	-
24	-	-	67·3	52·4	91·5

Activation Frequency (%)

postovulatory age of the ovulated oocytes increased from HCG+ 20h to HCG+ 24h (Fig. 9.1). The majority of oocytes, previously exposed to a dilute solution of ethanol *in vivo* at either HCG+ 4h, 6h, or 8h, developed as 1PN parthenogenones (Table 9.2; total incidence: 97.4%) following hyaluronidase activation at HCG+ 20h. A lower incidence of this parthenogenetic class was observed (total incidence: 86.7%) when hyaluronidase activation was induced at HCG+ 24h but these values were not found to differ significantly (X^2 = 0.78; P = 0.3 - 0.5) from each other (Fig. 9.2).

9.3.2 The influence of an intragastric injection of a dilute ethanol solution to female mice during the first meiotic division on the chromosome constitution of hyaluronidase activated 1PN parthenogenones

The influence of 1ml of distilled water, administered to female mice by intragastric injection during the first meiotic division, on the chromosome constitution of hyaluronidase-induced 1PN parthenogenones at metaphase of the first cleavage mitosis is presented in Table 9.4 Groups 1-3. This control study revealed that the baseline incidence of aneuploidy was 3.8%.

The intragastric injection of a 1ml 12.5% (v/v) solution of ethanol to female mice during the first meiotic division was found to increase the proportion of

TABLE 9·4

The influence of an intragastric injection of distilled water to F1 hybrid female mice during the first meiotic division on the chromosome constitution of hyaluronidase-induced 1PN parthenogenones.

Group	Administration of distilled water	Postovulatory age of oocytes at activation (h)	Number of chromosome spreads	Number of non-analysable preparations	Chromosome Constitution						Frequency of aneuploidy
					19	20	21	22	23	24	
1	1ml dist.water at HCG+ 4h i.g.	22	47	2	0	43	0	0	1	1	4·4
2	1ml dist. water at HCG+ 6h i.g.	22	30	3	1	26	0	0	0	0	3·7
3	1ml dist. water at HCG+ 8h i.g.	22	39	6	0	32	1	0	0	0	3.1
Total			106	11	1	101	1	0	1	1	3·8

TABLE 9·5

The influence of an intragastric injection of a dilute ethanol solution to F1 hybrid female mice during the first meiotic division on the chromosome constitution of hyaluronidase-induced 1PN parthenogenones.

Group	Administration of ethanol solutions	Postovulatory age of oocytes at activation	Number of chromosome preparations	Number of non analysable preparations	Chromosome Constitution							Frequency of Aneuploidy (%)
					19	20	21	22	23	24	25	
1	1ml 12·5% ethanol at HCG+ 4h.	20	48	4	1	32	3	0	0	0	0	11·1
		22	59	7	2	47	4	1	0	0	0	12·9
		24	40	5	2	31	1	1	0	0	0	11·4
		Total	147	16	5	110	8	2	0	0	0	12·0
2	1ml 12·5% ethanol at HCG+ 6h.	20	70	9	2	54	2	3	0	0	0	11·5
		22	120	29	4	80	3	0	2	1	1	10·1
		24	57	9	4	40	1	2	1	0	0	16·7
		Total	247	47	10	174	6	5	3	1	1	13·1
3	1ml 12·5% ethanol at HCG+ 8h.	20	34	7	3	19	3	1	0	1	0	29·6
		22	25	3	3	18	1	0	0	0	0	18·2
		24	91	18	7	59	4	1	1	1	0	19·2
		Total	150	28	13	96	8	2	1	2	0	21·3

hyaluronidase-induced 1PN parthenogenones that exhibited numerical chromosome anomalies (Table 9.5). The intragastric injection of a dilute solution of ethanol to female mice at HCG+ 4h was observed to increase the proportion of hyaluronidase-induced 1PN parthenogenones that exhibited an aneuploid chromosome constitution from the baseline control value of 3.8% to a total value (Group 1) of 12.0%. The increase in the postovulatory age of activated oocytes within this group was not found to influence the incidence of aneuploidy ($X^2 = 2.6 \times 10^{-3}$; P= 0.90 - 0.95). The majority of malsegregation events (86.7%) involved the loss or gain of only one chromosome.

The intragastric injection of ethanol to female mice at HCG+ 6h was also found to increase the proportion of hyaluronidase-induced 1PN parthenogenones that exhibited an aneuploid chromosome constitution from the control value of 3.8% to a total value of 13.1% (Group 2). The increase in the postovulatory age of the activated oocytes within this group was not found to significantly increase the incidence of aneuploidy ($X^2 = 1.0$; P= 0.3 -0.5). The majority of malsegregation events (61.5%) involved the loss or gain of only one chromosome. The incidence of aneuploidy observed in Groups 1 and 2 was not found to be significantly differant ($X^2 = 6.9 \times 10^{-2}$; P= 0.7 - 0.8). Representative air-dried chromosome preparations of these parthenogenones are illustrated in Fig. 9.3.

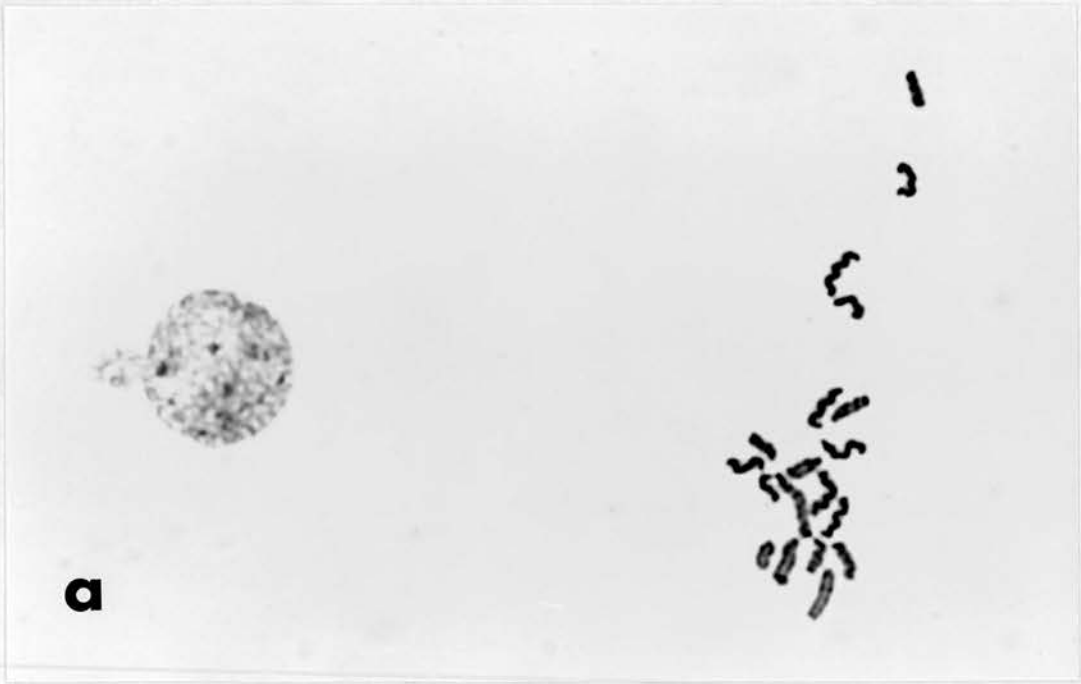


Fig. 9.3. Representative air-dried chromosome of hyaluronidase activated 1PN parthenogenones at metaphase of the first cleavage mitosis that exhibit (a) 19, (b) 20, and overleaf, (c) 21 and (d) 24 chromosomes respectively. The hyaluronidase activated oocytes were recovered from a F1 hybrid female mice that received an i.g. injection of dilute ethanol at HCG+ 6h and subsequently exposed to hyaluronidase at HCG+ 22h.

c

d

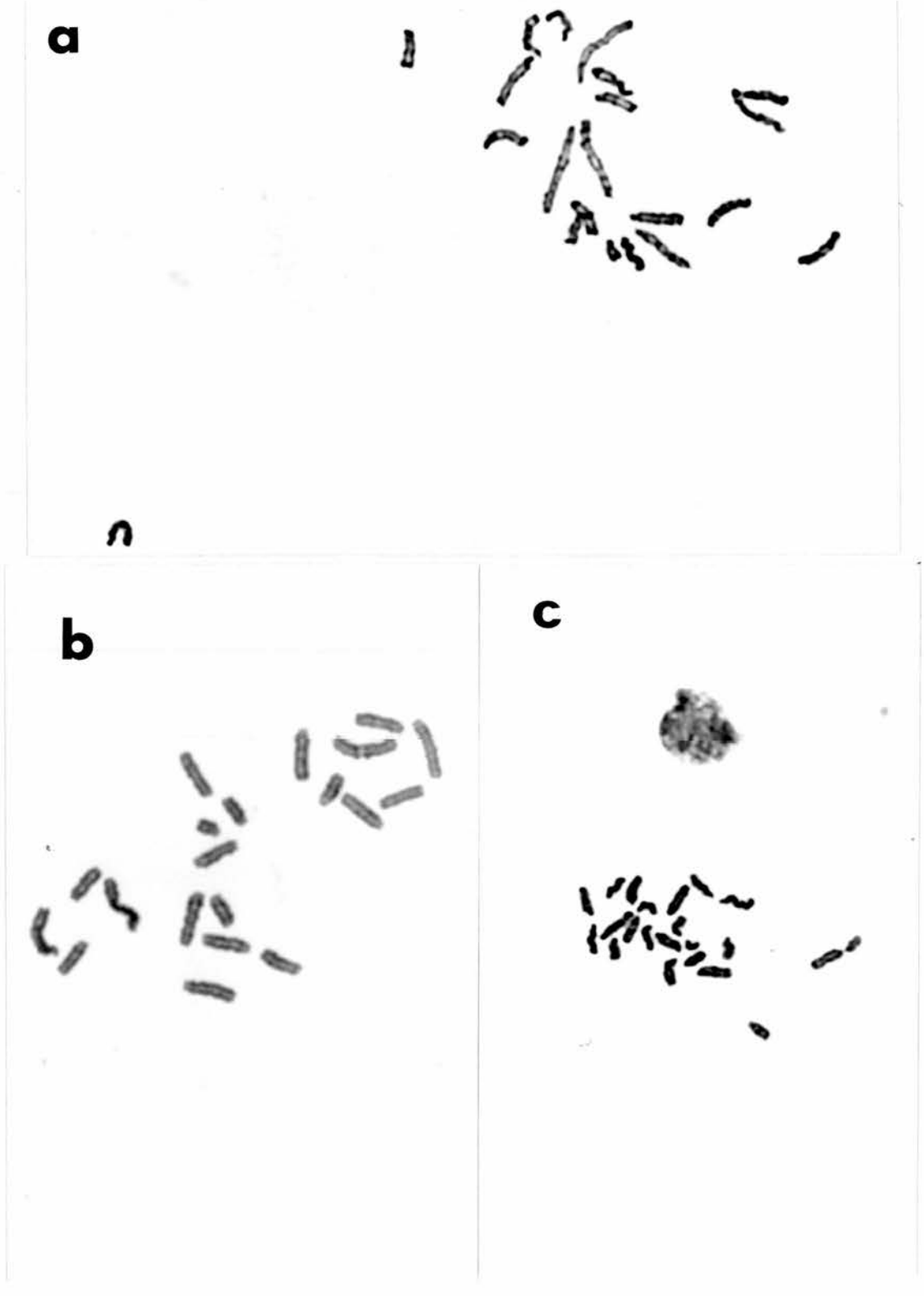


Fig. 9.4. Representative air-dried chromosome spreads of 1PN parthenogenones at metaphase of the first cleavage mitosis that exhibit (a) 21, (b) 20 and (c) 21 chromosomes respectively. The hyaluronidase activated oocytes were isolated from F1 hybrid female mice that received an i.g. injection of ethanol at HCG+ 8h and subsequently exposed to hyaluronidase at HCG+ 22h (a, b) and 24h (c).

The intragastric injection of a dilute solution of ethanol to female mice at HCG+ 8h was found to increase the proportion of hyaluronidase-induced 1PN parthenogenones that exhibited an aneuploid chromosome constitution to a total value of 21.3%. The incidence of aneuploidy within Group 3 was in the range of 29.6% to 19.2% as the postovulatory age of the activated oocytes increased from HCG+ 20h to HCG+ 24h. The majority of malsegregation events in this group also involved the loss or gain of only one chromosome (80.8%). The total incidence of aneuploidy within this group differed significantly from that observed in Groups 1 and 2 ($P < 0.05$). Representative air-dried chromosome spreads are presented in Fig. 9.4.

9.3.3. The karyotypic analysis of hyaluronidase activated oocytes that were exposed to ethanol during the first meiotic division

The karyotypic analysis of the aneuploid chromosome spreads that were prepared from hyaluronidase activated oocytes previously exposed to ethanol *in vivo* at HCG+ 4h 6h and 8h and induced to initiate parthenogenesis at HCG+ 20h is presented in Table 9.6.and Table 9.7. The karyotypic analysis of those oocytes that were exposed to ethanol *in vivo* at HCG+ 4h and induced to initiate parthenogenesis at HCG+ 22h is also presented in Table

Table 9.6

The incidence of specific chromosome malsegregation observed in ethanol exposed primary oocytes that developed as hyaluronidase-induced 1PN parthenogenones.

Chromosome	Chromosome constitution		Incidence
	Hyperhaploid	Hypohaploid	
4	1	1	2
6	1	1	2
12	0	1	1
13	1	0	1
15	1	0	1
17	2	0	2
18	0	1	1
19	1	0	1

Table 9.7

The specific chromosome constitution of ethanol exposed primary oocytes that develped as hyaluronidase-induced 1PN parthenogenones.

Chromosome constitution of hyperhaploid and hypohaploid 1PN parthenogenones	Incidence
1ml 12.5% ethanol at HCG+ 4h Hyaluronidase activation at HCG+ 20h	
21, X; + 13	1
21, X; + 17	1
19, X; - 4	1
19, X; - 18	1
1ml 12.5% ethanol at HCG+ 4h Hyaluronidase activation at HCG+ 22h	
22, X; + 17, 15	1
1ml 2.5% ethanol at HCG+ 6h Hyaluronidase activation at HCG+ 20h	
21, X; + 6	1
21, X; + 19	1
21, X; + 1 chromosome, either 15 or 18	1
1ml 12.5% ethanol at HG+ 8h Hyaluronidase activation at HCG+ 20h	
21, X; +4	1
19, X; -6	1
19, X; -12	1

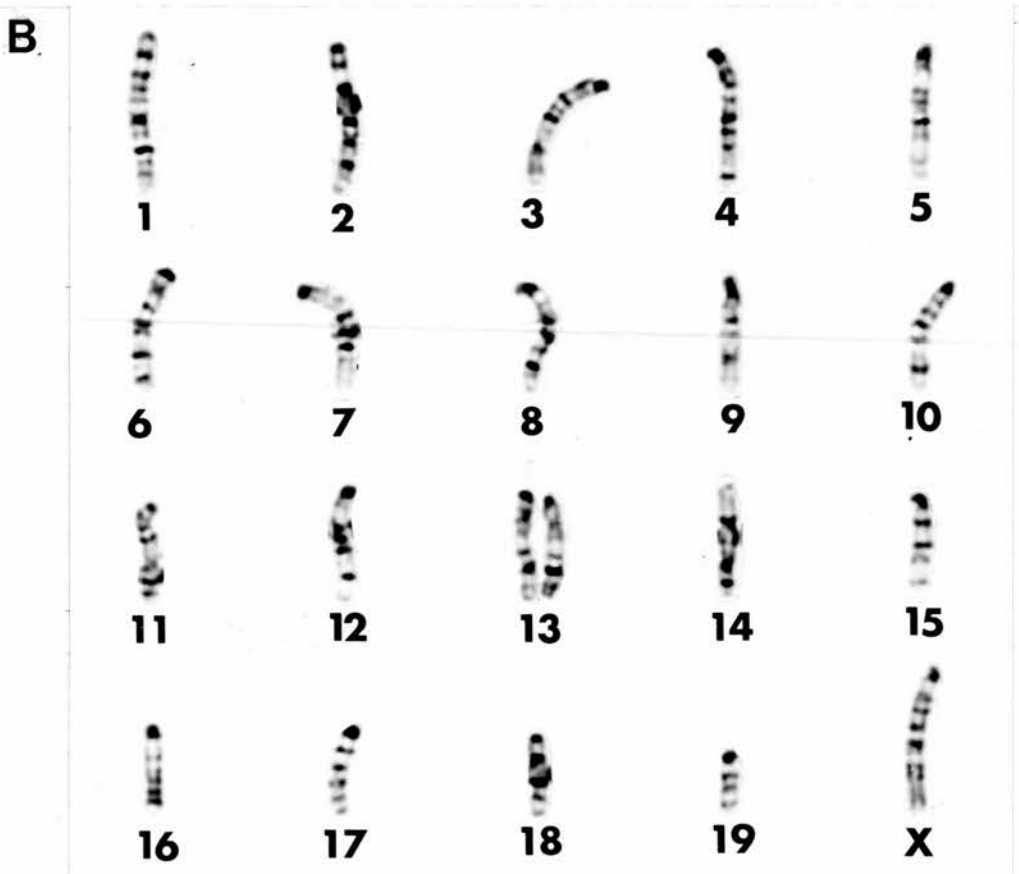


Fig. 9.5 (A) Giemsa-banded, air-dried chromosome spread from a hyperhaploid (N = 21) 1PN parthenogenone at metaphase of the first cleavage mitosis that developed following the hyaluronidase activation (at HCG+ 20h) of a F1 hybrid oocyte recovered from a female mouse that received an i.g. injection of 1ml 12.5% ethanol at HCG+ 4h. (B) Karyotypic analysis demonstrates that this parthenogenone is disomic for chromosome 13.

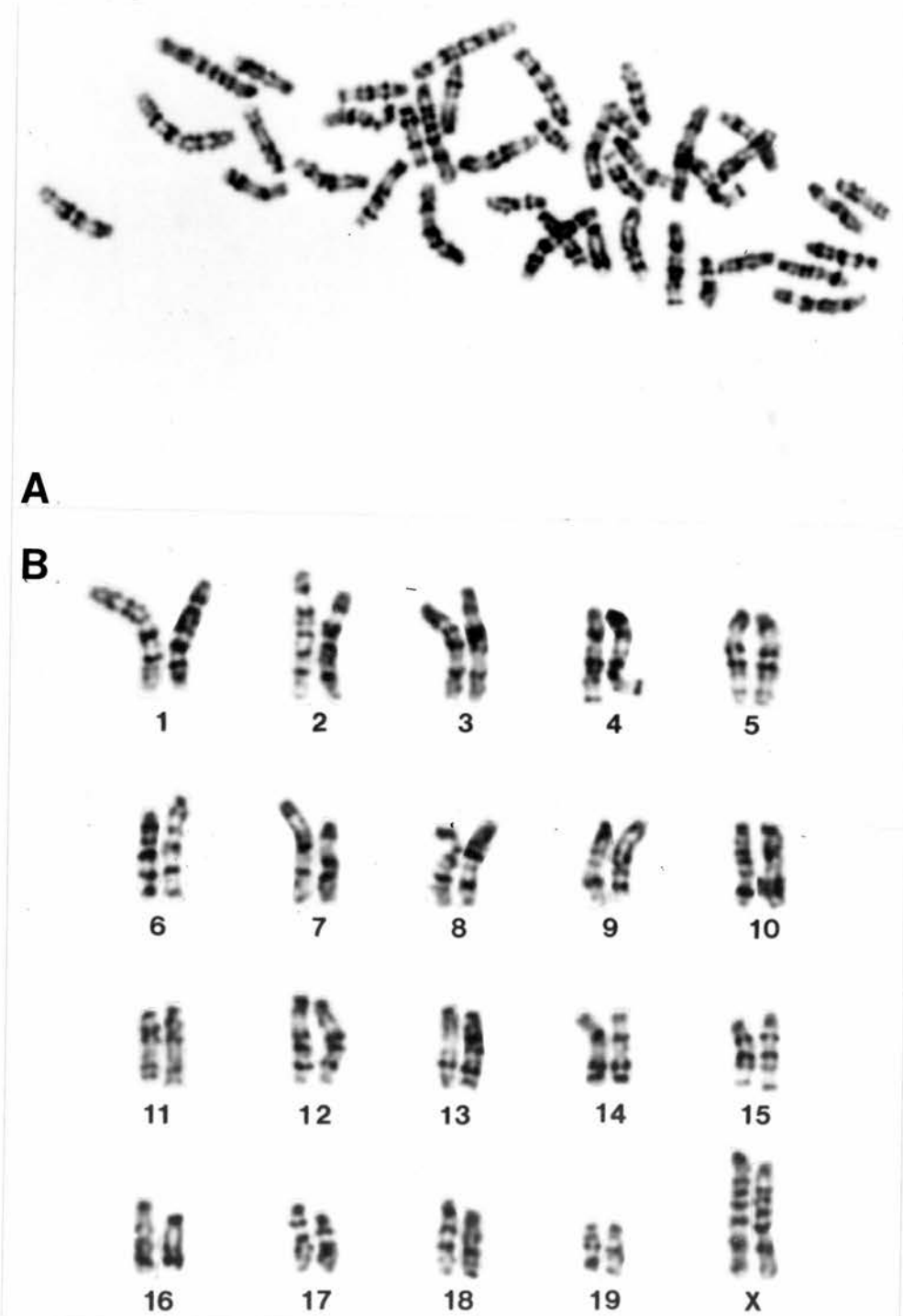


Fig. 9.6 (A) Giemsa-banded, air-dried chromosome spread from a hyaluronidase activated 2PN parthenogenone at metaphase of the first cleavage mitosis that developed following the hyaluronidase activation of an oocyte recovered from a F1 hybrid female mouse that received an i.g. injection of 1ml 12.5% ethanol at HCG+ 6h. (B) Karyotypic analysis demonstrates that this 2PN parthenogenone is euploid.

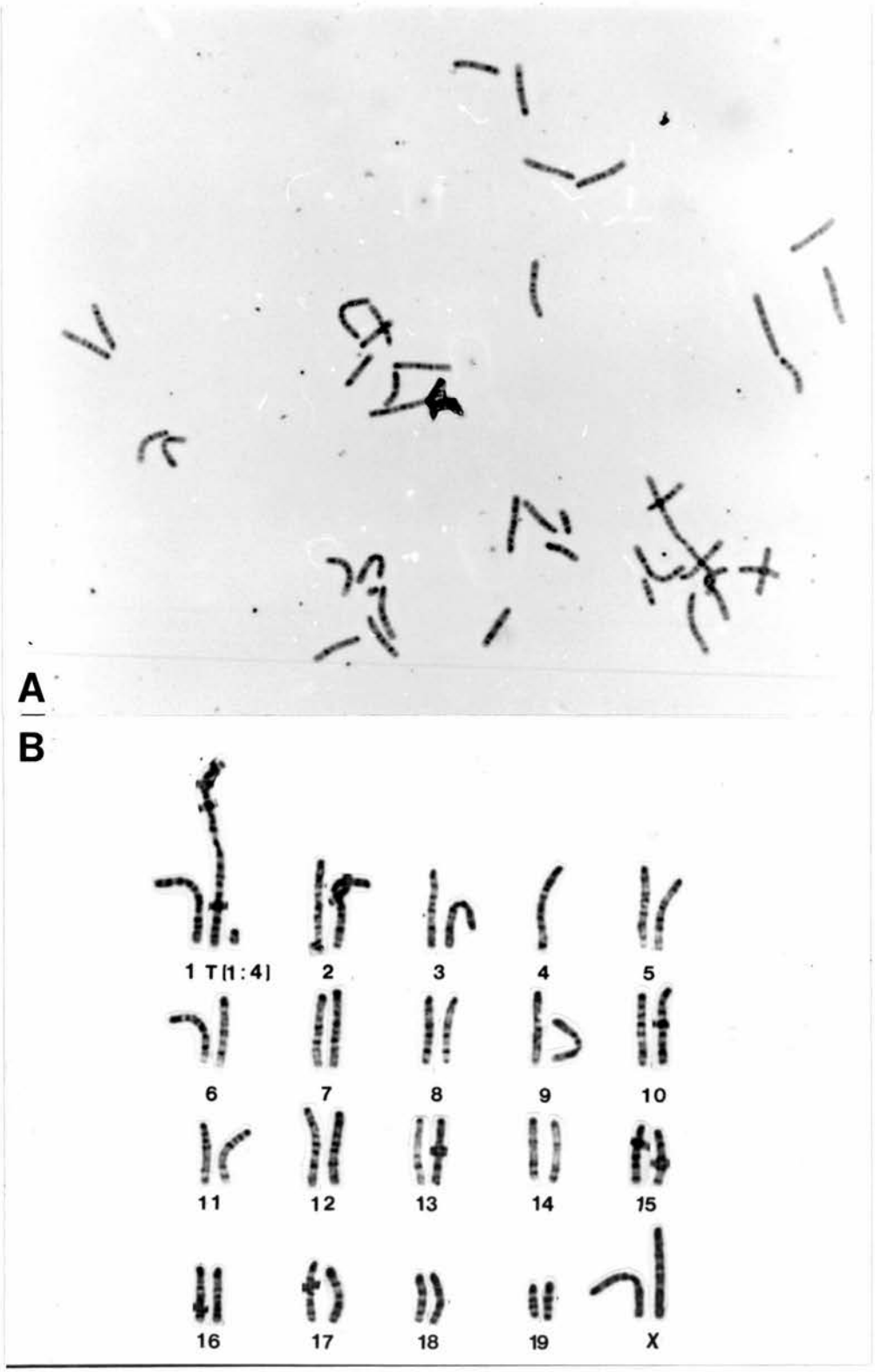


Fig. 9.7 (A) Giemsa-banded, air-dried chromosome preparation from a hyaluronidase activated 2PN parthenogenone at metaphase of the first cleavage mitosis. (B) Karyotypic analysis demonstrates that this parthenogenone was heterozygous for a non-reciprocal translocation between chromosomes 1 & 4. The translocation has also induced the formation of a centric fragment from chromosome 4.

9.6 and 9.7 The number of aneuploid chromosome preparations that were available for G-band analysis was not as large as that achieved in Chapter 4. The chromosomes that were involved in malsegregation events were 4, 6, 12, 13, 15, 17, 18 and 19 (Fig. 9.5). The karyotypic analysis of 24 randomly chosen G-banded chromosome spreads of euploid 1PN parthenogenones has found that all were cytogenetically balanced. The analysis of the chromosome constitution of 15 2PN parthenogenones that were exposed to ethanol in vivo at HCG+ 4h and induced to initiate parthenogenesis at HCG+ 22h has found that none exhibited either hypodiploidy or hyperdiploidy (Fig. 9.6). However one 2PN parthenogenone was found to be heterozygous for a nonreciprocal centric fusion between chromosomes 1 and 4. This translocation also induced the development of a small centric fragment of chromosome 4 (Fig 9.7).

9.3.4 The influence of an intragastric injection of a dilute ethanol solution to female mice during the first meiotic division on the frequency of hyaluronidase activated oocytes induced to develop as 2PN parthenogenones in vitro

The results presented in Table 9.8 Groups 1 and 2 clearly demonstrate that the ovulated oocytes, isolated from female mice administered 1ml of distilled water (control) at HCG+ 4h or HCG+ 6h, exhibited a high

TABLE 9·8

The influence of an intragastric injection of either distilled water or a dilute ethanol solution to F1 hybrid female mice during the first meiotic division on the frequency of hyaluronidase activated cytochalasin D-induced 2PN parthenogenones.

Group	Administration of dist. water or ethanol	Postovulatory age of oocytes at activation (h)	Number of ovulated oocytes	Number of activated oocytes	Class of Parthenogenone				Activation frequency (%)
					1PN	2PN	IC	1PND	
1	1ml of dist. water at HCG+ 4h, i.g.	22	138	121	0	114	2	5	87·7
2	1ml of dist. water at HCG+ 6h, i.g.	22	47	31	0	27	2	2	87·1
3	1ml of 12·5% ethanol at HCG+ 4h, i.g.	22	195	92	5	87	0	0	47·2
4	1ml of 12·5% ethanol at HCG+ 6h, i.g.	22	187	92	5	87	0	0	49·2

incidence of parthenogenetic activation (87.7% and 87.1%, respectively) when incubated in M16 medium supplemented with 3mg/ml of hyaluronidase for 10-15 min. at HCG+ 22h. The majority of activated oocytes developed as 2PN parthenogenones as their incubation in M16 medium supplemented with 1ug/ml of cytochalasin D for 4h after activation inhibited the extrusion of the second polar body. In groups 4 and 5 a small proportion (5.8% and 12.9% respectively) of the activated oocytes developed as IC or 1PND parthenogenones. The results presented in Table 9.8 Groups 3 and 4 clearly demonstrate that the intragastric injection of a 1ml 12.5% (v/v) ethanol solution to female mice at either HCG+ 4h or HCG+ 6h significantly reduced the proportion of ovulated oocytes that initiated hyaluronidase-induced activation at HCG+ 22h to 47.2% and 49.2% respectively. The majority of activated oocytes also developed as 2PN parthenogenones as their incubation in M16 supplemented with Cytochalasin D inhibited the extrusion of the second polar body.

9.3.5 The influence of an intragastric injection of a dilute ethanol solution to female mice during the first meiotic division on the chromosome constitution of hyaluronidase activated CD-induced 2PN parthenogenones

The cytogenetic analysis of the hyaluronidase activated oocytes induced to develop as 2PN parthenogenones

TABLE 9·9

The influence of an intragastric injection of either distilled water or a dilute ethanol solution to F1 hybrid female mice during the first meiotic division on the chromosome constitution of hyaluronidase activated cytochalasin D-induced 2PN parthenogenones

Group	Administration of dist. water or ethanol	Number of chromosome preparations	Number of nonanalysable preparations	Chromosome Constitution						Frequency of aneuploidy (%)	Frequency of hyperdiploidy (%)
				38	39	(20/20)	40	41	42		
1	1ml dist. water at HCG+ 4h, i.g.	86	6	2	5	40	32	0	0	8·8	0·0
2	1ml of 12·5% ethanol at HCG+ 4h, i.g.	97	6	1	4	37	44	0	0	5·8	0·0
3	1ml of 12·5% ethanol at HCG+ 6h, i.g.	101	14	1	5	40	35	1	0	8·5	1·2

The incidence of numerical chromosome anomalies in Group 1 is not significantly different to that observed in Group 2: $X^2 = 0·57$, $P = 0·3 - 0·5$

Group 3; $X^2 = 5·2 \times 10^{-2}$, $P = 0·9 - 0·8$

following exposure to cytochalasin D is presented in Table 9.9. Chromosome spreads of euploid 2PN parthenogenones at metaphase of the first cleavage mitosis exhibit two distinct groups of 20 chromosomes (20 - 20) (Fig. 9.8) or a single group of 40 chromosomes if the boundary between these two groups is indistinct. If the dose of ethanol administered during the first meiotic division induces nondisjunction, the two pronuclear chromosome sets of the 2PN parthenogenones are expected to exhibit a (19 - 19) or (21 - 21) chromosome constitution at metaphase of the first cleavage mitosis. Presegregation errors (also termed premature chromatid separation) during the first meiotic division would expected to induce the development of 2PN parthenogenones that exhibit a (21 - 20) or (19 - 20) chromosome constitution at metaphase of the first cleavage mitosis. A proportion of the 2PN parthenogenones that exhibit a single group of 39 chromosomes may also result from the mechanical loss of chromosomes incurred during the fixation procedure.

The influence of 1ml of distilled water, administered to female mice by intragastric injection at HCG+ 4h, on the chromosome constitution of hyaluronidase activated cytochalasin D-induced 2PN parthenogenones at metaphase of the first cleavage mitosis is presented in Table 9.9. This control study has found that the "baseline" incidence of aneuploidy was 8.8%. All aneuploid

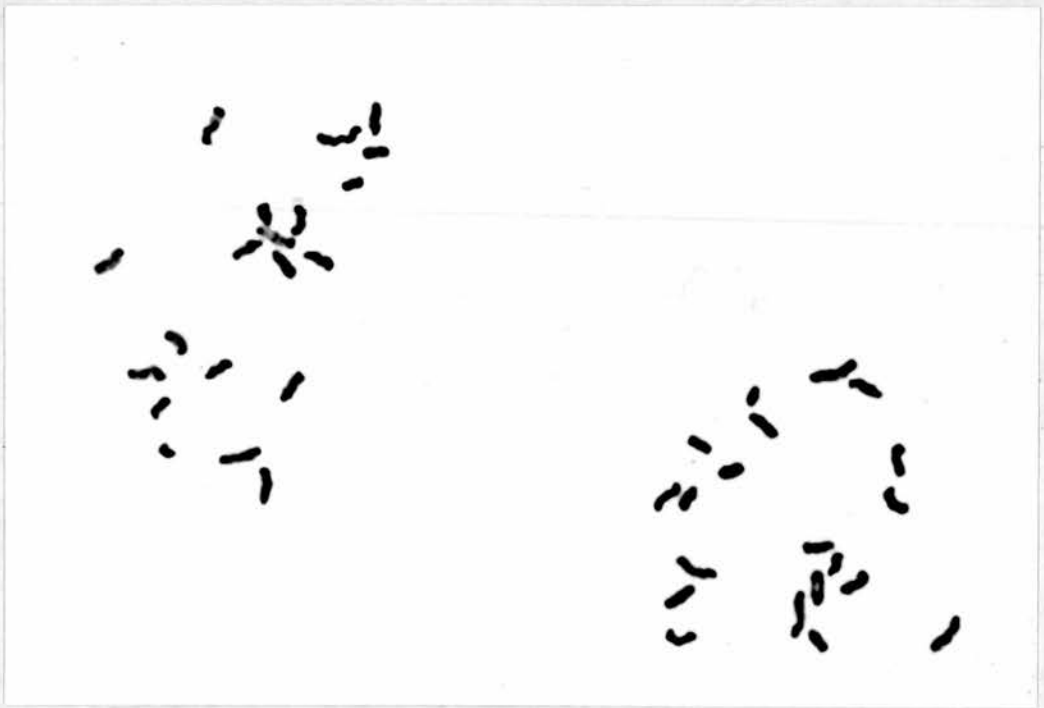


Fig. 9.8 Representative air-dried chromosome spread that exhibits the two distinct haploid pronuclear chromosome sets (20 - 20) from a hyaluronidase activated, cytochalasin-D induced, 2PN parthenogenone at metaphase of the first cleavage mitosis.

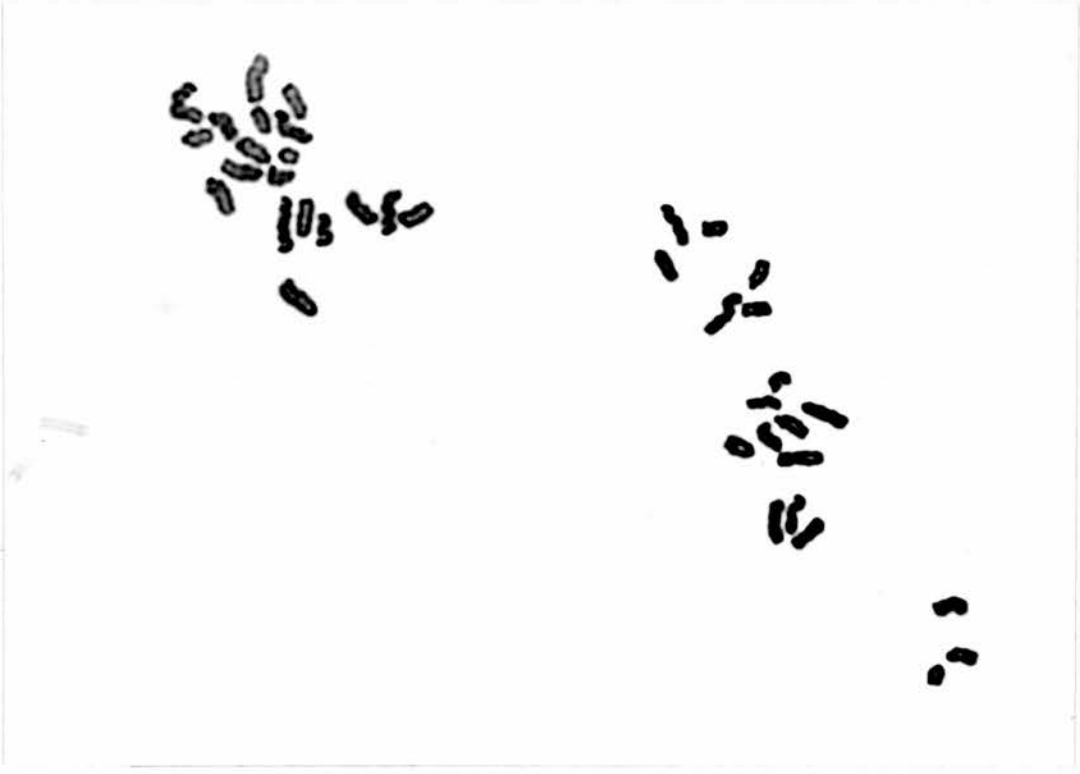


Fig. 9.9 Air-dried chromosome spread that exhibits two distinct hypohaploid pronuclear chromosome sets (19 - 19) from a hyaluronidase activated, cytochalsin-D induced, 2PN parthenogenone at metaphase of the first cleavage mitosis.

parthenogenones exhibited a hypodiploid chromosome constitution. The majority of these exhibited a single group of 39 chromosomes.

The intragastric injection of a 1ml 12.5% ethanol (v/v) solution to female mice at either HCG+ 4h or HCG+ 6h was not found to increase the frequency of hyaluronidase activated cytochalasin D-induced 2PN parthenogenones that exhibited an aneuploid chromosome constitution at metaphase of the first cleavage mitosis (Table 9.9, Groups 2 - 3). A cytochalasin D-induced 2PN parthenogenone with the numerical chromosome constitution of (19 - 19) is presented in Fig. 9.9

9.3.6 The influence of an intraperitoneal injection of a standard dose of Avertin to female mice during the first meiotic division on the frequency of parthenogenetic activation in vitro

The results presented in Table 9.10 Group 1 demonstrate that a high incidence of parthenogenetic activation was observed (80.8%) when ovulated oocytes, isolated from female mice that received an intragastric injection of 0.4ml of Dulbecco's PBS (control) at HCG+ 6h, were incubated briefly in M16 medium supplemented with 3mg/ml of hyaluronidase at HCG+ 20h. The majority (88.1%) of the activated oocytes were observed to develop as 1PN parthenogenones.

TABLE 9·10

The influence of an intraperitoneal injection of the standard dose of Avertin anaesthesia to F1 hybrid female mice on the frequency of hyaluronidase-induced parthenogenesis.

Group	Administration of Avertin anaesthesia	Postovulatory age of oocytes at activation	Number of ovulated oocytes	Number of activated oocytes	Class of Parthenogenone				Activation frequency (%)
					1PN	2PN	IC	1PND	
1	0·4ml of PBS at HCG+ 6h, i.p.	20	52	42	37	7	0	0	80·8
2	X 1·0 Std. Avertin* dose. HCG+ 6h, i.p.	20	114	81	75	6	0	0	71·1

* The standard dose of Avertin anaesthesia is 0·02ml of a 1·2% Avertin solution per g/b.wt. of mouse.

The incidence of parthenogenetic activation in Group 1 is not significantly greater than that observed in Group 2. $X^2 = 0·25$, $P = 0·7 - 0·5$.

The proportionate incidence of the 1PN class of parthenogenone in Group 1 is not significantly different to that observed in Group 2. $X^2 = 2·2$, $P = 0·2 - 0·1$.

A reduced incidence of parthenogenetic activation (71.1%) was observed in Group 2 when ovulated oocytes, isolated from female mice that received a standard dose of Avertin anaesthesia by intraperitoneal injection at HCG+ 6h, were exposed to 3mg/ml of hyaluronidase in M16 medium at HCG+ 20h. The majority (92.6%) of the activated oocytes developed as 1PN parthenogenones. The frequency of activation and the proportionate incidence of the 1PN pathway in Group 2 was not found to differ significantly to that observed in the control study.

9.3.7 The influence of an intraperitoneal injection of a standard dose of Avertin to female mice during the first meiotic division on the chromosome constitution of hyaluronidase activated 1PN parthenogenones

The influence of an intraperitoneal injection of 0.4ml of Dulbecco's PBS (control), administered to female mice at HCG+ 6h, on the chromosome constitution of hyaluronidase activated 1PN parthenogenones at metaphase of the first cleavage mitosis is presented in Table 9.11 Group 1. This control study has found that the "baseline" incidence of aneuploidy was 3.6%. The results presented in Group 2 reveal that the administration of a standard dose of Avertin to female mice at HCG+ 6h significantly increased the proportion of hyaluronidase activated 1PN parthenogenones that exhibited an aneuploid chromosome

TABLE 9·11

The influence of an intraperitoneal injection of a standard dose of Avertin anaesthesia to female mice during the first meiotic division on the chromosome constitution of hyaluronidase-induced 1PN parthenogenones.

Group	Administration of Avertin anaesthesia	Number of chromosome preparations	Number of nonanalysable preparations	19	20	21	22	23	24	25	Frequency of aneuploidy (%)
1	0·4ml of PBS at HCG+ 6h, i.p.	32	4	1	27	0	0	0	0	0	3·6
2	X 1·0 Std. Avertin dose at HCG+ 6h,i.p.	49	4	4	34	4	0	1	0	1	22·7

constitution to 22.7%.

9.3 DISCUSSION

The ovulated oocytes isolated from female mice that received an intragastric injection of a dilute ethanol solution during the first meiotic division exhibited a significantly lower frequency of hyaluronidase-induced parthenogenesis than that observed in the control study. However, the ability of these oocytes to initiate hyaluronidase-induced parthenogenesis was found to improve as the time interval between ethanol administration and hyaluronidase activation increased from 12h - 18h. It appears that the advancing postovulatory age of the ovulated oocytes exposed to ethanol during the first meiotic division permits an increasing proportion to "recover" from the inhibitory effects of this agent and initiate hyaluronidase-induced parthenogenesis.

The majority of activated oocytes in the control series developed as 1PN parthenogenones. The proportionate incidence of this class was significantly reduced when the postovulatory age of the oocytes increased from HCG+ 20h to HCG+ 24h. A postovulatory age-related decrease in the incidence of this class was also observed in Chapter 3 (O'Neill and Kaufman, 1988). The majority of the hyaluronidase activated oocytes that had been previously exposed to ethanol in vivo also developed as 1PN

parthenogenones. However, an increase in the postovulatory age of the ethanol exposed oocytes prior to activation was not found to significantly decrease the proportionate incidence of the 1PN class. The results of this study would seem to indicate that the ethanol exposed oocytes that have maintained or "regained" the ability to initiate parthenogenesis through the period of postovulatory ageing are those in which the peripheral location and integrity of the meiotic spindle apparatus has been retained. The lower incidence of activation and the overall absence of 2PN parthenogenones demonstrates that the intragastric dose of ethanol interferes with the activation of those aged oocytes that would normally fail to extrude the second polar body. The ovarian oocytes that were exposed to Avertin in vivo exhibited a high incidence of hyaluronidase-induced parthenogenesis. Furthermore, in vivo exposure to this agent was not found to interfere with the proportionate incidence of the four main pathways of parthenogenesis. The majority of the activated oocytes developed as 1PN parthenogenones. However, analysis of their chromosome constitution at metaphase of the first cleavage mitosis has found that a significant number exhibited aneuploidy.

A reduced incidence of fertilisation was not observed when female mice that received an intragastric dose of ethanol shortly after the administration of HCG were mated with fertile males (O'Neill and Kaufman,

1987a). However, the oocytes were not examined *in vitro* until at least 6-8h after the expected time of fertilisation and it is not known if *in vivo* ethanol exposure interfered transiently with the ability of sperm to penetrate the oocyte. This same study also found that ethanol exposure during the first meiotic division or at conception did not increase the incidence of triploidy. It would appear that the ability of an intragastric dose of ethanol to interfere with the activation of the second meiotic division is restricted to the events associated with induction of hyaluronidase-induced parthenogenesis. This phenomenon further highlights that the mechanism of parthenogenetic activation is not homologous with the events that occur at fertilisation. The nature of the ethanol-induced biochemical or biophysical changes that interfere with oocyte maturation and subsequently reduce the frequency of parthenogenetic activation are not known. In Chapter 3 it was proposed that hyaluronidase-induced parthenogenesis was an electrophysiochemical response within the oocyte to the rapid dispersal of the adhering cumulus cells. Ethanol-induced alterations to the relationship between the zona pellucida and the cumulus cells may be one of the factors that interferes with the initiation of hyaluronidase-induced parthenogenesis.

The cytogenetic analysis of hyaluronidase activated 1PN parthenogenones at metaphase of the first cleavage

that the incidence of aneuploidy was
mitosis has consistently observed in the range of 2-4% (O'Neill and Kaufman, 1988, Chapter 3) and in the control series of this study a similar "background" incidence of aneuploidy was observed. The cytogenetic analysis of the hyaluronidase-induced 1PN parthenogenones from the ovulated oocytes previously exposed to ethanol in vivo found that a significant proportion exhibited an aneuploid chromosome constitution. However. the administration of a dilute ethanol solution at either HCH+ 4h or HCG+ 6h was not found to influence a significant difference in the proportion of 1PN parthenogenones that exhibited aneuploidy. Furthermore, the incidence of aneuploidy was not influenced by the increasing postovulatory age of the activated oocytes. These findings indicate that each stage of the first meiotic division may be equally susceptible to the influence of ethanol. The higher incidence of aneuploid 1PN parthenogenones that were observed when ethanol was administered to female mice at HCG+ 8h may indicate, however, that the oocytes at this stage of meiosis are more susceptible to the influence of ethanol. It is also likely that these results may have been due to the smaller size of the sampled population of oocytes.

The karyotypic analysis of the aneuploid hyaluronidase activated 1PN parthenogenones that developed from oocytes exposed to a dilute ethanol solution in vivo at either HCG+ 4h, 6h or 8h cannot clearly establish whether

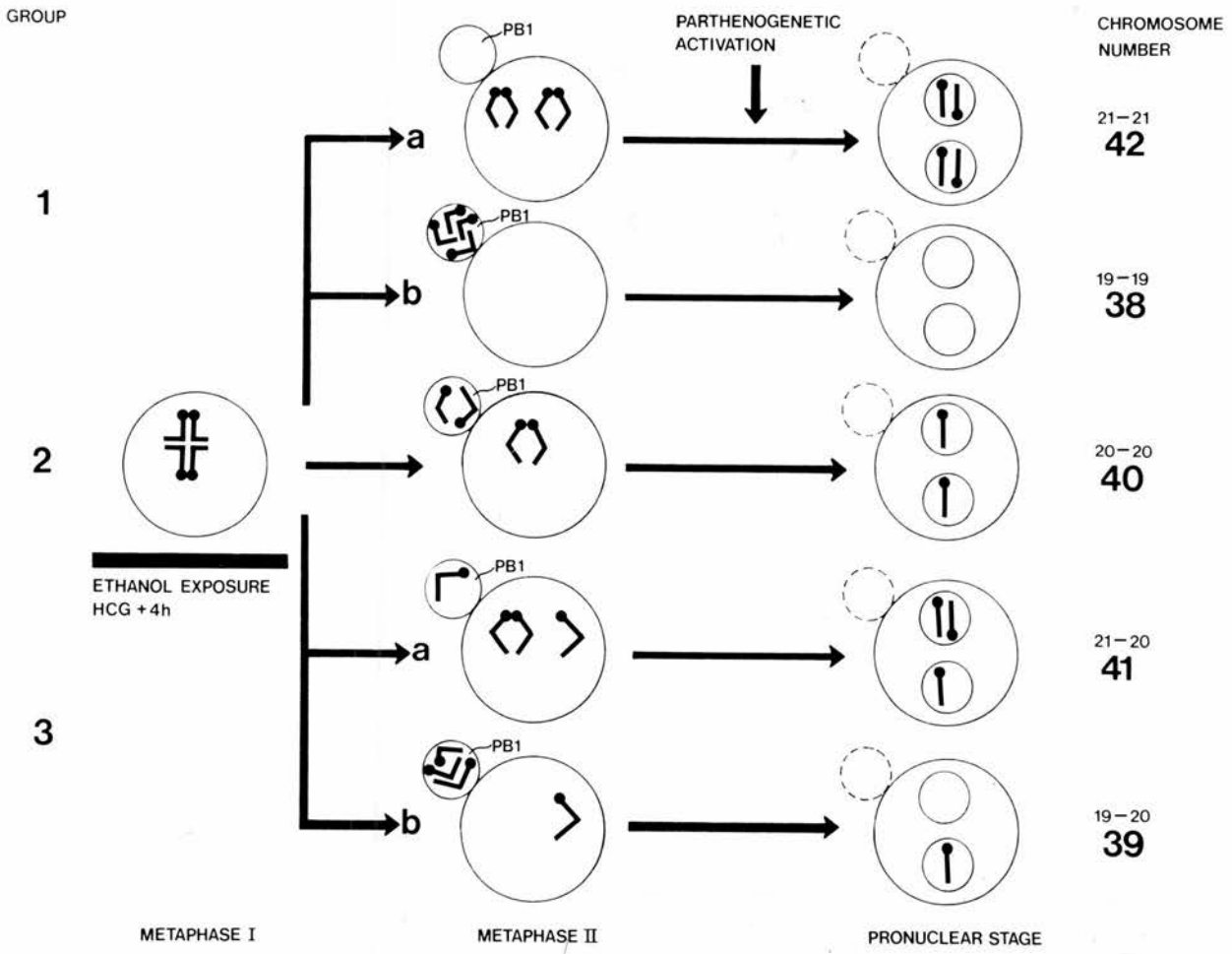
chromosome segregation errors were random events or provide information as to whether specific chromosomes were selectively involved in malsegregation. Only a proportion of the total number of aneuploid parthenogenones exhibited quality G-band patterns from which the disomic chromosome pair could be identified. In this study the chromosomes that were involved in segregation errors during meiosis were found to be those that were also more frequently involved in malsegregation events when the aneuploid metaphase chromosome preparations of in vitro ethanol-induced 1PN parthenogenones were karyotypically analysed (O'Neill and Kaufman, 1989a, Chapter 4).

The subtraction of the "background" incidence of postovulatory age-related chromosome segregation errors from the total incidence of aneuploidy produces an adjusted value of the true incidence of ethanol-induced chromosome segregation errors. These adjusted values of aneuploidy do not differ significantly to those observed in the metaphase chromosome preparations of fertilised one-cell embryos that were isolated from female mice that received an intragastric dose of ethanol during the first meiotic division (O'Neill and Kaufman, 1987a). These results strongly indicate that ethanol induces chromosome segregation errors during the completion of the first meiotic division and that the aneuploid 1PN parthenogenones develop from activated aneuploid ovulated

oocytes. A similar increase in the incidence of aneuploidy has been observed in the metaphase chromosome spreads of fertilised one-cell embryos isolated from female mice that received an intragastric injection of a dilute ethanol solution at either HCG+ 2h or HCG+ 4h (O'Neill and Kaufman, 1987a; reviewed, Kaufman and O'Neill, 1988). However, these studies did not directly examine the chromosome constitution of the ovulated oocytes that were exposed to ethanol in vivo during the first meiotic division. Furthermore, these studies have failed to conclusively demonstrate that ethanol exposure in vivo induces chromosome segregation errors during the first meiotic division.

The present study has also analysed the developmental potential and chromosome constitution of hyaluronidase activated oocytes that developed as 2PN parthenogenones, either spontaneously, or following their brief exposure to culture medium supplemented with cytochalasin D. The cytogenetic analysis of this parthenogenetic class permits an examination of the chromosome constitution of both genomic products of the second meiotic division. This can demonstrate the occurrence and the incidence of chromosome malsegregation during the first meiotic division as numerical chromosomal anomalies are represented in each of two genomic products of 2PN parthenogenones. The specific chromosome constitution of each haploid genome can also assist in the identification

Fig. 9.10. Illustration of one technique that can be used to analyse the various events that may occur following exposure to ethanol (or other trisomogenic agents) during the first meiotic division. In this approach, first cleavage metaphase chromosome preparations of 2PN parthenogenones are analysed following in vitro exposure to hyaluronidase, an activating stimulus that does not induce chromosome malsegregation. The parthenogenones shown in Group 2 develop as normal euploid diploids, having a euploid haploid set of chomosomes in each pronucleus. In the parthenogenones that develop along the Groups 1a and 1b malsegregation induced by in vivo exposure to ethanol during the first meiotic division would result in the development of parthenogenones with complements of either 42 or 38 chromosomes. Alternately, aneuploid parthenogenones could also develop (Groups 3a, 3b) if premature centromeric division had been induced following in vivo ethanol exposure during the first meiotic division.


GROUP
PB1
PARTHENOGENETIC ACTIVATION
CHROMOSOME NUMBER
1
a
21–21
42
PB1
b
19–19
38
2
PB1
20–20
40
ETHANOL EXPOSURE
HCG +4h
PB1
a
21–20
41
3
PB1
b
19–20
39
METAPHASE I
METAPHASE II
PRONUCLEAR STAGE

of the mechanism by which segregation errors arise. This system of analysis has been presented in full by Kaufman and O'Neill (1988) and represented in Fig. 9.10.

The ovulated oocytes isolated from female mice that received an intragastric injection of distilled water exhibited a high incidence of hyaluronidase-induced activation and cytochalasin D-induced 2PN parthenogenesis. However, as observed previously, the intragastric administration of a dilute ethanol solution to female mice at either HCG+ 4h or HCG+ 6h significantly reduced the ability of the ethanol exposed oocytes to initiate parthenogenesis. The analysis of the chromosome constitution of hyaluronidase activated cytochalasin-D induced 2PN parthenogenones that developed from oocytes exposed to ethanol <u>in vivo</u> during the first meiotic division has found that they did not exhibit aneuploidy at a frequency higher than that observed under control conditions. Furthermore, numerical chromosomal anomalies were not observed in the "spontaneous" 2PN parthenogenones that developed from the ethanol exposed ovarian oocytes. This clearly demonstrates that the intragastric injection of a dilute ethanol solution to female mice during the first meiotic division does not significantly increase the incidence of chromosome segregation errors that occur during the completion of the first meiotic division. These results strongly indicate that the ethanol exposed ovarian oocytes that

subsequently developed as aneuploid 1PN parthenogenones or aneuploid fertilised one-cell embryos (O'Neill and Kaufman, 1987a) exhibit an increased incidence of numerical chromosome anomalies due to chromosome segregation errors that arise during the completion of the second meiotic division. The direct examination of the ovulated oocytes isolated from female mice exposed to Avertin during the first meiotic division has found that they exhibit aneuploidy (Kaufman, 1977b). These conclusions strongly indicate that ethanol and Avertin interact differently with the meiotic spindle of the primary oocyte.

It was also observed in Chapter 7 that the ovulated primary oocytes of LT/Sv strain mice exhibited a high incidence of parthenogenetic activation when exposed briefly to a dilute solution of ethanol in vitro. The majority of activated oocytes exhibited a single diploid pronucleus following the extrusion of a polar body (1DPN). The cytogenetic analysis of this unique class of parthenogenone has found that in vitro ethanol-induced parthenogenetic activation failed to induce chromosome segregation errors during the completion of the "first" and only meiotic division of these oocytes. The inability of ethanol to induce chromosome segregation errors when the ovulated primary oocytes of LT/Sv mice are exposed to ethanol in vitro or when F1 ovarian oocytes are exposed to this agent in vivo indicates that the meiotic spindle

apparatus of these oocytes would appear to be more resistent to the trisomogenic potential of ethanol than the spindle apparatus of ovulated secondary oocytes.

The intragastric injection of a dilute ethanol solution to female mice at the time of conception (Kaufman, 1983b; Kaufman and Bain, 1984; O'Neill and Kaufman, 1987a) can increase the proportion of fertilised one-cell embryos that exhibit aneuploidy. Ethanol-induced parthenogenetic activation in vitro can also significantly increase the proportion of 1PN parthenogenones that exhibit aneuploidy at metaphase of the first cleavage mitosis. The mechanism by which ethanol interferes with the segregation of chromosomes during the completion of the second meiotic division in fertilised oocytes and ethanol-induced parthenogenones is believed to be similar (Chapter 4).

F1 female mice have been reported to rapidly metabolise an intragastric dose of ethanol within six hours (O'Neill and Kaufman, 1987a). The results of this study strongly indicate that increased incidence of aneuploidy observed in the hyaluronidase-induced 1PN parthenogenones that developed from oocytes exposed to ethanol during the first meiotic division was due to chromosome segregation errors that occurred during the completion of the second meiotic division. These events, therefore, cannot be induced by the direct interference of ethanol with the meiotic spindle apparatus of the ovulated oocyte. The

development of aneuploid 1PN parthenogenones must be due to anomalies that arise during the formation of the meiotic spindle apparatus of ovulated oocytes. The induction of aneuploidy following the parthenogenetic activation of postovulatory aged ethanol exposed oocytes also indicates that these spindle anomalies persist during the period of postovulatory ageing. Similarly, these results also indicate that the aneuploid fertilised one-cell mouse embryos that developed following the exposure of primary oocytes to ethanol in vivo (Kaufman and Bain, 1984; O'Neill and Kaufman, 1987a) were also the product of chromosome segregation errors during the completion of the second meiotic division.

The absence of metaphase chromosome spreads from ethanol exposed 2PN parthenogenones that exhibited pronuclear chromosome sets with a (21-20) or (19-20) constitution indicates that ethanol-induced aneuploidy is not due to the presegregation of chromatids in the primary oocytes exposed to ethanol in vivo. It is also possible that ethanol exposure in vivo induces the presegregation, but equational separation, of sister chromatids during the first meiotic division. The presence of independently segregating chromatids ("monads") at the completion of the second meiotic division has the potential to increase the proportion of aneuploid 1PN parthenogenones. However, metaphase chromosome spreads that exhibited pronuclear sets of chromosomes with complementary aneuploidy (19 -

21) were not observed. This novel mechanism of chromatid missegregation has been reported to occur when maize microsporocytes were exposed to ethylene glycols. In the same studies, ethanol was reported to disrupt the progression of meiosis in these cells but was not found to induce this form of genomic imbalance (Maguire, 1974).

The induction of aneuploidy during the completion of the second meiotic division in 1PN parthenogenones previously exposed to ethanol during the first meiotic division may be due to an interaction of the metabolites of ethanol, specifically acetaldehyde, with components of the meiotic spindle apparatus in ovulated oocytes. Acetaldehyde binds to the lysine residues of the tubulin protein and interferes with the polymerisation of cytoskeletal microtubules of cells exposed to ethanol in vivo (Tuma, Jennet and Sorrell, 1987; Sorrel and Tuma, 1987). The development of aneuploid parthenogenones from activated oocytes previously exposed to ethanol during the first meiotic division may be due to acetaldehyde-induced disorder in the structure or function of the second meiotic spindle apparatus during the activation of the ovulated oocyte.

Chapter 10

Conclusions and future studies

Cytogenetic and ultrastructural analyses of parthenogenetically activated mouse oocytes were performed in this investigation in order to investigate the nature and origin of ethanol-induced meiotic chromosome segregation errors. These parthenogenetic models were found to be efficient test systems to examine the trisomogenic potential of ethanol. In future studies, they could easily be adapted to examine the trisomogenic or clastogenic capacity of other suspect agents. Furthermore, the analysis of the proportionate incidence of the four main pathways of parthenogenetic development can also be used to assess the ability of exogenous stimuli to interfere with cytological events such as polar body extrusion. Overall, this investigation has confirmed the observations made by Kaufman (1985) and Kaufman and O'Neill (1988) that the exposure of mouse oocytes to dilute ethanol solutions, either prior to or at the time of conception, can increase the proportion of one-cell mouse embryos that exhibit aneuploidy.

The cytogenetic and ultrastructural analysis of *in vitro* ethanol-induced 1PN parthenogenones (Kaufman, 1982; O'Neill and Kaufman, 1989a, 1989b) was used as a model

system to examine the ability of ethanol to interfere with chromosome segregation during the completion of the second meiotic division. Cytogenetic analyses of the 1PN class of parthenogenone revealed that the duration of ethanol exposure was directly related to the incidence of aneuploidy. These findings indicated that ethanol interfered with the ability of the meiotic spindle apparatus to effect the balanced segregation of chromosomes. The karyotypic analysis of these parthenogenones at metaphase of the first cleavage mitosis demonstrated that numerical chromosomal anomalies were not the product of ethanol-induced damage to chromosomal structure. Furthermore, the fact that ethanol exposure inhibited second polar body extrusion in a significant proportion of activated oocytes indicated that this agent also disrupted actin-dependent events. These findings demonstrated that ethanol, per se, did not specifically interfere with the functioning of the microtubular filaments of the second meiotic spindle apparatus. These conclusions were confirmed by the ultrastructural analyses of ethanol activated oocytes. Ethanol exposure did not induce structural alterations to individual spindle elements. Structural anomalies to the second meiotic spindle apparatus and disruption of chromosome movement were only observed in activated oocytes at least 15 - 30 minutes after they had been removed from the ethanol stimulus. As ethanol exhibits the physiochemical properties of an anaesthetic agent, it

was concluded that the ability of ethanol to induce aneuploidy during the second meiotic division was due to its capacity to disrupt the Ca^{2+}-mediated regulation of cytoskeletal, but more particularly, microtubular function.

In contrast, ethanol-induced activation of the ovulated primary oocytes of LT/Sv strain mice was not found to induce the development of 1DPN parthenogenones that possessed an aneuploid chromosome constitution. This observation has stongly indicated that the interaction between ethanol and the meiotic spindle apparatus of activated primary oocytes differed to that which occurred when LT/Sv and F1 hybrid secondary oocytes were exposed to ethanol <u>in vitro</u>.

The subsequent cytogenetic analyses of the 1PN and 2PN hyaluronidase-induced parthenogenones that developed from those primary oocytes that were exposed to ethanol during the first meiotic division revealed that this agent did not interfere with the balanced segregation of chromosomes at this particular meiotic division. These studies found that the intragastric injection of a dilute ethanol solution to female mice during this period increased the incidence of chromosome malsegregation during the completion of the second meiotic division. These findings indicated that the mechanisms responsible for the development of aneuploid 1PN parthenogenones

following the in vivo exposure of the first meiotic division to ethanol differed to that which induced the development of in vitro ethanol-induced aneuploid 1PN parthenogenones. These results have indicated that the first meiotic division was either resistant to ethanol-induced disruption of meiotic spindle functions or that the mechanics of the spindle apparatus in the primary oocyte was able to compensate for ethanol-induced disorder. In these studies, the female mice had metabolised the dose of ethanol several hours before exposure to hyaluronidase and it was concluded that the primary metabolite of ethanol, acetaldehyde, was the active compound that induced the development of aneuploid 1PN parthenogenones. It was proposed that the formation of stable complexes between acetaldehyde and the lysine residues of tubulin proteins disrupted the ability of the second meiotic spindle to effect the balanced segregation of chromosomes. Furthermore, these alterations to the second meiotic spindle apparatus appearred to be relatively stable as, in these studies, they persisted through the period of postovulatory ageing. In subsequent studies, immunocytochemical or ultrastructural analyses of the first and second meiotic spindle apparatus of oocytes exposed to ethanol under these conditions could be used to identify the processes associated with the induction of aneuploid hyaluronidase-induced 1PN parthenogenones. However, the use of biochemical and biophysical techniques to analyse microtubule dynamics in

isolated oocytes may become, in future studies, the methodologies that will be best suited to establish the precise nature of the alterations to the structure and functioning of the meiotic spindle apparatus.

The karyotypic analyses of the aneuploid parthenogenones in each of these studies found that the smaller meiotic chromosomes of the mouse were predisposed more frequently to malsegregation events during the completion of the second meiotic division than other members of the mouse genome. Genomic factors such as the possession of NORs or the degree of centromeric heterochromatin did not appear to directly influence the malsegregation of particular chromosomes. Only a proportion of the total number of parthenogenones and fertilised one-cell embryos that were available for karyotypic analysis in these studies exhibited quality G-band patterns. Karyotypic studies of both euploid and aneuploid mouse parthenogenones and fertilised one-cell embryos should be continued as they will provide fundamental information necessary to further a greater understanding of the conditions that influence the malsegregation of particular chromosomes. The initial aim of the karyotypic studies presented in Chapter 9 was to examine the pattern of ethanol-induced chromosome malsegregation during the first meiotic division and to determine whether these events corresponded with those that occurred in ethanol activated oocytes. However this was not possible as the numerical chromosome anomalies in

the hyaluronidase-induced 1PN parthenogenones derived from ethanol exposed primary oocytes arose during the completion of the second meiotic division. Ultimately, the susceptibility of particular chromosomes to displacement and subsequent malsegregation may be influenced by factors such as their spatial organisation within the spindle apparatus and the genetic control of chromosome movement during meiotic anaphase. It is significant that the ultrastructural study of ethanol activated oocytes encountered both divergent arrays of microtubules at the periphery of the spindle apparatus and disorganised chromosome movement.

The findings of this animal model strongly indicate that the consumption of ethanol by women during the resumption of the first meiotic division or at fertilisation could result in the development of aneuploid conceptuses. The cytogenetic analyses of human oocytes and preimplantation embryos, and the identification of the parental origin of chromosome polymorphisms in trisomic abortuses and offspring, have strongly indicated that only 5%, or at most 10%, of human aneuploid conditions occur as a result of chromosome segregation errors during the completion of the second meiotic division. These present studies have found that ethanol-induced malsegregation events arose only during the completion of the second meiotic division whether exposure to ethanol occurred during the first or at the completion of the second meiotic division.

Furthermore, ethanol is likely to be only one of the many factors that have the potential to increase the incidence of chromosome malsegregation at this particular meiotic division of the human oocyte. It should therefore be considered that this agent may not be one of the more significant factors responsible for the high incidence of human meiotic aneuploidy. In contrast to the normal patterns of human alcohol consumption, the mice in this study were only exposed to a single intragastric dose of ethanol. There remains the possibility that the repeated exposure of human primary oocytes to ethanol, or its metabolites could interefere with chromosome segregation during the first meiotic division. However, when the fetotoxic, teratogenic and clastogenic properties of ethanol are also considered in conjunction with the potential of this agent to induce aneuploidy, it is clearly evident that the consumption of ethanol severely impairs the reproductive potential of the human species.

REFERENCES

Allison, A.C., Hulands, G.H., Nunn, J.F., Kitching, J.A. and MacDonald, A.C. (1970). The effect of inhalational anaesthetics on the microtubular system in Actinosphaerium nucleofilum. J. Cell Sci. 7: 483 - 499.

Ambramzuk, J. and Sawicki, W. (1975). Pronuclear synthesis of DNA in fertilized and parthenogenetically activated mouse eggs.
Exptl. Cell Res. 92: 361 - 372.

Anderson, R.A., Beyler, S.A. and Zaneveld, L.J.D. (1978). Alterations of male reproduction induced by chronic ingestion of ethanol: Development of an animal model.
Fertil. Steril. 30: 103 - 105.

Anderson, R.A., Reddy, J.M., Joyce, C., Willis, B.R., Van der Ven, H. and Zaneveld, L.J.D. (1982). Inhibition of mouse sperm capacitation by ethanol.
Biol. Reprod. 27: 833 - 840.

Andrews T., Dunlop, W. and Roberts D.F. (1984). Cytogenetic studies in spontaneous abortion.
Hum. Genet. 66: 77 - 84.

Angell, R.A., Aitken, P.F.A., van Look, P.F.H, Lumsden, M.A. Templeton, A.A. (1983). Chromosome abnormalities in human embryos after in vitro fertilisation.
Nature, Lond, 303: 336 - 338.

Angell, R.A., Templeton, A.A. and Aitken, R.J. (1986). Chromosome studies in human in vitro fertilization.
Hum. Genet. 72: 333 - 339.

Asakawa, T. and Dukelow, W.R. (1982). Chromosome constitution analyses after in vitro fertilization of squirrel monkey (Saimiri sciureus) oocytes.
Biol. Reprod. 26: 579 - 583.

Asakawa, T., Ishikawa, M., Shimizu, T. and Dukelow, W.R. (1988). The chromosomal normality of in vitro-fertilized rabbit oocytes. Biol Reprod. 38: 292 - 295.

Austin, C.R. (1970). Ageing and reproduction: Postovulatory deterioration of the egg.
J. Reprod. Fert. suppl. 12: 39 - 53.

Bajinòczky, K. and Méhès, K. (1988). Parental centromere separation sequence and aneuploidy in the offspring.
Hum. Genet. 78: 286 - 288.

Bałakier, H. and Tarkowski, A.K, (1976). Diploid parthenogenetic mouse embryos produced by heat shock and cytochalasin B. J. Embryol. exp. Morph. 35: 25 - 39.

Bandriff, B., Gordon, L., Ashworth, L., Watchmaker, G., Moore, I.I., Wyrobek, A. and Carrono, A.V. (1985). Chromosomes of human sperm: Variability amoung normal individuals. Hum. Genet. 70: 18 - 24.

Beerman, F., Francke, U. and Hansmann, I. (1986). High susceptibility for diploidy in ovulated oocytes from XO mice. Hum. Genet. 72: 323 - 327.

Beerman, F., Hummler, E., Francke, U. and Hansmann, I. (1988). Maternal modulation of the inheritable meiosis error Dipl. 1 in mouse oocytes is associated with the type of mitochondrial DNA. Hum. Genet. 79: 338 - 340.

Bond, D.J. (1987). Mechanisms of aneuploid induction. Mutation Res. 181: 257 - 266.

Bond, D.J. and Chandley, A.C. (1983). Aneuploidy. Oxford University Press, Oxford.

Boué, J., Boué, A. and Lazar, P. (1975). The epidemiology of human spontaneous abortions with chromosomal anomalies. In, Aging Gametes. Their biology and pathology. Ed., J. Blandau. Karger, Basel. pp. 330 - 348.

Boué, J., Boué, A. and Lazar, P. (1976). Retrospective and prospective epidemiological studies of 1500 karyotyped spontaneous human abortions. Teratology 12: 11 - 26.

Braden, A.W.H. (1957). The relationship between the diurnal light cycle and the time of ovulation in mice. J. Exp. Biol. 34: 177 - 188.

Braden, A.W.H., Austin, C.R. and David, H.A. (1954). The reaction of the zona pellucida to sperm penetration. Aust. J. Biol. Sci. 7: 391 - 409.

Brinkley, B.R. and Rao. P.N. (1973). Nitrous oxide: effects on the mitotic apparatus and chromosome movement in Hela cells. J. Cell Biol. 58: 96 - 106.

Brook, J.D., Gosden, R.G. and Chandley, A.C. (1984). Maternal ageing and aneuploid embryos - Evidence from the mouse that biological and not chronological age is the important influence. Hum. Genet. 66: 41 - 45.

Butler, M.G. and Sanger, W.G. (1981). Increased frequency of sister-chromatid exchanges in alcoholics.

Mutation Res. 85: 71- - 76.

Chandley, A.C. (1980). The origin of chromosomal abherrations in man and their potential for survival and reproduction in the adult human population.
Ann. Genet. Paris 24: 5 - 11.

Chandley, A.C. (1982). The origin of aneuploidy. In, Human Genetics, Part B. Medical Aspects. Eds. Bonne-Tamir, B., Cohen, T. and R.G. Goodman. Alan R. Liss, New York. pp. 337 - 347

Chernoff, G.F. (1977). The fetal alcohol syndrome in mice: An animal model. Teratology 15: 223 - 229.

Committee on Standardized Genetic Nomenclature for Mice. (1972). Standard karyotype of the mouse, Mus musculus.
J. Hered. 63: 69 - 72.

Cox, S.M., Rao, P.N. and Brinkley, B.R. (1977). Differential effects of nitrous oxide on the spindle and cytoplasmic microtubule complex. J. Cell Sci. 75: 291a.

Crowley, P.H., Gulati, D.K., Hayden, T.L., Lopez, P. and Dyer, R. (1979). A chiasma-hormonal hypothysis relating Down's syndrome and maternal age.
Nature, Lond. 280: 417 - 419.

Cuthbertson, K.S.R., Whittingham, D.G., Cobbold, P.H. (1981). Free Ca^{2+} increases in exponential phases during mouse oocyte activation. Nature, Lond. 294: 754 - 756.

Czeizel, A., Bognār, Z. and Rochenbauer, M. (1984). Some epidemiological data on spontaneous abortions in Hungary. J. Epidem. Comm. Health 38: 143 - 148.

Danford, N. and Parry, J.M. (1986). Induction of sex chromosome aneuploidy in mice by vincristine sulphate. I.R.C.S. Med. Sci. Biochem. 14: 15 - 17.

Daniel, A. and Roane, D. (1987). Aneuploidy is not induced by ethanol during spermatogenesis in the Chinese hamster. Cytogenet. Cell Genet. 44: 43 - 48.

de Boer,P. and Tates, A.D. (1983). Radiation-induced nondisjunction. In, Radiation-induced Chromosome Damage in Man. Series, Progress and Topics in Cytogenetics. Eds. Ishihara, T and M.S. Sasaki. Alan R. Liss, New York. Vol. 4. pp 299 - 325.

Dev, V.G., Tantravahi, D., Miller, A. and Miller, O.J. (1977). Nucleolus organizers in Mus musculus sub-species and in the Rag mouse cell line. Genetics 86: 389 - 398.

Donahue, R.P. (1972). Cytogenetic analysis of the first cleavage division in mouse embryos.
Proc. Natl. Acad. Sci. U.S.A. 69: 74 - 77.

Donahue, R.P. and Karp, L.E. (1973). Chromosomal anomalies after fertilization of aged, postovulatory mouse oocytes. Am J. Hum. Genet. 25: 24a.

Dyban, A.P. and Khozhai, L.I. (1980). Parthenogenetic development of ovulated mouse ova under the influence of ethyl alcohol. Bull. Exp. Biol. Med. 89. 528 - 530.

Dyban, A.P. and Baranov, V.S. (1987). Cytogenetics of Mammalian Embryonic Development. Oxford University Press, Oxford.

Edwards, R.G. (1983). Chromosomal abnormalities in human embryos.
Nature, Lond, 303: 283.

Edwards, R.G. and Gates, A.H. (1959). Timing of the stages of the maturation divisions, ovulation, fertilisation and the first cleavage of eggs of adult mice treated with gonadotrophins.
J. Endocrin. 18: 292 - 304.

Eichenlaub-Ritter, U., Chandley, A.C. and Gosden, R.G. (1986). Alterations to the microtubular cytoskeleton and increased disorder of chromosome alignment in spontaneously ovulated mouse oocytes aged in vivo.
Chromosoma 94: 337 - 345.

Eichenlaub-Ritter, U., Chandley, A.C. and Gosden R.G. (1988). The CBA mouse as a model for age-related aneuploidy in man: studies of oocyte maturation, spindle formation and chromosome alignment during meiosis.
Chromosoma 96: 220 - 226.

Eisen, A. and Reynolds, G.T. (1985). Source and sinks for the calcium released during fertilization of single sea urchin eggs. J. Cell Biol. 100: 1522 - 1527.

Eppig, J.J. (1978). Granulosa cell-deficient-follicles. Occurrence, structure and relationship to ovarian teratocarcinogenesis in strain LT/Sv mice. Differentiation 12: 111 - 120.

Epstein, C.J. (1985). The mouse trisomies. Experimental systems for the study of aneuploidy. In, Issues and Reviews in Teratology. Vol. 3. Ed., H. Kalter. Plenum Press, New York. pp. 171 - 217.

Eusebi, F. and Siracusa, G. (1983). An electo-physiological study of parthenogenetic activation in mammalian oocytes. Devl. Biol. 96: 386 - 395.

Figueroa, M.L. and Vig, B.K. (1983). Sequence of centromere separation: Lack of Colcemid effect on the Chinese hamster genome.
Cytogenet. Cell Genet. 36: 627 - 632.

Fitzgerald, P.H., Archer, S.A. and Morris, C.M. (1986). Evidence for the repreated primary non-disjunction of chromosome 21 as a result of premature centromere division (P.C.D.). Hum. Genet. 72: 58 - 62.

Ford, C.E. (1975). The time in development at which gross genome unbalance is expressed. In, The Early Development of Mammals. Eds., Balls, M. and A.E. Wild. Cambridge University Press, Cambridge. pp. 285 - 384.

Ford, J.H. and Lester, P. (1982). Factors affecting the displacement of human chromosomes from the metaphase plate. Cytogenet. Cell Genet. 33: 327 - 332.

Ford, J.H. and Roberts, C. (1983a). Displacement of chromosomes in mitosis: A technique for assessing differential chromosome error.
Cytogenet. Cell Genet. 36: 537 - 541.

Ford, J.H. and Roberts, C.G. (1983b). Chromosome displacement and spindle tubule polymerization. 1. The effects of alterations in pH on displacement frequency. Cytobios 37: 163 - 169.

Ford, J.H. and Roberts, C.G. (1984). Chromosome displacement and spindle tubule polymerization. 2. Effect of alterations of extracellular calcium on displacement parameters. Cytobios 39: 81 - 88.

Franks, N.P. and Lieb, W.R. (1982). Molecular mechanisms of general anaesthesia. Nature, Lond. 300: 487 - 492.

Fraser, L.R. and Dandekar, P.V. (1973). The effects of aging on in vitro fertilization of rabbit eggs and subsequent embryonic development.
J. exp. Zool. 184: 303 - 312.

Freire-Maia, N. (1982). Zygotic loss and mutational load. In, Human Genetics, Part A. The Unfolding Genome. Eds., Bonne-Tamir, B., Cohen, T. and R.G. Goodman. Alan R. Liss, New York. pp. 299 - 306

Gabarrrón, J., Jimenez, A. and Glover, G. (1988). Premature centromere division dominantly inherited in a

subfertile family. Cytogenet. Cell Genet. 43: 69 - 71.

Gallimore. P.H. and Richardson, C.R. (1973). An improved banding technique exemplified in the karyotype analysis of two strains of rat. Chromosoma, 41: 259 - 263.

Gavaler, J.S. and Van Thiel D.H. (1987). Reproductive consequences of alcohol abuse: males and females compared and contrasted. Mutation Res. 186: 269 - 277.

German, G. (1968). Mongolism, delayed fertilization and human sexual behaviour. Nature, Lond. 217: 516 - 518.

Gosden, C. (1986). Prenatal karyotyping amniotic fluid cells or chorion villus samples. In, Chorion Villus Sampling. Eds. Liu, D.T.Y., Symonds, E.M. and M.S. Golbus. Chapman and Hall, London. pp 257 - 272.

Gropp, A. (1982). Value of an animal model for trisomy. Virchows Arch (Pathol. Anat.) 395: 117 - 131.

Grudzinskas, J.G. and Nysenbaum, A.M. (1985). Failure of human pregnancy after implantation. In, In Vitro Fertilization and Embryo Transfer Techniques. Ann. N. Y. Acad. Sci. Vol. 442. Eds., Seppala. M. and R.G. Edwards. pp. 38 - 44

Halmesmaki, E., Raivio, K.O. and Ylikorkala, O. (1987). Patterns of alcohol consumption during pregnancy. Obstet. and Gynaecol. 69: 594 - 597.

Hamilton, W.J. and Day, F.T. (1945). Cleavage stages of the ova of the horse with notes on ovulation. J. Anat. 79: 127 - 130.

Hansmann, I and El-Nahaas, E. (1979). Incidence of nondisjunction in mouse oocytes. Cytogenet. Cell Genet. 24: 115 - 121.

Hansmann, I. and Probeck, H.D. (1979). Chromosomal imbalance in ovulated oocytes from Syrian hamsters (Mesocricetus auratus) and Chinese hansters (Cricetulus griseuus). Cytogenet. Cell Genet. 23: 70 - 76.

Harlap, S. and Shiono, P.A. (1980). Alcohol, smoking and the incidence of spontaneous abortion in the first and second trimester. Lancet 2: 173 - 176.

Harsanyi, Z., Granek, I.A. and MacKenzie, D.W.R. (1977). Genetic damage induced by ethyl alcohol in Aspergillus nidulans. Mutation Res. 48: 51 - 74.

Hassold, T., Chen, N., Funkhouser, J., Jooss, T., Manuel, B., Matsuura, J., Matsuyama, A., Wilson, C., Yamane, A. and Jacobs, P.A. (1980a). A cytogenetic study of 1000 spontaneous abortions. Ann. Hum. Genet. 44: 151 - 178.

Hassold, T. and Chiu, D. (1985). Maternal-age specifc rates of numerical chromosome abnormalities with specific reference to trisomy. Hum. Genet. 70: 11 - 17.

Hassold, T., Chiu, D. and Yamane J.A. (1984). Parental origin of autosomal trisomies.
Ann. Hum. Genet. 48: 129 - 144.

Hassold, T. and Jacobs P.A. (1984). Trisomy in man.
Ann. Rev. Genet. 18: 69 - 97.

Hassold T., Jacobs, P., Kline, J., Stein, Z. and Warburton, D. (1980b). Effect of maternal age on autosomal trisomies. Ann. Hum. Genet. 44: 29 - 36.

Hassold, T., Jacobs, P.A. and Pettay, D. (1987). Analysis of nucleolar organising regions in the parents of trisomic spontaneous abortions.
Hum. Genet. 76: 381 - 384.

Hassold, T., Kumlin, E., Takaesu, N. and Leppert, M. (1985b). Determination of the parental origin of sex chromosome monosomy using restriction fragment length polymorphisms. Am. J. Hum. Genet. 37: 965 - 972.

Hayden, T.L., Crowley, P.H. and Wilson, E. (1980). A model relating the incidence of meiotic trisomy to maternal age. J. Theor. Biol. 86: 123 - 136.

Heidam, L.Z. (1984). Spontaneous abortions amoung dental assistants, factory workers, painters and gardening workers: a follow up study.
J. Epidem. Comm. Health. 38: 149 - 155.

Henderson S.A. and Edwards, R.G. (1968). Chiasma frequency and maternal age in mammals.
Nature, Lond. 218: 22 - 28.

Hinkley, R.E. and Samson, F.E. (1972). Anaesthetic induced transformation of axonal microtubules.
J. Cell Biol. 53: 258 - 263.

Hinkley, R.E. and Telser, A.G. (1974). The effects of Halothane on cultured mouse neuroblastoma cells. Inhibition of morphological differentiation.
J. Cell Biol. 63: 531 - 540.

Hoek, J.B. (1987). Effects of ethanol on calcium

homeostasis in rat hepatocytes and its interaction with the phosphoinositide-dependant pathway of signal transduction. In, Alcohol and the Cell. Ann. N.Y. Acad. Sci. Vol. 492. Ed. E.Rubin. pp.212 - 223.

Hoffman, G.R., Dellarco, V.L. and Voytek, P.E. (1986). A review of the symposium on aneuploidy. Etiology and mechanisms. Environ. Mutagen. 8: 643 - 651.

Howlett, S.K., Webb, M., Maro, B. and Johnson, M.H. (1985). Meiosis II, mitosis I and the interlinking interphase: a study of the cytoskeleton in the fertilised mouse egg. Cytobios, 43: 295 - 305.

Hummler, E. and Hansmann, I. (1985). Preferential nondisjunction of specific bivalents in oocytes from Djungarian hamsters (Phodopus sungorus) following colchicine treatment.
Cytogenet. Cell Genet. 39: 161 - 167.

Hummler, E., Theuring, F. and Hansmann, I. (1987). Meiotic nondisjunction in oocytes from aged Djungarian hamsters correlates with an alteration in meiosis rate but not in univalent formation.
Hum. Genet. 76: 352 - 356.

Hunt, P.A. (1987). Ethanol-induced aneuploidy in male germ cells. Cytogenet. Cell Genet. 44: 7 - 10.

Igusa, Y. and Miyazaki, S.I. (1986). Periodic increase of cytoplasmic free calcium in fertilised hamster eggs measured with calcium-sensitive electrodes.
J. Physiol. 377: 193 - 205.

Iwamatsu, T. and Chang, M.C. (1971). Factors involved in the fertilization of mouse eggs in vitro.
J. Reprod. Fert. 26: 197 - 208.

Iwamatsu T. and Chang, M.C. (1972) Sperm pnetration in vitro of mouse oocytes at various times during maturation. J. Reprod. Fert. 31: 237 - 247.

Ito, S. and Winchester, R.T. (1963). The fine structure of the gastric mucosa of the bat.
J. Cell Sci. 16: 541 - 577.

Izant. J.G. (1983). The role of calcium ions during mitosis. Calcium participates in the anaphase trigger. Chromosoma. 88: 1 - 10.

Jackson, S.H. (1975). Anesthetics and cell multiplication. Clin. Anesth. 11: 75 - 92.

Jackson-Cook, C.K., Flannery, D.B., Corey, L.A., Nance, W.E. and Brown, J.A. (1985). Nucleolar organizer region variants as a risk factor for Down Syndrome. Am. J. Hum. Genet. 37: 1049 - 1061.

Jacobs, P.A. and Mayer, M. (1981). The origin of human trisomy: a study of heteromorphisms and satellite associations. Ann. Hum. Genet. 45: 357 - 365.

Jagiello, G. (1987). Chromosome analysis of oocytes in stimulated cycles. N. Eng. J. Med. 317: 318.

Jenderny, J. and Röhrborn, G. (1987). Chromosome analysis of human sperm. I First results with a modified method. Hum. Genet. 76: 385 - 388.

Jones, K.L. and Smith, D.W. (1975). The fetal alcohol syndrome. Teratology. 12: 1 - 10.

Juberg, R.C. (1983). Origin of chromosomal abnormalities: evidence for delayed fertilization in meiotic nondisjunction. Hum. Genet. 64: 122 - 127.

Juberg, R.C. and Mowry, P.N. (1983). Origin of Trisomy 21 Syndrome: All studies compiled, parental age analysis and international comparisons.
Am. J. Hum. Genet. 16: 111 - 116.

Juetten, J. and Bavister, B.D. (1983). Effects of egg aging on in vitro fertilization and first cleavage division in the hamster. Gamete Res. 8: 219 - 230.

Käfer, E. (1984). Disruptive effects of ethyl alcohol on mitotic chromosome segregation in diploid and haploid strains of Aspergillus nidulans.
Mutation Res. 135: 53 - 75.

Karasiewicz, J. (1981). Electron microscope studies of cytokinesis in metazoan cells. In, Mitosis / Cytokinesis. Eds., Zimmerman, A.M. and A. Forer. Academic Press, New York. pp. 419 - 436.

Karasiewicz, J. and Soltynska, M.S. (1986). Effects of cytochalasin B on the cleavage furrow in mouse blastomeres. Roux's Arch. Devl. Biol. 195: 137 - 141.

Kaufman, M.H. (1973a). Parthenogenesis in the mouse. Nature, Lond, 242: 475 - 476.

Kaufman, M.H. (1973b). Analysis of the first cleavage division to determine the sex-ratio and the incidence of chromosome anomalies at conception in the mouse.
J. Reprod. Fert. 35: 67 - 72.

Kaufman, M.H. (1975a). Parthenogenetic activation of mouse oocytes by Avertin anaesthesia.
J. Embryol. exp. Morph. 33: 941 - 946.

Kaufman, M.H. (1975b). The exerimental induction of parthenogenesis in the mouse. In, The Early Development of Mammals. B.S.D.B. Symp. 2. Eds., Balls, M. and A.E. Wild. Cambridge University Press, Cambridge. pp. 25 - 44.

Kaufman, M.H. (1977a). Effect of anaesthetic agents on eggs and embryos. In, Development in Mammals Vol.5. Ed., M.H. Johnson. North Holland, Amsterdam. pp. 137 - 163.

Kaufman, M.H. (1977b) Effect of anaesthesia on the outcome of pregnancy in mice. J. Reprod. Fert. 49: 167 - 168.

Kaufman, M.H. (1982). The chromosome complement of single-pronuclear haploid mouse embryos following activation by ethanol treatment.
J. Embryol. exp. Morph. 71: 139 - 154.

Kaufman, M.H. (1983a). Chromosome abnormalities.
Nature, Lond. 304: 482.

Kaufman, M.H. (1983b). Ethanol-induced chromosomal abnormalities at conception.
Nature, Lond, 302: 258 - 260.

Kaufman, M.H. (1983c). Early Mammalian Development. Parthenogenetic Studies. Cambridge University Press, Cambridge.

Kaufman, M.H. (1985). An hypothysis regarding the origin of aneuploidy in man: Indirect evidence from an experimental model. J. Med. Genet. 22: 171 - 178.

Kaufman, M.H. (1988). Hyatidiform mole: Genetic and practical implications. Hosp. Update. 14. 1415 - 1420.

Kaufman, M.H. amd Bain, I.M. (1984). Influence of ethanol on chromosome segregation during the first and second meiotic divisions in the mouse egg.
J. exp. Zool. 230: 315 - 320.

Kaufman, M.H. and Howlett, S.K. (1986). The ovulation and activation of primary and secondary oocytes in LT/Sv strain mice. Gamete Res. 14: 255 - 264.

Kaufman, M.H. and O'Neill, G.T. (1988). Aneuploidy Induced by Ethanol. In, Aneuploidy, Part B: Induction and Test Systems. Series, Progress and Topics in

Cytogenetics. Eds., Sandberg, A.A. and Vig B.K. Alan R. Liss. New York. pp 95 - 122.

Kaufman, M.H. and Speirs, S. (1987). The postimplantation development of spontaneous digynic triploid embryos in LT/Sv strain mice. Development 101: 383 - 391.

Kaufman, M.H. and Woollam, D.H.M. (1981). The passage to the foetus and liquor amnii of ethanol administered orally to the pregnant mouse.
Br. J. Exp. Path. 62: 357 - 361.

Kiehart, D.P. (1981). Studies on the in vivo sensitivity of spindle microtubules to calcium ions and evidence for a vesicular calcium sequestering system.
J. Cell Biol. 88: 604 - 617.

King, W.A., Bezard, J., Bousquet, D. Palmer, E. and Betteridge, K.J. (1987). The meiotic stage of preovulatory oocytes in mares. Genome 29 679 - 682.

Klassen, R.W. and Persaud, T.V.N. (1976). Experimental studies on the influence of male alcoholism on pregnancy and projeny. Exp. Path. Bd. 12: s38 - 45.

Kline, J., Shrout, P., Stein, Z., Susser, M. and Warburton, D. (1980). Drinking during pregnancy and spontaneous abortion. Lancet 2: 176 - 180.

La Fontaine, J.R. (1985). Chromosome segregation and spindle structure in crane fly spermatocytes collowing Colcemid treatment. Chromosoma 91: 329 - 336.

Liang, J.C., Sherron, D.A. and Johnston, D. (1986). Lack of correlation between mutagen induced chromosomal univalency and aneuploidy in mouse spermatocytes.
Mutation Res. 163: 285 - 298.

Lin, C.C., De Braekeleer, M. and Jamro, H. (1985). Cytogenetic studies in spontaneous abortion: the Calgary experience. Can. J. Genet. Cytol. 27: 565 - 571.

Lippman, A. and Aymé, S. (1984). Fetal death rates in mothers with trisomy 21 (Down Syndrome).
Ann. Hum. Genet. 48: 303 - 312.

Long, S.E. and Williams, C.V. (1980). Frequency of chromosomal abnormalities in early embryos of the domestic sheep (Ovis avies).
J. Reprod. Fert. 58: 197 - 201.

Longo, F.J. and Chen, D.Y. (1985). Development of cortical polarity in mouse eggs: Involvement of the

meiotic apparatus. Devl. Biol. 107: 382 - 394.

Luthardt, F.W., Palmer, C.G. and Yu, P.L. (1973). Chiasma and univalent frequencies in ageing female mice. Cytogenet. Cell Genet. 12: 68 - 79.

McClearn, G.E. and Rodgers, D.A. (1972). Differances in alcohol preference amoung inbreed strains of mice. Q. J. Stud. Alcohol. 20: 691 - 695.

McGaughey, R.W. and Chang, M.C. (1969). Inhibition of fertilization and production of herteroploidy in eggs of mice treated with colchicine. J. exp. Zool. 171: 465 - 480.

Mailhes, J.B., Preston, R.J. and Lavappa, K.S. (1986). Mammalian in vivo assays for aneuploidy in female germ cells. Mutation Res. 167: 139 - 148.

Mailes, J.B., Preston, R.J., Yuan, Z.P. and Payne, H.S. (1988). Analysis of mouse metaphase metaphase II oocytes as an assay for chemically induced aneupoidy. Mutation Res. 198: 145 - 152.

Mailes, J.B. and Yuan, Z.P. (1987). Differential sensitivity of mouse oocytes to colchicine-induced aneuploidy. Environ. Mol. Mutagen. 10: 183 - 188.

Magnuson, T. (1983). Genetic abnormalities and early mammalian development. In, Development in Mammals, Vol. 5. Ed, M.H. Johnson Elsevier, Amsterdam. pp. 209 - 249.

Maguire, M.P. (1976). The effect of ethanol on meiotic chromosome behaviour in maize. Caryologia. 29: 41 - 52.

Mankes, R.F., Le Fevre, R., Benitz, F., Rosenblum, I., Bates, H., Walker, A.I.T.and Abraham, R. (1982). Paternal effects of ethanol in the Long Evans rat. J. Toxicol. Environ. Health. 10: 871 - 878.

Marcum, J.M., Dedman, J.R., Brinkley, B.R. and Means, A.R. (1978). Control of microtubule assembly-dissassembly by calcium-dependant regulator protein. Proc. Natl. Acad. Sci. U.S.A. 75: 3771- 3775.

Maro, B., Howlett, S.K. and Webb M. (1985). Non-spindle microtubule organizing centers in metaphase II-arrested mouse oocytes. J. Cell Biol. 101: 1665 - 1672.

Maro, B., Johnson, M.H., Pickering, S.J. and Flach, G. (1984). Changes in actin distribution during fertilisation of the mouse egg.

J. Embryol. exp. Morph. 8: 211 - 237.

Maro, B., Johnson, M.H., Webb, M. and Flack, G. (1986). Mechanism of polar body formation in the mouse oocyte: an interaction between the chromosomes, the cytoskeleton and the plasma membrane.
J. Embryol. exp. Morph. 92: 11 - 32.

Marston, J.H. and Chang, M.C. (1964). The fertilizable life of ova and their morphology following delayed insemination in mature and immature mice.
J. exp. Zool. 155: 237 - 252.

Martin, R.H., Balkan, W. and Burns, K. (1983). Cytogenetic analysis of Q-banded pronuclear chromosomes in fertilized Syrian hamster eggs.
Cytogenet. Cell Genet. 35: 41 - 45.

Martin, R.H., Dill, F.J. and Miller, J.R. (1976). Nondisjunction in aging female mice.
Cytogenet. Cell Genet. 17: 150 - 160.

Martin-De Leon, P.A. and Boice, M.L. (1983). Spontaneous heteroploidy in one-cell mouse embryos.
Cytogenet. Cel Genet. 35: 57 - 63.

Maudlin, I. and Fraser L.R. (1978). Maternal age and the incidence of aneuploidy in first cleavage mouse embryos.
J. Reprod. Fert. 54: 423 - 426.

Méhès, K (1978). Non-random centromere division: a mechanism of non-disjunction causing aneuploidy.
Hum. Hered. 28: 255 - 260.

Mikkelsen, M., Poulsen, H., Grinsted, J. and Lange, A. (1980). Non-disjunction in trisomy 21: study of chromosomal heteromorphisms in 110 families.
Ann. Hum. Genet. 44: 17 - 28.

Miller, K.W., Firestone, L.L. and Forman, S.A. (1987) General anesthetic and specific effects of ethanol on acetlycholine receptors. In, Ethanol and the Cell. Ann. N.Y. Acad. Sci. Vol. 492. Ed. E Rubin. pp. 71 - 87.

Miller, O.J., Miller, D.A., Tantravahi, R. and Dev, V.G. (1978). Nucleolus organizer activity and the origin of Robertsonian translocations.
Cytogenet. Cell Genet. 20: 40 - 50.

Mirre, C., Hartung, M. and Stahl, A. (1980). Association of ribosomal genes in the fibrillar center of the nuleolus. A factor influencing translocation and

non-disjunction in the human meiotic oocyte.
Proc. Natl. Acad. Sci. U.S.A. 77: 6017 - 6021.

Mitelman, F. and Wadstein, J. (1978). Chromosome abherrations in chronic alcoholics.
Lancet, 1. 216.

Miyazaki, Y., Hashimoto, N., Yoshimoto, Y., Kishimoto, T., Igusa, Y. and Hiramoto, Y. Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization in golden hamster oocytes.
Devl. Biol. 118: 259 - 267.

Mooseker,M.S., Coleman, T.R. and Conzelman, K.A. (1986). Calcium and the regulation of cytoskeletal assembly, structure and contractibility. In, Calcium and the Cell. Eds. Evered, D and J. Whelen. Ciba Foundation Symposium 122. John Wiley, London. pp. 232 - 249.

Nesbitt, M.N. and Francke, U. (1973). A system of nomenclature for band patterns of mouse chromosomes.
Chromosoma 41: 145 - 158.

Niemierko, A. (1981). Postimplantation development of CB-induced triploid mouse embryos.
J. Embryol. exp. Morph. 66: 81 - 89.

Nijhoff, J.H. and de Boer, P. (1981). Spontaneous meiotic non-disjunction in mammals. A study evaluating the various experimental approaches. Genetica 56, 99 - 121.

Niikawa, N., Merrotto, G. and Kajii, T. (1977). Origin of acrocentric trisomies in spontaneous abortions.
Hum. Genet. 40: 73 - 78.

Niwa, K. and Chang, M.C. (1975). Fertilization of rat oocytes in vitro at various times before and after ovulation with special reference to fertilization of ovarian oocytes in culture.
J. Reprod. Fert. 43: 435 - 451.

Niwa, K., Miyake, M., Iritani, A., and Nishihawa, Y. (1976). Fertilization of rat oocytes cultured in vitro from various stages of maturation.
J. Reprod. Fert. 47: 105 - 106.

Obe, G., Natarajan, A.T., Meyers, M. and Den Hertog, A. (1979). Induction of chromosomal abherations of peripheral lymphocytes of human blood in vitro and of S.C.E.s in bone marrow cells in mice in vivo by ethanol and it's metabolite acetaldeheyde.
Mutation Res. 68: 291 - 294.

Obe, G., Gobel, D., Engeln, H., Herha, J. Natarajan, A.T. (1980). Chromosomal abherations in peripheral lymphocytes of alcoholics. Mutation Res. 73: 377 - 386.

Obe, G. and Anderson, D. (1987). Genetic effects of ethanol. Mutation Res. 186: 177 - 200.

Ohnishi, S.T., Flick, J.L and Rubin E. (1984). Ethanol increases calcium permeability of heavy sacroplasmic reticulum of skeletal muscle.
Arch. Biochem. Biophys. 233: 588 - 594.

Ohnishi, S.T. (1987). Effects of alcohol and halothane on the structure and function of sarcoplasmic reticulum. In, Alcohol and the cell . Ann. N. Y. Acad. Sci. Vol. 492. Ed. E. Rubin. pp. 138 - 144.

Okada, K., Yanagimachi, R. Yanagimachi, H. (1986). Development of a cortical granule-free area of cortex and the perivitelline space in the hamster oocyte during maturation and following ovulation.
J. Submicrosc. Cytol. 18: 233 - 247.

O'Neill, G.T. and Kaufman, M.H. (1987a). Cytogenetic analysis of first cleavage fertilised mouse eggs following in vivo exposure to ethanol shortly before or at the time of conception. Development 100, 441 - 448.

O'Neill, G.T. and Kaufman M.H. (1987b). Ovulation and fertilization of primary and secondary oocytes in LT/Sv strain mice. Gamete Res. 18: 27 - 36.

O'Neill, G.T. and Kaufman, M.H. (1988). Influence of postovulatory aging on chromosome segregation during the second meiotic division in mouse oocytes: a parthenogenetic analysis. J. exp. Zool. 248: 125 - 131.

O'Neill, G.T. and Kaufman,M.H. (1989a). Cytogenetic analysis of ethanol-induced parthenogenesis.
J. exp. Zool. 249: 182 - 192.

O'Neill, G.T., McDougall, R.D. and Kaufman, M.H. (1989b). Ultrastructural analysis of abnormalities in the morphology of the second meiotic spindle in ethanol-induced parthenogenones. Gamete Res. 22: 285 - 299.

O'Shea, K.S. and Kaufman, M.H. (1979). The teratogenenic effect of acetaldehyde: implications for the study of the fetal alcohol syndrome. J. Anat. 128: 65 - 76.

O'Shea, K.S. and Kaufman. M.H. (1981). Effect of acetaldehyde on the neuroepithelium of early mouse

embryos. J. Anat. 132: 107 - 118.

Onfelt, A. (1986). Mechanistic aspects on chemical induction of spindle disturbances and abnormal chromosome numbers. Mutation Res. 168: 249 - 300.

Pearson, A.P. and Enders R.K. (1943). Ovulation, maturation and fertilization in the fox.
Ant. Rec. 85: 69 - 84.

Plachot, M., de Grouchy, J., Junca, A., Mandelbaum, J., Turleau, C., Couillin, P., Cohen, J. and Salat-Baroux, J. (1987). From oocyte to embryo: A model, deduced from in vitro fertilization, for natural selection against chromosome abnormalities. Ann Genet, Paris. 30: 22 - 32.

Plachot, M., Veiga, A., Montagut, J., de Grouchy, J., Calderon, G., Lepetre, S., Junca, A., Santalo, J., Carles, E., Mandelbaum, J., Barri, P., Degoy, J., Cohen, J. Egozcue, J., Sabatier, J.C. and Salat-Baroux, J. (1988). Are clinical and biological parameters correlated with chromosomal disorders in early life: a multicentric study. Hum. Reprod. 3: 627 - 635.

Petrinelli, P., Antonelli. A. and Dallapicola, B. (1984). Premature centromere splitting in a presumtive mild form of Roberts syndrome. Hum. Genet. 66: 96 - 99.

Philips, R.J.S and Kaufman, M.H. (1974). Bare-patches, a new sex-linked gene in the mouse, associated with a high production of XO females. II. Investigations into the nature and mechanism of XO production.
Genet. Res. 24: 27 - 41.

Proctor, S.E., Watt, J.L. and Gray, E.S. (1986). Cytogenetic analysis in 100 spontaneous abortions in North East Scotland. Clin. Genet. 29: 101 - 103.

Randall, C.L. and Taylor, W.J. (1979). Prenatal ethanol exposure in mice: Teratogenic effects.
Teratology 19: 305 - 311.

Rodman, T.C. (1971). Chromosomes of the first polar body in mammalian meiosis. Exptl. Cell Res. 68: 205 - 210.

Rogers, B.J., Cash, M.K.M. and Vaughn, W.K. (1987). Ethanol inhibits human and hamster sperm penetration of eggs. Gamete Res. 16: 97 - 107.

Santalo, J., Estop, A.M. and Egozcue, J. (1986). The chromosome complement of first cleavage mouse embryos after in vitro fertilization.
J. In Vitro. Fert. Embryo. Transfer. 3: 99 - 105.

Sato, K. and Blandau R.J. (1979). Second meiotic division and polar body formation in mouse eggs fertilized in vitro. Gamete Res. 2: 283 - 293.

Sax, K. and Sax, H.J. (1966). Radiomimetric beverages, drugs and mutagens.
Proc. Natl. Acad. Sci. U.S.A. 55: 1431 - 1435.

Schliwa, M. (1981). Proteins associated with cytoplasmic actin. Cell 25: 587 - 690.

Searle, A.G. (1981). Numerical variants and structural rearrangements. In, Genetic Variants and Strains of the Laboratory Mouse. Ed, M.C. Green. Fisher, Stuttgart. pp. 324 - 357.

Selletto, C. and Kuriyama, R. (1988). Distribution of centriolar material in multipolar spindles induced by Colcemid treatment in Chinese hamster ovary cells.
J. Cell Sci. 98: 57 - 65.

Sengoku, K. and Dukelow, W.R. (1988). Gonadotrophin effects on chromosomal normality of hamster preimplantation embryo. Biol. Reprod. 38: 150 - 155.

Siracusa, G., Whittingham, G.G., Codoesu, M. and De Felici, M. (1978). Local anesthetics and phenothiazine tranquillizers induce parthenogenetic activation of the mouse oocyte. Devl. Biol. 65: 531 - 535.

Smith, A.L. and Lodge, J.R. (1987). Interactions of aged gamate: In vitro fertilization using in vitro-aged sperm and in vivo-aged ova in the mouse. Gamete Res. 16: 47 - 56.

Sorrell, M.F. and Tuma, D.J. (1987). The functional implications of acetaldehyde binding to cell constituents. In, Ann. N. Y. Acad. Sci. Vol. 492. Ed. E. Rubin. pp. 50 - 62.

Speed, R.M. and Chandley, A.C. (1983). Meiosis in the fetal mouse ovary: II Oocyte development and age related aneuploidy. Does a production line exist?
Chromosoma 88: 184 - 189.

Speirs, S and Kaufman, M.H. (1988). Effect of exogenous hormones on the ovulation of primary and secondary oocytes in LT/Sv strain mice. Gamete Res. 21: 179 - 184.

Stevens, L.C. (1975). Comparative development of normal

and parthenogenetic mouse embryos, early testicular and ovarian teratomas and embryoid bodies. In, Teratomas and differentiation. Eds., Sherman, M.I. and D. Solter. Academic Press, New York. pp. 17 - 32.

Stevens, L.C. and Varnum, D.S. (1974). The development of teratomas from parthenogenetically activated mouse oocytes. Devl. Biol. 37: 369 - 380.

Streissguth, A.P., Landesman-Dwyer, S., Martin, J.C. and Smith, D.W. (1980). Teratogenic effects of ethanol in humans and laboratory animals. Science 209, 533 - 361.

Sugawara, S. and Mikamo, K. (1980). An experimental approach to the analysis of mechanisms of meiotic non-disjunction and anaphase lagging in primary oocytes. Cytogenet. Cell Genet. 28: 251 - 264.

Sugawara, S and Mikamo, K. (1983). Absence of correlation between univalent formation and meiotic nondisjunction in aged female Chinese hamsters.
Cytogenet. Cell Genet. 35: 34 - 40.

Sugawara, S. and Mikamo, K. (1986). Maternal aging and nondisjunction: A comparative study of two chromosomal techniques on the formation of univalents in first meiotic anaphase oocytes of the mouse.
Chromosoma 93: 321 - 325.

Sulik, K.K., Johnston, M.C. and Webb, M.A. (1981). Fetal alcohol syndrome. Embryogenesis in a mouse model. Science 214, 936 - 938.

Surani, M.A.H. and Kaufman, M.H. (1977). Influence of extracellular Ca^{2+} and Mg^{2+} ions on th second meiotic division of mouse oocyte: Relevance to obtaining haploid and diploid parthenogenetic embryos.
Devl. Biol. 59: 86 - 90.

Szöllösi, D. (1967). Development of cortical granules and the cortical reaction in rat and hamster eggs.
Anat. Rec. 159: 431 - 436.

Szöllösi, D. (1971). Morphological changes in mouse eggs due to aging in the fallopian tube.
Am. J. Anat. 130: 209 - 226.

Szöllősi, D. (1975). Mammalian eggs in the fallopian tube. In, Aging Gametes. Their biology and pathology. Ed. R.J. Blandau. Karger, Basel. pp. 99 - 121.

Tarkowski, A.K. (1966). An air-drying method for chromosome preparations from mouse eggs.
Cytogenetics 5: 394 - 400.

Tarkowski, A.K., Witkowska, A and Nowicka, J (1970). Experimental parthenogenesis in the mouse. Nature, Lond. 226: 162 - 165.

Tates, A.D., de Vogel, N. and Neuteboom, I. (1980). Cytogenetic effects in hepatocytes, bone marrow cells and blood lymphocytes of rats exposed to ethanol in drinking water. Mutation Res. 79: 285 - 288.

Tease, C. and Fisher, G. (1986). Oocytes from young and old female mice respond differently to colchicine. Mutation Res. 173: 31 - 34.

Teichert-Kuliszcwska, K., Israel, Y. and Cinader, B. (1988). Alcohol dehydrogenase is not a major derterminant of alcohol preferance in mice. Alcohol 5: 45 - 48.

Templado, C., Benet, J., Genesca, A., Navarro, J., Cabalin, M.R., Miro, R. and Egozcue, J. (1988). Human sperm chromosomes. Hum. Reprod. 3: 133 - 138.

Thibault, C., Szöllősi, D. and Gérard, M. (1987). Mammalian oocyte maturation. Réprod. Nutr. Dévèlop. 27: 865 - 897.

Tuma, D.J., Jennet, R.B. and Sorrell, M.F. (1987). The interaction of acetaldehyde with tubulin. In, Ann. N. Y. Acad. Sci. Vol. 492. Ed. E. Rubin. pp. 277 - 286.

Van Blerkom, J. and Bell, M.N. (1984). Mitochondrial reorganization during resumption of arrested meiosis in the mouse oocyte. Am. J. Anat. 171: 335 - 355.

Van Blerkom, J. and Bell, H. (1986). Regulation of development in fully grown mouse oocytes: chromosome mediated temporal and spatial differentiation of the cytoplasm and the plasma membrane. J. Embryol. exp. Morph. 93: 213 - 238.

Van der Stricht, O. (1923). Étude comparée des ovules des mammifères aux différentes périodes de l'ovogenèse, d'après les travaux de Laboratoire d'Histologie et d'Embryologie de l'Universitē de Gand. Arch. de Biol. Paris. 33: 229 - 300.

Vickers, A. (1969). Delayed fertilisation and chromosomal anomalies in mouse embryos. J. Reprod Fert. 20: 69 - 76.

Vig, B.K. (1983). Sequence of centromere separation: Occurance, possible significance and control. Cancer Genet. and Cytogenet. 8: 249 - 274.

Warburton, D. and Fraser, F.C. (1964). Spontaneous abortion risks in man: Data from reproductive histories

collected in a medical genetics unit.
Am. J. Hum. Genet. 16: 1 - 25.

Wasserman, P.M. and Fujiwara, K. (1978). Immunofluorescent antitubulin staining of spindles during meiotic maturation of mouse oocytes in vitro.
J. Cell Sci. 29: 171 - 188.

Watt, J.L., Templeton, A.A., Messinis, I., Bell, L., Cunningham, P. and Duncan, R.O. (1987). Trisomy 1 in an eight-cell human pre-embryo. J. Med. Genet. 24: 60 - 64.

Webb, M., Howlett, S.K and Maro, B. (1986). Parthenogenesis and cytoskeletal organisation in ageing mouse oocytes. J. Embryol. exp. Morph. 95: 131 - 145.

Whittingham, D.G. (1971). Culture of mouse ova.
J.Reprod. Fert. suppl. 14: 7 - 21.

Wright, J.J., Waterson, E.J., Barrison, I.C., Toplis, P.J., Lewis, IG., Gordon, M.G., MacRae, K.D., Morris, N.F. and Murray-Lyon, I.M. Alcohol consumpyion, pregnancy and low birth weight. Lancet, 1: 663 - 665.

Yoshida, M.C. and Kodama, Y. (1983). C-band patterns of chromosomes in 17 strains of mice.
Cytogenet. Cell Genet. 35: 51 - 56.

Zamboni, L. (1970). Ultrastructure of mammalian oocytes and ova. Biol. Reprod. suppl. 2: 44 - 63.

APPENDIX:

Development 100, 441–448 (1987)
Printed in Great Britain © The Company of Biologists Limited 1987

Cytogenetic analysis of first cleavage fertilized mouse eggs following *in vivo* exposure to ethanol shortly before and at the time of conception

G. T. O'NEILL and M. H. KAUFMAN*

Department of Anatomy, University of Edinburgh, University Medical School, Teviot Place, Edinburgh EH8 9AG, UK

* For reprints

Summary

In this study, the chromosome constitution of mouse eggs exposed *in vivo* to a dilute solution of ethanol during specific stages of the first and second meiotic divisions was determined at the first cleavage mitosis. Exposure to ethanol prior to the completion of the second meiotic division induced an incidence (7–10 %) of aneuploidy involving only one chromosome in 98 % of malsegregation events. This investigation provides indirect evidence that ethanol may induce aneuploidy by disrupting the functioning of the meiotic spindle. Karyological analyses of chromosome spreads prepared at the first cleavage metaphase suggest that only a small proportion of the total chromosome complement may be induced to undergo malsegregation.

Key words: chromosome analysis, first cleavage, cytogenetic analysis, ethanol-induced aneuploidy, mouse egg.

Introduction

Recent studies (Kaufman, 1983*a*; Kaufman & Bain, 1984) have demonstrated that the intragastric administration of a dilute solution of ethanol can induce malsegregation of chromosomes during the first and second meiotic divisions of the mouse egg. Chromosome counts at metaphase of the first cleavage mitosis revealed that 14–22 % of the zygotes exhibited an abnormal chromosome number and that the malsegregation event was restricted to the female-derived set. The frequency of induced aneuploidy, in the female gamete, was found to be dependent on the dose of ethanol, but, more particularly, is affected by the timing of its administration. However, in one other limited study (Washington, Cain, Cacheiro & Generoso, 1985) concerning the effect of a dilute solution of ethanol upon the malsegregation of chromosomes at the second meiotic division, the observed incidence of heterodiploidy was not significantly different from that observed in controls. Although there is no direct evidence that ethanol is a spindle-active agent, it has been hypothesized that it probably disrupts normal chromosome segregation by interfering with the components of the meiotic spindle apparatus (Kaufman, 1985). Similar results have been achieved when spindle-active drugs such as colchicine (McGaughey & Chang, 1969; Sugawara & Mikamo, 1980; Hummler & Hansmann, 1985; Tease & Fisher, 1986) and vincristine sulphate (Danford & Parry, 1986) were administered to rodents in low doses. Anaesthetics, which share a variety of physiological effects with those of ethanol, are also capable of disrupting chromosome segregation during meiosis (Kaufman, 1977) and of interfering with the integrity of microtubules when cell lines in tissue culture are exposed to these agents (Hinkley & Samson, 1972; Brinkley & Rao, 1973; Hinkley & Telser, 1974). The spontaneous incidence of chromosomal anomalies in gametes and zygotes is thought to be very low in the mouse (Donahue, 1972; Röhrborn, 1972; Kaufman, 1973; Hansmann & El-Nahaas, 1979).

In the present investigation, first cleavage metaphase chromosome preparations were G-banded to provide a karyotypic analysis of chromosome malsegregation. The incidence of chromosome malsegregation during the first and second meiotic divisions, induced by the oral administration of ethanol, was observed to be about 10 % and 7 %, respectively. Although these values are lower than those observed by Kaufman (1983*a*) and Kaufman & Bain (1984), the present incidence of chromosome malsegregation is

still significantly higher than that observed in control studies. Karyological analysis has indicated that certain chromosomes may be involved preferentially in malsegregation events.

Materials and methods

8- to 12-week-old (C57BL×CBA) F_1 hybrid female mice, mean weight 21·8 g, were injected with 5 i.u. of pregnant mares' serum gonadotrophin (PMSG) followed 48 h later by 5 i.u. of human chorionic gonadotrophin (HCG) to induce superovulation. Shortly after the HCG injection, the females were caged with (C57BL×CBA) F_1 hybrid males. Checks for the presence of vaginal plugs were made early the next morning and their presence was taken as evidence of mating.

The female mice that had successfully mated were lightly anaesthetized with ether at various times in relation to the HCG injection and either 1 ml of 12 % (by volume) analar-quality ethyl alcohol or 1 ml of distilled water (control) was injected into the stomach *via* a fine plastic tube. The mice recovered from the anaesthetic effects of the ether almost immediately after the plastic tube was removed from the stomach. Following the ethanol administration, the mice were drowsy for $1\frac{1}{2}$–$2\frac{1}{2}$ h and were left to recover in a warm environment. No deaths resulted from the ethanol administration. An intragastric injection of a dilute solution of ethanol or of distilled water was administered at one of the following times: 2, 4, $13\frac{1}{2}$, 16, 18 or 20 h after the HCG injection. The intragastric dose of ethanol gave a blood alcohol level of about 200–230 mg 100 ml^{-1} after 30 min. This blood alcohol level remained constant for about 2 h and then fell rapidly, so that, after approximately 6 h no blood alcohol could be detected. Blood samples were analysed using a Lion Alcolmeter Model AE-D1 (Lion Laboratories Ltd). Females with vaginal plugs were autopsied at 21 h after the HCG injection, and their eggs were isolated and examined for the presence of pronuclei. Fertilized eggs were then transferred to M16 culture medium (see Kaufman, 1983*b*).

After about 27 h following the injection of HCG, eggs were examined at 1 h intervals to establish whether the pronuclear membranes had broken down. Those eggs in which this had occurred were harvested and chromosome spreads prepared by the air-drying method described by Tarkowski (1966). The slides were left for at least one week before any attempt was made to stain them. The chromosome spreads were then G-banded using a modification of the trypsin ASG method (Gallimore & Richardson, 1973).

The female and male chromosomes were usually readily identified as they exhibited different degrees of condensation and intensity of staining (Donahue, 1972). Karyograms were prepared according to the Standardised Genetic Nomenclature for Mice (Nesbitt & Francke, 1973). Two illustrations of normal first cleavage G-banded spreads are presented in Figs 1 and 2.

Results

The results of the first cleavage analysis of fertilized eggs exposed *in vivo* to 1 ml of a 12 % solution of ethanol are presented in Table 1. The results of the control experiments when eggs were exposed *in vivo* to 1 ml of distilled water are presented in Table 2.

The ratio of hypodiploidy to hyperdiploidy (for terminology see Beatty, 1957) was 1·0:0·6. The higher incidence of hypodiploidy suggests that some

Table 1. *Chromosome constitution of eggs from (C57BL×CBA) F_1 hybrid mice analysed at first cleavage mitosis following ethanol administration at various times in relation to HCG injection for inducing superovulation: all females received 1 ml of 12 % solution of ethanol*

Group	Time of ethanol administration after HCG injection (h)	Total number of embryos examined	Number of preparations not analysable	Chromosome number 39	40	41	42	Triploid	Percentage of aneuploidy (excluding triploidy)	Adjusted percentage of aneuploidy (excluding triploidy)*
1	+2	73	12	5	52	3	0	1	13·3	10·0
2	+4	246	50	11	172	9	1	3	10·8	10·3
3	+13·5	249	46	11	182	7	0	3	9·0	7·0
4	+16	69	16	2	50	1	0	0	5·6	3·7
5	+18	66	16	2	48	0	0	0	4·0	0·0
6	+20	75	17	3	53	1	0	1	7·0	3·5
Total		778	157	34	555	21	1	8		

* The adjusted percentage of aneuploidy is determined from $\frac{\text{Number of hyperdiploid chromosome spreads}}{\text{Total number of chromosome spreads (excluding triploids)}} \times 2$

Groups (1–3): mean = 9·1 ± 1·5 (S.D.$_n$)
Groups (4–6): mean = 2·4 ± 1·7 (S.D.$_n$)
Table 1: Group (1): values are not significantly different from Table (1): Group (2): $\chi^2 = 1{\cdot}7$ $P = 0{\cdot}20$–$0{\cdot}10$
Table 1: Group (1): values are not significantly different from Table (1): Group (3): $\chi^2 = 0{\cdot}9$ $P = 0{\cdot}80$–$0{\cdot}70$
Table 1: Group (2): values are not significantly different from Table (1): Group (3): $\chi^2 = 0{\cdot}3$ $P = 0{\cdot}70$–$0{\cdot}50$
Table 1: Group (1–3): values are significantly different from Table 1: Group (4–6): $\chi^2 = 4{\cdot}85$ $P = 0{\cdot}05$–$0{\cdot}02$

Table 2. *Chromosome constitution of eggs from (C57BL×CBA) F_1 hybrid mice analysed at first cleavage mitosis following the administration of 1 ml distilled water (control) at various times after the HCG injection for inducing superovulation*

Group	Time of administration of 1 ml of distilled water after HCG injection (h)	Total number of embryos examined	Number of preparations not analysable	Chromosome number 39	40	41	42	Triploid	Percentage of aneuploidy (excluding triploidy)	Adjusted percentage of aneuploidy (excluding triploidy)*
1	+2	53	7	1	43	0	0	1	4·4	4·4
2	+4	58	6	1	49	0	0	2	2·0	0·0
3	+13·5	58	12	0	45	1	0	0	1·6	3·2
4	+16	18	3	0	14	0	0	1	0·0	0·0
5	+18	26	7	0	19	0	0	0	0·0	0·0
6	+20	33	3	1	29	0	0	0	3·3	0·0
Total		246	38	3	199	1	0	4		

* The adjusted percentage of aneuploidy is determined from $\frac{\text{Number of hyperdiploid chromosome spreads}}{\text{Total number of chromosome spreads}} \times 2$

Group (1–3): mean = 2·5 ± 1·8 ($S.D._n$)
Group (4–6): mean = 0·0
Table 1 (Group 1–3) are significantly different from Table 2 (Group 1–3) $\chi^2 = 7{\cdot}7$ $P < 0{\cdot}01$
Table 1 (Group 4–6) are not significantly different from Table 2 (Group 4–6) $\chi^2 = 1{\cdot}05$ $P = 0{\cdot}50–0{\cdot}30$

hypodiploid chromosome preparations are the product of mechanical chromosome loss incurred during the spreading procedure. The analysis of hyperdiploid chromosome preparations is a more reliable indication of chromosome malsegregation events. In this study the overall frequency of aneuploidy is derived from twice the incidence of hyperdiploidy i.e.

$$\frac{\text{No. of hyperdiploid chromosome spreads}}{\text{Total no. of chromosome spreads (excluding triploids)}} \times 2$$

This adjusted value implies that the chromosomes involved in malsegregation are probably not preferentially included or excluded from the egg and reduces the possibility of including invalid hypodiploid chromosome counts in the final analysis. Chromosome preparations that could not be counted due to, for example, overlapping of chromosomes or those with less than 39 chromosomes were recorded as 'not analysable'.

The total incidence of aneuploidy (excluding the triploids) under control conditions was 1·3 % and this level of background aneuploidy is comparable to previously recorded values for spontaneous aneuploidy in the mouse (Donahue, 1972; Kaufman, 1973; Hansmann & El Nahaas, 1979).

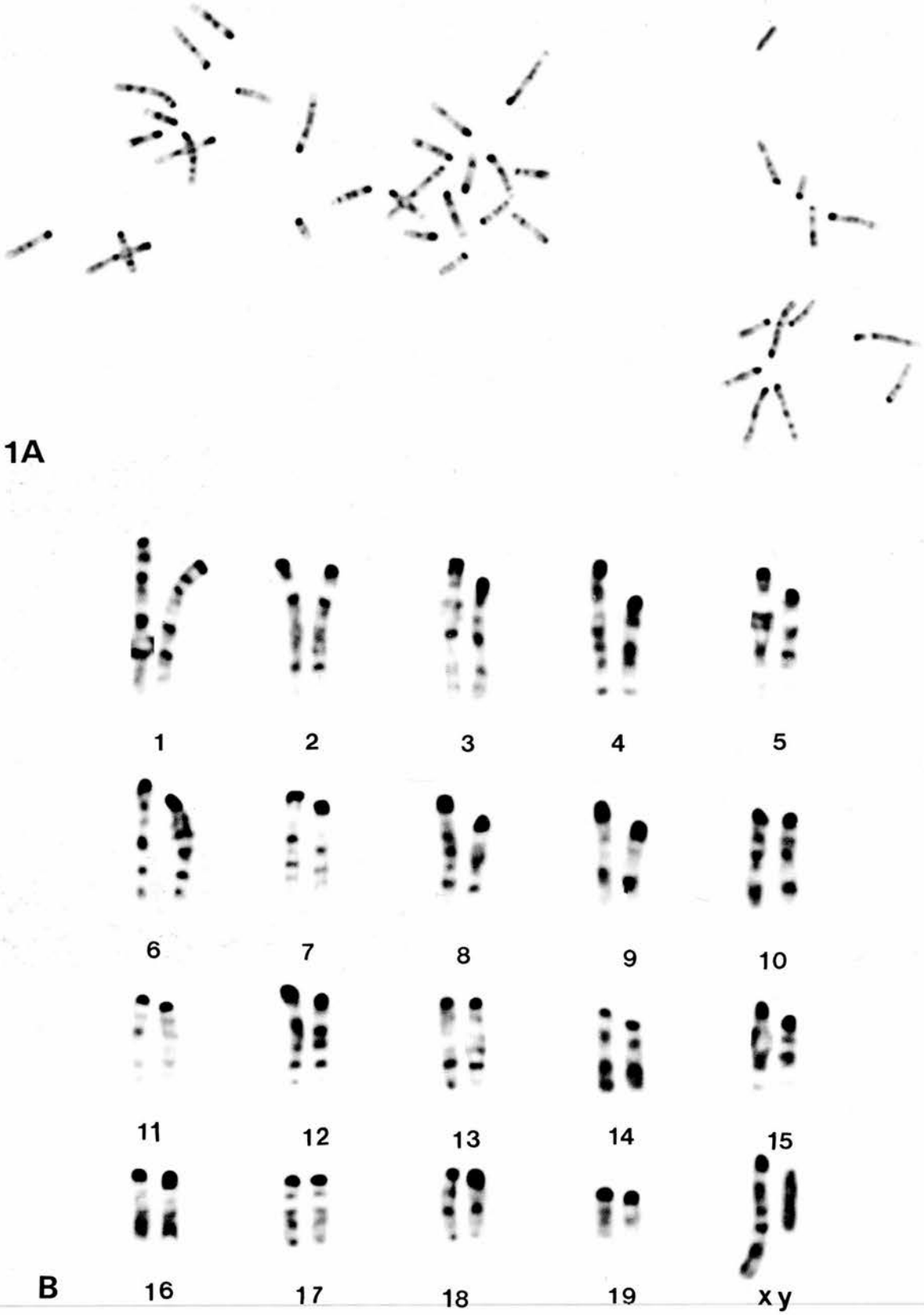
In contrast, when oocytes are exposed to a dilute solution of ethanol *in vivo* a higher level of aneuploidy is observed. When exposure occurred prior to the completion of the second meiotic division (groups 1–3, Table 1) the incidence of aneuploidy varied from 7·0–10·3 % ($\bar{X} = 9{\cdot}1 \pm S.D._n$ 1·5) in the different experimental groups. These results do not differ significantly from each other (see Table 1) and are significantly higher than comparable control values ($P < 0{\cdot}01$). These results demonstrate that *in vivo* exposure to ethanol may induce a significant increase in the level of aneuploidy when administered prior to the completion of the second meiotic division.

When exposure to a dilute solution of ethanol occurred after the expected completion of the second meiotic division (groups 4–6, Table 1), the observed frequency of aneuploidy decreased to 0·0–3·7 % ($\bar{X} = 2{\cdot}4 \pm S.D._n$ 1·7). These values do not differ significantly from comparable control levels of spontaneous aneuploidy ($P = 0{\cdot}50–0{\cdot}30$).

These results imply that ethanol induces a significant increase in the incidence of aneuploidy when maturing oocytes are exposed to this agent prior to the second meiotic division. After the completion of the second meiotic division and the subsequent development of the female pronucleus, the incidence of aneuploidy is not significantly different from that observed in control experiments.

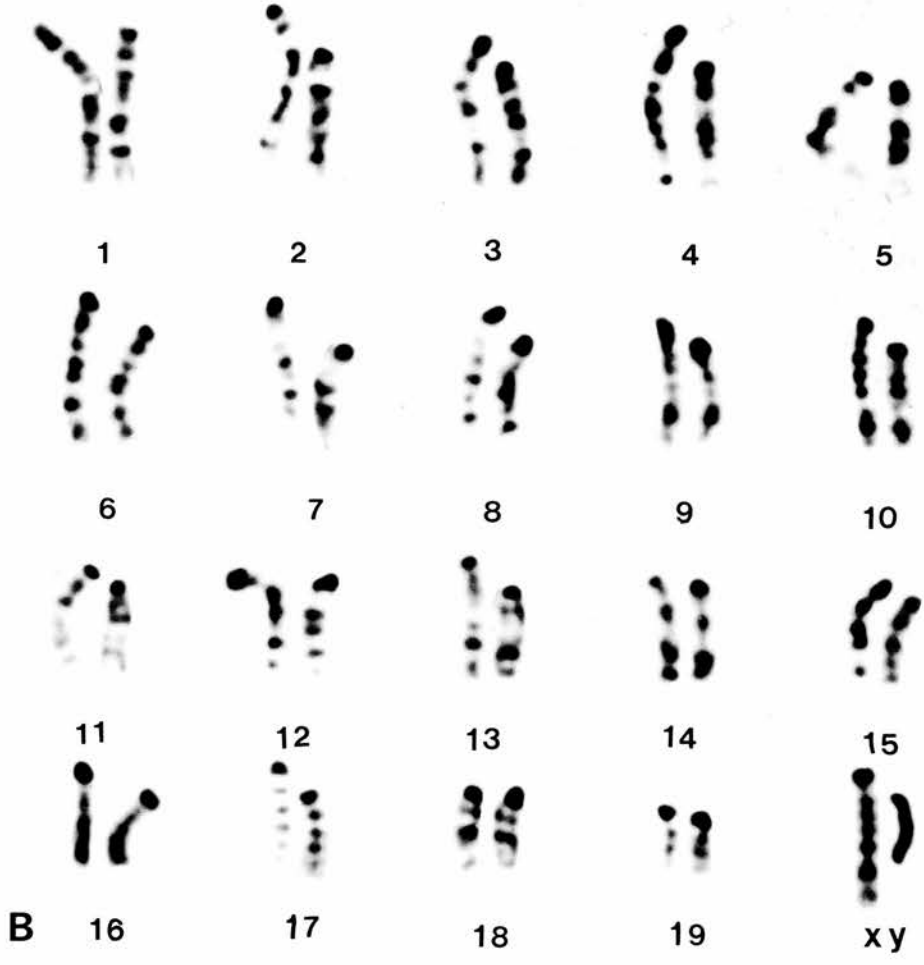
Furthermore, the incidence of aneuploidy induced by the *in vivo* exposure of oocytes to ethanol prior to the completion of the second meiotic division (groups 1–3, Table 1) is significantly greater than the incidence of aneuploidy observed after the expected completion of the second meiotic division ($P =$ 0·05–0·02).

The incidence of triploidy did not appear to be influenced by the *in vivo* exposure of oocytes to ethanol. Values of 1·9 % and 1·3 % were recorded from the control and experimental series respectively. There was, in addition, no apparent relationship between ethanol exposure and the type of triploidy observed. Of the ten triploid preparations, two were due to dispermy, six to digyny, while the origins of the remaining two could not be determined.



Figs 1A, 2A. Air-dried chromosome spreads from two normal fertilized mouse eggs at the first cleavage metaphase, each displaying 40 chromosomes. Giemsa-banded preparation.
Figs 1B, 2B. Karyograms of the Giemsa-banded chromosome spreads illustrated in Figs 1A, 2A.

2A



B

Karyogram analysis of chromosome preparations exhibiting a non-euploid number was unable to provide a definitive answer to the question as to whether specific chromosomes were more frequently involved in ethanol-induced malsegregation than others (see Discussion). In each of the experimental and control groups, only 15–20 % of the metaphase preparations exhibited high quality G-banded spreads and, unfortunately, there were no aneuploid preparations that exhibited high-quality band patterns. Furthermore, all of the banded chromosome spreads that exhibited the euploid number were all normal. There were, in addition, no examples of non-disjunction involving two or more chromosomes (where chromosome pair A migrates to one pole and chromosome pair B migrates to the other) in which the total number of chromosomes present in the spread would have been normal but the zygote would have been genetically unbalanced.

A further 20–25 % of metaphase preparations exhibited G-band patterns of variable quality. Karyogram analysis of three hyperdiploid metaphase spreads indicated that chromosomes 15, 16 and 18, respectively, were involved in malsegregation. In the remaining seven metaphase spreads that were observed to have an aneuploid chromosome number and which exhibited low quality G-band patterns, it was unfortunately not possible to identify the specific chromosomes involved in the malsegregation event. However, it was determined that chromosome 19 was not involved in malsegregation and the larger chromosomes of the genome, namely chromosomes 1, 2 and 3, could be readily identified in each spread.

Discussion

The present investigation confirms earlier reports that a dilute solution of ethanol, administered orally to the female mouse, may induce chromosomal malsegregation at both the first and second meiotic divisions of the egg (Kaufman, 1985). About 10 % of the oocytes exposed to ethanol in the present study showed evidence of chromosomal malsegregation when exposure occurred prior to the extrusion of the first polar body. Exposure to the same level of ethanol during the second meiotic division produced a similar result, with 7 % of the resultant zygotes exhibiting aneuploidy. These similar levels of malsegregation imply that both the first and second meiotic divisions are equally susceptible to the effects of ethanol.

The incidence of chromosomal malsegregation fell significantly when exposure to ethanol occurred after the extrusion of the second polar body and the completion of the second meiotic division. This observation would seem to indicate that ethanol probably interferes directly with components of the meiotic spindle apparatus that are involved in normal chromosome segregation.

The *in vivo* exposure of gametes to ethanol did not appear to influence the incidence of triploidy, as the control and experimental incidence was found to be 1·9 % and 1·3 % respectively, similar to previous findings reported by others (Vickers, 1969; Donahue, 1972; Kaufman & Bain, 1984). The incidence of digyny was more frequent than that of dispermy in the present series, though there appeared to be no obvious relationship between exposure to ethanol and the type of triploidy observed. In the mouse, digynic triploids that result from failure of extrusion of the second polar body are more frequently observed than dispermic triploids (Vickers, 1969).

Although the mechanism of ethanol-induced chromosomal malsegregation has yet to be fully determined, it should be appreciated that several possible mechanisms may give rise to aneuploid zygotes (see Bond & Chandley, 1983). Our results have demonstrated that exposure of female gametes to ethanol may induce the development of both hypodiploid and hyperdiploid eggs. However, the observed incidence of hypodiploidy was slightly greater than the incidence of hyperdiploidy. The validity of including hypodiploid findings to establish an estimate of the incidence of aneuploidy has been questioned (see, for example, Washington *et al.* 1985) as it is likely that a proportion of these aneuploid spreads may result from the mechanical loss of one or more chromosomes during the spreading procedure. For this reason, an 'adjusted' (and consequently lower) value for the frequency of aneuploidy has been derived from the observed incidence of hyperdiploid spreads alone. As only one zygote was spread on each slide, the presence of one or two extra chromosomes unquestionably represents a more accurate indication of the incidence of aneuploidy than might be obtained from the total incidence of hypo- and hyperdiploid spreads encountered.

Cytogenetic analysis of the chromosome constitution of zygotes at metaphase of the first cleavage division provides an accurate estimate of the incidence of both spontaneous and ethanol-induced chromosomal anomalies. On the other hand, analysis of the chromosome constitution of embryos at later developmental stages provides a less accurate means of assessing the initial incidence of aneuploidy due to the progressive loss of the monosomic and trisomic populations of embryos (see Magnuson, 1983; Epstein, 1985).

Clearly, this investigation has demonstrated that the first cleavage metaphase is probably not the most suitable developmental stage to pursue a karyotypic analysis of malsegregation events, since only 15–20 %

of chromosome preparations could be accurately karyotyped. The problem is largely a technical one, since the presence of residual cytoplasm adjacent to the chromosome spread tended to inhibit the development of uniform chromosome band patterns. As each embryo was only represented by a single chromosome spread, only about 15–20% of the embryos examined in this study were consequently available for karyotypic analysis. As indicated earlier, all the karyograms prepared from chromosome spreads that exhibited high quality chromosome band patterns were found to be completely normal. A further 20–25% of the chromosome spreads examined exhibited band patterns of variable quality and it was within this series that both hyperdiploid and hypodiploid mitoses were encountered.

Our results suggest that the smaller heterochromatic chromosomes of the mouse genome, except chromosome 19, are possibly more frequently involved in malsegregation than the other chromosomes of the complement, whereas the larger chromosomes of the genome appear to be less prone to malsegregation.

In a similar karyological analysis of the spontaneous incidence of chromosome malsegregation observed at the first cleavage metaphase, Martin-De Leon & Boice (1983) concluded that chromosome 19 was most frequently involved in malsegregation events. However, this is in marked contrast to our findings in the present study, as even in the chromosome spreads that exhibited low quality G-bands, chromosome 19 could be readily recognized and was never observed to be involved in malsegregation. In addition, the validity of their karyological analysis must be open to grave doubt, as all but three of the chromosome pairs in their published representative karyograms (Martin-De Leon & Boice, 1983) have been incorrectly analysed (personal communication E. P. Evans). Furthermore, the likelihood that they accurately karyotyped 15 of 17 chromosome spreads that have either lost or gained a single chromosome is doubted.

A recent limited cytogenetic study of *in vitro* fertilized human embryos that were analysed during the first and second cleavage divisions (Angell, Templeton & Aitken, 1986) has revealed that the majority of numerical chromosomal anomalies encountered involved the chromosomes of groups D and G. Other findings from analyses of spontaneous human abortuses (Chandley, 1982; Lin, De Braekeleer & Jamro, 1985) also indicated that the smaller heterochromatic chromosomes, especially numbers 16 and 21, were most frequently involved in autosomal non-disjunction.

Our results suggest that, as in man, the small acrocentric chromosomes of the mouse may also be particularly prone to undergo malsegregation during meiosis. It is possible that ethanol consumption during meiosis, and at about the time of conception in man, may also tend to increase the 'spontaneous' or 'baseline' level of malsegregation of the small acrocentric chromosomes. Detailed cytogenetic studies of early-cleavage-stage human conceptuses following their exposure either *in vivo* or *in vitro* to ethanol or other spindle-active agents will obviously be required to confirm or refute this hypothesis.

G. T. O'Neill is supported by a Bonnar Scholarship from the Faculty of Medicine, University of Edinburgh. The work is supported by a grant (to MHK) from the National Fund for Research into Crippling Diseases (Action Research for the Crippled Child), and an equipment grant (to M.H.K.) from the Scottish Home and Health Department ref. no. K/MRS/50/C866. We would like to thank Dr E. P. Evans for his advice and encouragement during the preparation of this manuscript and for assistance with the interpretation of the G-banded chromosome preparations.

References

Angell, R. R., Templeton, A. A. & Aitken, R. J. (1986). Chromosome studies in human *in vitro* fertilization. *Hum. Genet.* **72**, 333–339.

Beatty, R. A. (1957). *Parthenogenesis and Polyploidy in Mammalian Development.* London: Cambridge University Press.

Bond, D. J. & Chandley, A. C. (1983). *Aneuploidy.* Oxford University Press.

Brinkley, B. R. & Rao, P. N. (1973). Nitrous oxide: effects on the mitotic apparatus and chromosome movement in Hela cells. *J. Cell Biol.* **58**, 96–106.

Chandley, A. C. (1982). The origin of aneuploidy. In *Human Genetics, Part B, Medical Aspects*, pp. 337–347. New York: Alan R. Liss.

Danford, N. & Parry, J. M. (1986). Induction of sex chromosome aneuploidy in mice by vincristine sulphate. *I.R.C.S. Med. Sci.* **14**, 15–16.

Donahue, R. P. (1972). Cytogenetic analysis of the first cleavage division in mouse embryos. *Proc. natn. Acad. Sci. U.S.A.* **69**, 74–77.

Epstein, C. J. (1985). The mouse trisomies. Experimental systems for the study of aneuploidy. In *Issues and Reviews in Teratology*, vol. 3 (ed. H. Kalter), pp. 171–218. New York: Plenum Press.

Gallimore, P. H. & Richardson, C. R. (1973). An improved banding technique exemplified in the karyotype analysis of two strains of rat. *Chromosoma* **41**, 259–263.

Hansmann, I. & El-Nahass, E. (1979). Incidence of nondisjunction in mouse oocytes. *Cytogenet. Cell Genet.* **24**, 115–121.

Hinkley, R. E. & Samson, F. E. (1972). Anaesthetic induced transformation of axonal microtubules. *J. Cell Biol.* **53**, 258–263.

Hinkley, R. E. & Telser, A. G. (1974). The effects of halothane on cultured mouse neuroblastoma cells.

Inhibition of morphological differentiation. *J. Cell Biol.* **63**, 531–540.

HUMMLER, E. & HANSMANN, I. (1985). Preferential nondisjunction of specific bivalents in oocytes from Djungarian hamsters (Phodopus sungorus) following colchicine treatment. *Cytogenet. Cell Genet.* **39**, 161–167.

KAUFMAN, M. H. (1973). Analysis of the first cleavage division to determine the sex-ratio and incidence of chromosome anomalies at conception in the mouse. *J. Reprod. Fert.* **35**, 67–72.

KAUFMAN, M. H. (1977). Effect of anaesthetic agents on eggs and embryos. In *Development in Mammals*, vol. 1 (ed. M. H. Johnson), pp. 137–163. Amsterdam: North-Holland.

KAUFMAN, M. H. (1983*a*). Ethanol-induced chromosomal abnormalities at conception. *Nature, Lond.* **302**, 258–260.

KAUFMAN, M. H. (1983*b*). *Early Mammalian Development: Parthenogenetic Studies.* Cambridge: Cambridge University Press.

KAUFMAN, M. H. (1985). An hypothesis regarding the origin of aneuploidy in man: indirect evidence from an experimental model. *J. Med. Genet.* **22**, 171–178.

KAUFMAN, M. H. & BAIN, I. M. (1984). Influence of ethanol on chromosome segregation during the first and second meiotic divisions in the mouse egg. *J. exp. Zool.* **230**, 315–320.

LIN, C. C., DE BRAEKELEER, M. & JAMRO, H. (1985). Cytogenetic studies in spontaneous abortion: the Calgary experience. *Can. J. Genet. Cytol.* **27**, 565–570.

MAGNUSON, T. (1983). Genetic abnormalities and early mammalian development. In *Development in Mammals*, vol. 5 (ed. M. H. Johnson), pp. 209–249. Amsterdam: Elsevier.

MARTIN-DE LEON, P. A. & BOICE, M. L. (1983). Spontaneous heteroploidy in one-cell mouse eggs. *Cytogenet. Cell Genet.* **35**, 57–63.

MCGAUGHEY, R. W. & CHANG, M. C. (1969). Inhibition of fertilization and production of heteroploidy in eggs of mice treated with colchicine. *J. exp. Zool.* **171**, 465–480.

NESBITT, M. N. & FRANCKE, U. (1973). A system of nomenclature for band patterns of mouse chromosomes. *Chromosoma* **41**, 145–158.

RÖHRBORN, G. (1972). Frequencies of spontaneous non-disjunction in metaphase II oocytes of mice. *Hum. Genet.* **16**, 123–125.

SUGAWARA, S. & MIKAMO, K. (1980). An experimental approach to the analysis of mechanisms of meiotic nondisjunction and anaphase lagging in primary oocytes. *Cytogenet. Cell Genet.* **28**, 251–264.

TARKOWSKI, A. K. (1966). An air drying method for chromosome preparations from mouse eggs. *Cytogenetics* **5**, 394–400.

TEASE, C. & FISHER, G. (1986). Oocytes from young and old female mice respond differently to colchicine. *Mutation Res.* **173**, 31–34.

VICKERS, A. D. (1969). Delayed fertilization and chromosomal anomalies in mouse embryos. *J. Reprod. Fert.* **20**, 69–76.

WASHINGTON, W. J., CAIN, K. T., CACHEIRO, N. L. A. & GENEROSO, W. M. (1985). Ethanol-induced late fetal death in mice exposed around the time of fertilization. *Mutation Res.* **147**, 205–210.

(*Accepted 19 March 1987*)

Gamete Research 18:27–36 (1987)

Ovulation and Fertilization of Primary and Secondary Oocytes in LT/Sv Strain Mice

G.T. O'Neill and M.H. Kaufman

Department of Anatomy, University Medical School, Edinburgh, Scotland

In this study, the chromosome constitution of both unfertilized oocytes and fertilized eggs isolated from the oviducts of LT/Sv strain mice were analyzed. Air-dried chromosome preparations from unfertilized oocytes revealed that about one-third of those examined were ovulated as primary oocytes. These were arrested at metaphase of the first meiotic division and exhibited the characteristic "tetrad" chromosome configuration. The remaining two-thirds of the unfertilized oocytes were ovulated at metaphase of the second meiotic division. The fertilized eggs were isolated from the oviducts of LT/Sv females previously mated to (C57BL × CBA) F1 hybrid males. Analysis of the fertilized eggs at metaphase of their first cleavage mitosis revealed that about one-third of the eggs examined were digynic triploids, whereas the remaining two-thirds had the normal diploid chromosome constitution. In the triploids, the 40 female chromosomes present (mouse, n = 20) were derived from a single diploid pronucleus formed after the extrusion of a first polar body, and following the monospermic fertilization of primary oocytes. The female pronuclear-derived chromosomes invariably exhibited "homologous pairing," and these were associated at their centromeres. The ovulation, penetration, and subsequent fertilization of primary oocytes is an extremely unusual phenomenon in mammals and only appears to occur on a regular basis in LT/Sv mice. The premature "cytoplasmic maturation" of these oocytes is of interest, as they clearly have the same developmental capacity as secondary oocytes. The significance of these observations in relation to folliculogenesis and litter size in LT/Sv mice is discussed.

Key words: primary oocytes, secondary oocytes, triploidy

INTRODUCTION

Spontaneous parthenogenesis is an extremely rare phenomenon in mammals, occurring with any degree of regularity only in the LT/Sv strain of the mouse [see Stevens and Varnum, 1974]. Several investigators have indicated that the spontaneous activation of ovarian eggs in mature females is causally related to the high incidence of ovarian teratomas that have been observed in this strain. A proportion of the ovulated oocytes may also become activated spontaneously, and similar events may also be observed when their eggs are isolated from the oviduct and transferred to tissue culture medium. Pre- and early postimplantation stages of parthenogenetic

Received January 15, 1987; accepted April 2, 1987.

Address reprint requests to Professor M.H. Kaufman, Department of Anatomy, University Medical School, Teviot Place, Edinburgh, EH8 9AG, Scotland.

development have also been recognized both within the ovary and within the reproductive tract [Stevens and Varnum, 1974; Stevens, 1975; Eppig, 1978; reviewed by Kaufman, 1983].

A preliminary study carried out in order to determine the developmental pathways taken shortly after activation by ovulated LT/Sv oocytes [Kaufman, 1983] clearly demonstrated that a high proportion of the spontaneously activated eggs extruded a polar body and developed a single pronucleus. This pathway is usually associated with the development of haploid parthenogenones [Kaufman, 1983]. However, chromosome spreads prepared from the activated eggs that had progressed to metaphase of the first cleavage mitosis indicated that both haploid and diploid parthenogenones had developed. The latter observation was particularly surprising, as the presence of a polar body is almost invariably associated with haploid development. Furthermore, and even more curiously, the diploid chromosome preparations observed appeared to exhibit evidence of "homologous chromosome pairing." In some of the chromosome preparations, clear evidence of terminal end-to-end associations were still visible at the centromere regions.

A subsequent cytogenetic and nucleardensitometric study [Kaufman and Howlett, 1986] revealed that LT/Sv females regularly ovulated both primary and secondary oocytes. Ovulated eggs at both stages of meiotic maturation were found to be capable of spontaneous and induced parthenogenetic activation, and the development of single pronuclear eggs. Previous studies, in which the chromosome constitution of naturally ovulated eggs was analyzed (authors, unpublished observations), have clearly indicated that the incidence of the ovulation of primary oocytes did not appear to be influenced by the administration of exogenous hormones. Of particular interest, however, was the associated relationship observed between nuclear volume and ploidy. Two populations of single-pronuclear activated eggs could be distinguished on account of the significant difference between their nuclear diameters. Those with a large pronuclear volume were invariably found to be diploid, whereas those eggs in which the pronuclear volume was significantly smaller were invariably found to be haploid. In the former group, the activation of primary oocytes resulted in the separation of bivalents and the formation of a single diploid pronucleus. The "homologous pairing" of chromosomes observed at metaphase of the first cleavage mitosis is believed to be related to the absence of the centromeric division of chromosomes prior to the formation of the diploid pronucleus. In consequence, the chromosomes that are retained within the egg each still have two morphologically distinct chromatids prior to DNA replication. Following the S-phase, the DNA in each chromatid is replicated, a nucleardensitometric reading of 4C is recorded within the pronucleus, and at the first cleavage mitosis each member of an "homologous pair" is clearly seen to be divided into two distinct chromatids.

It was further hypothesized that if primary oocytes were capable of being activated parthenogenetically, they might also be capable of undergoing monospermic fertilization with the development of digynic triploid conceptuses. In the present study, we have confirmed that this hypothesis is indeed correct and have demonstrated not only that the primary oocytes are capable of monospermic penetration, but occur at a similar frequency to that observed during normal fertilization of secondary oocytes. By this means, triploid embryos of a particularly unusual type were commonly encountered. We believe that the presence of these embryos almost certainly represents one of the factors that account for the low litter size observed in LT/Sv strain mice.

The penetration of primary oocytes by spermatozoa is extremely unusual in mammals, having previously been described only in the dog (see Discussion), and in this species sperm head decondensation probably does not occur until the maturing oocyte has progressed to metaphase of the second meiotic division. In this study, we present the first evidence that we are aware of that both sperm penetration and normal sperm head decondensation can occur prior to the completion of the first meiotic division. We believe that this observation represents yet another example of the unique behavior of the eggs from this strain of mice.

MATERIALS AND METHODS

Eight- to ten-week-old female LT/Sv mice (MRC Carshalton) were given an intraperitoneal (i.p.) injection of pregnant mares' serum gonadotrophin (PMSG) (5 i.u.) followed 46 hr later by an i.p. injection of human chorionic gonadotrophin (HCG) (5 i.u.) to induce superovulation. After the HCG injection, the females were caged with (C57BL×CBA)F1 hybrid males. Early the next morning, the female mice were checked for the presence of vaginal plugs, and these were taken as evidence of mating.

At 20–22 hr after the HCG injection, the female mice were sacrificed by cervical dislocation. Their oviducts were dissected out and placed into Dulbecco's supplemented phosphate-buffered saline (PBS) maintained at 37°C. The eggs were released from the oviduct and transferred to drops of M16 tissue culture medium containing 4 mg/ml bovine serum albumin [Whittingham, 1971] equilibrated with 5% CO_2 in air at 37°C.

The eggs were then examined for the presence of pronuclei. Fertilized eggs were recognized by the presence of at least two pronuclei, whereas spontaneously activated eggs usually exhibited a single pronucleus. Eggs that were neither fertilized nor activated did not exhibit pronuclei. The latter eggs were isolated at this stage from culture, and metaphase chromosome spreads were prepared by the air-drying technique of Tarkowski [1966].

At 27–32 hr after the HCG injection, the fertilized eggs were observed to enter metaphase of the first cleavage mitosis. This was signaled by the breakdown of the pronuclear membranes. These eggs were harvested, and metaphase chromosome spreads were prepared as described above.

The female mice that did not mate with F1 males were also sacrificed by cervical dislocation at approximately 16 hr after the HCG injection. Their oviducts were removed, and the cumulus masses containing the ovulated oocytes were released into supplemented PBS at 37°C. The oocytes were then transferred to tissue culture medium containing 0.5 mg/ml of hyaluronidase to remove the adhering cumulus cells. The absence of pronuclei and sperm confirmed that neither fertilization nor spontaneous activation had occurred in this group. Air-dried chromosome preparations were subsequently prepared from these eggs. All chromosome preparations were stained with Giemsa R66 (G. T. Gurr). In the fertilized eggs, it was generally possible to distinguish between the male and female chromosome sets by their differential degrees of condensation [Donahue, 1972].

RESULTS

The chromosome analysis of unfertilized eggs clearly demonstrated that both primary and secondary oocytes, arrested at metaphase of the first meiotic and second

meiotic divisions, respectively, were ovulated by LT/Sv females (see Table 1). Chromosome preparations of primary oocytes exhibited the characteristic "tetrad" chromosome configuration, whereas secondary oocytes exhibited the "dyad" chromosome configuration usually observed in recently ovulated eggs. An analysis of the findings presented in Table 1 reveals that of the total number of ovulated oocytes recovered from the oviducts of the LT/Sv females, 33% had been ovulated as primary oocytes.

The results of the chromosome analysis of the oocytes isolated from LT/Sv females that had mated to (C57BL×CBA)F1 hybrid males are shown in Table 2. The results demonstrate that 79% of the oviductal oocytes had in fact been fertilized, and that 34% of these were triploid.

A representative example of a typical triploid air-dried chromosome preparation is shown in Figure 1. Note that under normal circumstances, when a secondary oocyte is fertilized, two distinct haploid chromosome sets may be observed during the early stages of the first cleavage mitosis, one being of paternal and the other of maternal origin. Despite the different degrees of condensation of the chromosomes, their gross morphology is identical. In those instances in the present study where primary oocytes were successfully fertilized, however, one distinct diploid and one distinct haploid chromosome spread were invariably present, the former group being of maternal and the latter group of paternal origin. In addition to the different degrees of condensation of the two sets of chromosomes, the morphological appearance of the individual chromosomes in the two sets was quite dissimilar. Whereas the haploid paternally derived chromosomes were morphologically normal and identical to those seen in normal fertilized eggs, the maternally derived diploid set of chromosomes were very unusual in appearance. These chromosomes were either present in pairs, united at their centromere region(s) (see Fig. 1), or slightly separated but still recognizable as belonging to a particular "pair." Indeed, their morphological appearance was identical

TABLE 1. Chromosome Constitution of Ovulated Oocytes Isolated From Superovulated Virgin LT/Sv Females as Determined From Air-Dried Chromosome Preparations

Total number of ovulated oocytes examined	Number of oocytes ovulated at metaphase of meiosis I	Number of oocytes ovulated at metaphase of meiosis II	Number of spontaneously activated oocytes	Number of nonanalyzable preparations
24	8	16	0	0

TABLE 2. Chromosome Constitution of Ovulated Oocytes Isolated From Superovulated LT/Sv Females Previously Mated to F1 Hybrid Males as Determined From Air-Dried First Cleavage Metaphase Preparations

Total number of ovulated oocytes examined	Number of unfertilized oocytes at metaphase of		Number of fertilized oocytes exhibiting a diploid (20♀/20♂) chromosome constitution	Number of fertilized oocytes exhibiting a triploid (40♀/20♂) chromosome constitution[a]	Number of spontaneously activated oocytes	Number of nonanalyzable preparations
	Meiosis I	Meiosis II				
52	0	2	27	14	6	3

[a]For typical morphological appearance, see chromosome preparation illustrated in Figure 1.

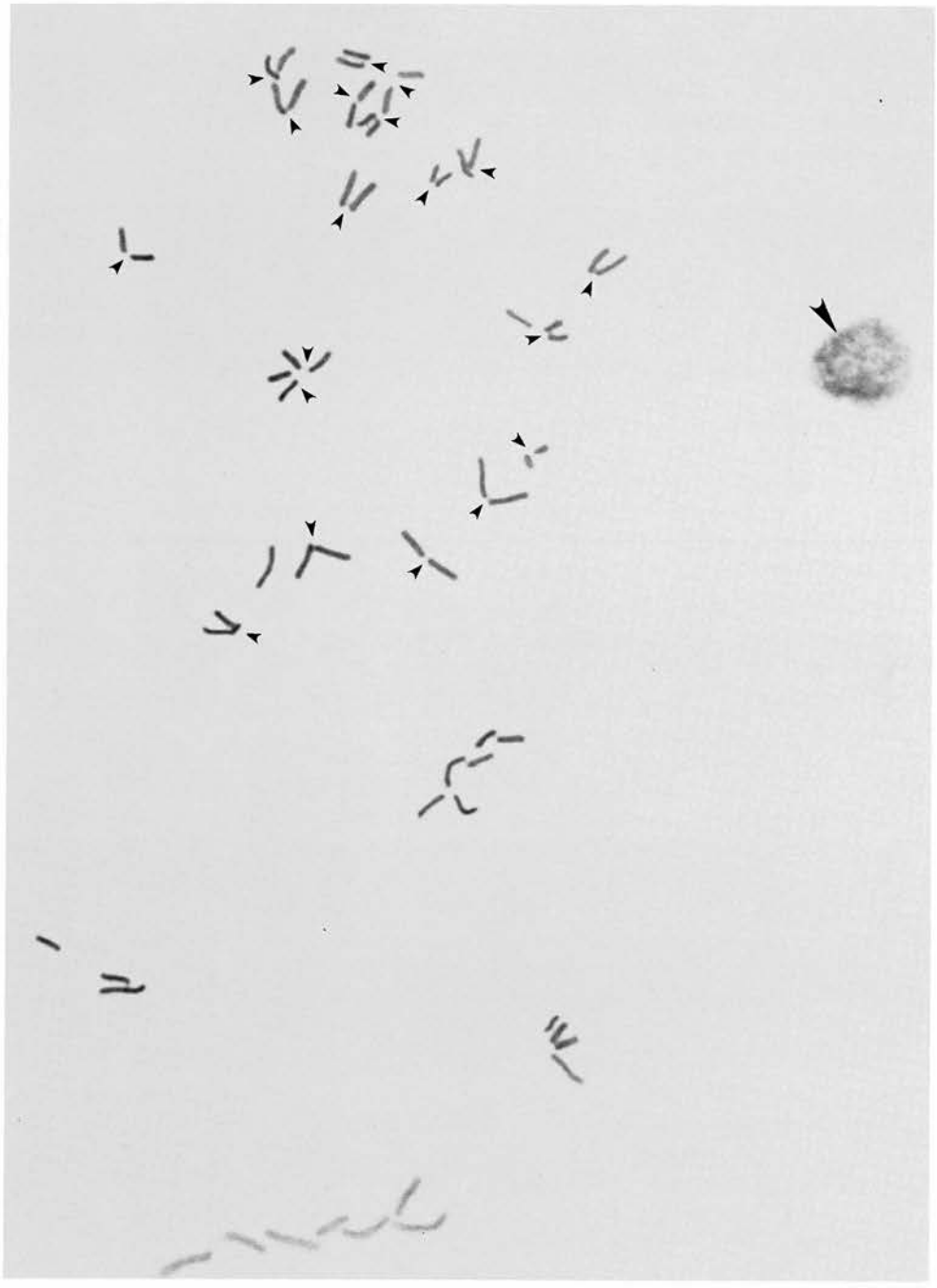


Fig. 1. Giemsa-stained air-dried preparation of the first cleavage mitosis in a typical digynic triploid egg from an LT/Sv strain mouse. The male pronuclear-derived haploid set contains 20 individual chromosomes, whereas the female pronuclear-derived set contains 40 chromosomes associated in "homologous pairs." The small arrowheads point to the centromeric region of 19 of the homologous pairs. The twentieth pair has separated, clearly an artifact of the spreading technique, and the two individual components may be seen toward the bottom and to the right of the group. An additional artifact of fixation is the less condensed appearance of the 8 male pronuclear-derived chromosomes on the bottom of the figure. This might have been due to their more peripheral location effected by the spreading procedure. Note in particular that the polar body chromatin is present in the form of a clearly recognizable nuclear mass (large arrowhead). This has a similar appearance to that of a normal second polar body nucleus, which is most unusual, as the chromatin of the first polar body almost invariably disperses without progressing to form a nucleus.

to those previously observed when primary oocytes from LT/Sv mice had been activated parthenogenetically, and their chromosomes analyzed at the first cleavage mitosis, where evidence of "homologous chromosome pairing" had been observed [see Kaufman, 1983; Kaufman and Howlett, 1986].

Another unusual feature observed in air-dried preparations of digynic triploid embryos was the morphological appearance of the polar body chromatin. The latter was invariably found to be present in the form of a single discrete nuclear mass (Fig. 1), very closely resembling the nuclear morphology characteristic of the second polar body in normal fertilized and parthenogenetically activated embryos [Kaufman, 1983]. Under normal circumstances, the chromosomes of the first polar body tend to remain as a compact group and invariably degenerate rather than progress to form a recognizable nuclear mass [Rodman, 1971; Abramczuk and Sawicki, 1975; Rodman and Barth, 1979]. In the present situation, the appearance of the chromatin mass has all the features of that seen when air-dried preparations are made of the second polar body.

DISCUSSION

Analysis of the chromosome constitution of unfertilized oviductal eggs has confirmed the previous observation that LT/Sv female mice regularly ovulate both primary and secondary oocytes [Kaufman, 1983; Kaufman and Howlett, 1986]. We have observed that about one-third of the oviductal oocytes exhibit the "tetrad" chromosome configuration and are ovulated as primary oocytes. Normally, such configurations are only observed when chromosome spreads are prepared from ovarian oocytes that have matured to diakinesis/metaphase of the first meiotic division. In the present study, only two-thirds of the oviductal eggs exhibited the normal "dyad" chromosome configurations characteristic of spreads prepared from unfertilized oviductal oocytes that have failed to progress beyond metaphase of the second meiotic division.

Furthermore, examination of the chromosome constitution of fertilized eggs analyzed at metaphase of the first cleavage mitosis revealed that eggs ovulated as primary or secondary oocytes were both capable of monospermic penetration and pronuclear development. Detailed chromosome counts revealed that about 34% of the fertilized eggs were triploid and resulted from the fertilization of primary oocytes. The remainder of the fertilized eggs examined had a normal diploid constitution and clearly resulted from the fertilization of normal secondary oocytes.

The fertilized triploid eggs isolated from the oviducts of LT/Sv mice are of an unusual type and developmentally different from the other classes of digynic triploid embryos that have previously been observed in analyses of the first cleavage preparations isolated from other strains of mice [Donahue, 1972; Kaufman, 1973; Martin-DeLeon and Boice, 1983]. The triploid eggs isolated in the present study originated from the fertilization of primary oocytes, and consequently exhibited only two pronuclei. The male-derived pronucleus was haploid, whereas the female pronucleus, since it was formed after the extrusion of a *first* rather than a *second* polar body, was invariably diploid. The first polar body is extruded at the completion of the first meiotic division, whether this occurs—as normally—prior to ovulation, or—as in the present case—some hours after the activation process induced by fertilization. The second polar body is extruded at the completion of the second meiotic division.

Digynic triploid eggs isolated from other strains of mice generally exhibit three haploid pronuclei and develop following the monospermic fertilization of a secondary oocyte in which second polar body extrusion has failed to occur. In the latter group, the egg generally contains one male-derived pronucleus and two female-derived haploid pronuclei. Often, failure of extrusion of the second polar body and/or polyspermy may be related to the postovulatory aging of the egg within the oviduct prior to fertilization [Marston and Chang, 1964; Vickers, 1969; Szöllösi, 1971; Kaufman, 1983]. Triploidy in the LT/Sv strain, however, results from the fertilization of primary oocytes and is apparently unrelated to the various changes that occur due to postovulatory aging of the oocyte. The origin and developmental pathways of the various classes of triploid embryos described above are represented diagrammatically in Figure 2.

A third feature that differentiates LT/Sv triploid embryos from the other classes of triploids induced following the fertilization of postovulatory aged secondary oocytes is the presence of "homologous chromosome pairing," which may be observed at the first cleavage metaphase. This phenomenon, as far as we are aware, has not previously been observed in other digynic triploids, as the maternally derived first cleavage chromosomes normally observed originate from two distinct pronuclei formed after the centromeric division of dyads in secondary oocytes. By contrast, the diploid female-derived pronucleus in the LT/Sv triploids is formed after the separation of bivalents, following the extrusion of the first rather than the second polar body. The chromosomes do not separate at their centromeres, and they seem to maintain the capacity to retain the morphological features characteristic of "homologous chromosome pairing" when they enter metaphase of the first cleavage division. This

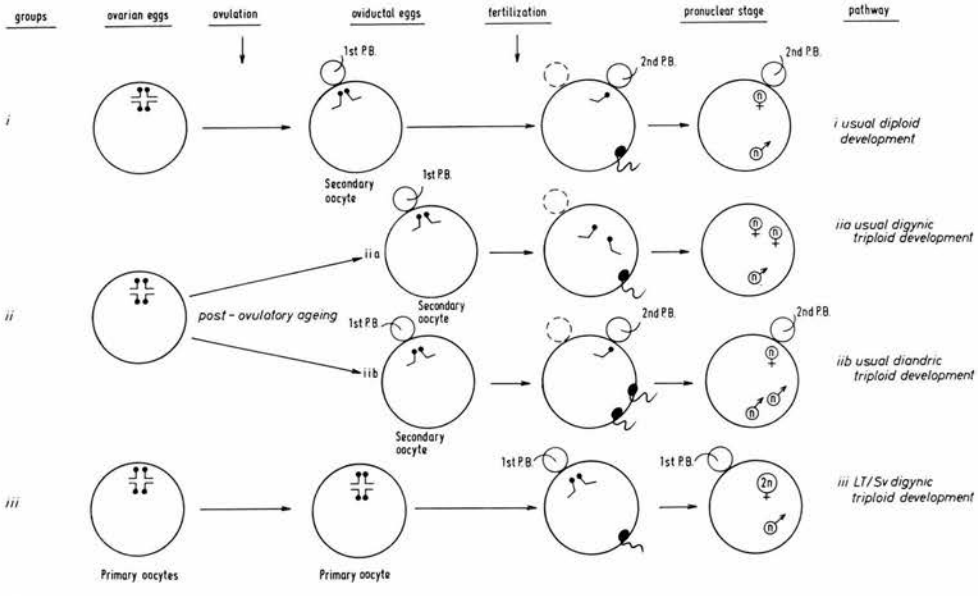


Fig. 2. Usual pathways of development taken following the fertilization of normal secondary oocytes (i) and primary oocytes (iii). Also illustrated are two possible pathways taken by fertilized secondary oocytes as a result of the influence of postovulatory aging changes that occur in the oocyte (iia, iib; see Kaufman, 1983). The LT/Sv digynic triploid eggs encountered in the present study developed along pathway iii.

feature, of "homologous chromosome pairing," was first observed when chromosome spreads prepared from diploid parthenogenones of LT/Sv origin were analyzed at the first cleavage mitosis [Kaufman, 1983; Kaufman and Howlett, 1986].

Previous attempts to fertilize primary oocytes (of ovarian origin) of rodents in vitro has been singularly unsuccessful in that only a small percentage were capable of progressing as far as metaphase of the first cleavage mitosis [Iwamatsu and Chang, 1971, 1972; Niwa and Chang, 1975; Niwa et al, 1976]. Eggs penetrated by sperm prior to anaphase of the first meiotic division were thought to be incapable of eliciting a proper "zona reaction" [see Braden et al, 1954]. Sperm head decondensation, polar body extrusion, and pronuclear formation were apparently also disorganized. In order to explain their observations, these authors have hypothesized that the primary oocyte, prior to ovulation, needs to undergo a period of "cytoplasmic maturation" necessary for normal fertilization to occur. Our results, however, have clearly demonstrated that ovulated primary oocytes, albeit from LT/Sv mice, are capable of progressing to metaphase of the first cleavage division with a frequency equal to that of normal secondary oocytes. Furthermore, these results would seem to indicate that ovulated primary oocytes from LT/Sv strain mice have necessarily undergone some form of "cytoplasmic maturation" within the ovary prior to ovulation, and that this phenomenon has occurred without the need for the oocyte to complete its first meiotic division.

In three mammalian species, namely the dog [Van der Stricht, 1923], the fox [Pearson and Enders, 1943], and the horse [Hamilton and Day, 1945], the egg is normally ovulated as a primary oocyte. These early investigations have clearly demonstrated, however, that meiotic maturation continues in the oviduct, and that sperm penetration does not in fact occur until after the egg has matured to metaphase of the second meiotic division. Van der Stricht [1923] reported that in the dog, sperm may occasionally enter the egg before meiotic maturation has been completed, but sperm head decondensation was observed to occur only in secondary oocytes. It may be concluded from these observations that the final stages of "cytoplasmic maturation" of the oocyte are probably completed when the oocyte is within the oviduct and that the primary oocytes of the dog, fox, and horse are not normally competent to initiate "premature" embryonic development. In contrast, whereas the ovulated primary oocytes of LT/Sv origin have the chromosomal constitution of normal ovarian eggs, either as a result of hormonal imbalance or more likely for reasons that have yet to be established, they clearly have the same initial developmental capacity as secondary oocytes.

Eppig [1978] has drawn attention to the fact that a proportion of the ovarian follicles in LT/Sv females are morphologically unusual in that they have a deficiency in their granulosa cell population. These granulosa-cell-deficient (or G.C.D.) follicles have previously been shown to be the location of spontaneous parthenogenetic activation of ovarian eggs and teratoma formation [Stevens and Varnum, 1974]. However, the G.C.D. follicles are probably not capable (for purely mechanical reasons) of ovulating their oocyte, and we propose that a possible third class of "unstable" follicles may exist that are morphologically normal in appearance but are capable of developing an aberrant relationship with the developing oocyte contained within them. The oocytes ovulated from these "unstable" follicles would be those that are ovulated as primary oocytes, but have the initial developmental potential of normal secondary oocytes.

The only other mice that regularly ovulate primary oocytes are from the NMRI/Han strain, which bear the inheritable trait "diploidy" [Jenderny et al, 1980]. In this strain, following exogenous gonadotrophin stimulation, up to 2–4% of the oocytes may be ovulated as primary oocytes [Bartels et al, 1984; Beermann and Hansmann, 1986]. When NMRI/Han males, known to be carriers of this trait, were bred with Ta/O females and their F1 offspring injected with gonadotrophins, chromosome analysis revealed very high levels of diploidy (about 25%) in oocytes ovulated by the XO females, but low levels (about 1%) in their XX littermates [Beermann et al, 1986]. Various hypotheses were proposed to explain this phenomenon, for example, that in the XO females, it was their XO status that increased the susceptibility of certain follicles to respond prematurely to gonadotrophin stimulation, with the ovulation of primary oocytes. Clearly, in the present study, where about one-third of the ovulated eggs were primary oocytes, the underlying mechanism is likely to be different, as all of the LT/Sv females had a normal XX chromosome constitution. It seems likely, therefore, that at least in LT/Sv mice, other as yet undetermined factors must induce the ovulation of a significant number of "cytoplasmically mature" primary oocytes.

We have clearly demonstrated in this study that both primary and secondary oocytes are capable of monospermic fertilization. Therefore, we believe it is reasonable to conclude that the hypothesis proposed by Kaufman and Howlett [1986], namely, that the ovulation of primary oocytes invariably has an effect on litter size in LT/Sv strain mice because of the prenatal loss of the significant triploid class of embryos, is likely to be substantially correct. Later litters may be small, and this low fertility is probably due in large part to the high frequency of ovarian teratomas in these mice.

ACKNOWLEDGMENTS

G.T.O'N. is supported by a Bonnar Scholarship from the Faculty of Medicine, University of Edinburgh. This work is supported by grants (to M.H.K.) from the National Fund for Research into Crippling Diseases (Action Research for the Crippled Child), the Cancer Research Campaign, and an equipment grant (to M.H.K.) from the Scottish Home and Health Department, ref. no. K/MRS/50/C886.

REFERENCES

Abramczuk J, Sawicki W (1975): Pronuclear synthesis of DNA in fertilized and parthenogenetically activated mouse eggs. Expl Cell Res 92:361–372.

Bartels I, Jenderny J, Hansmann I (1984): Control of meiosis by somatic cells in mice: Inheritance of the meiosis I error "diploidy" and nondisjunction in sensitive NMRI/Han oocytes ovulated from chimaeras. Cell Differ 15:189–194.

Beermann F, Hansmann I (1985): The influence of androgens and an aromatase inhibitor on oocyte maturation and meiosis. Acta Endocrin (suppl) 267:93–94.

Beermann F, Franke U, Hansmann I (1986): High susceptibility for diploidy in ovulated oocytes from XO mice. Hum Genet 72:323–326.

Braden AWH, Austin CR, David HA (1954): The reaction of the zona pellucida to sperm penetration. Aust J Biol Sci 7:391–409.

Donahue RP (1972): Cytogenetic analysis of the first cleavage division in mouse embryos. Proc Nat Acad Sci USA 69:74–77.

Eppig JJ (1978): Granulosa cell deficient follicles. Occurrence, structure and relationship to ovarian teratocarcinogenesis in strain LT/Sv mice. Differentiation 12:111–120.

Hamilton WJ, Day FT (1945): Cleavage stages of the ova of the horse with notes on ovulation. J Anat 79:127–130.

Iwamatsu T, Chang MC (1971): Factors involved in the fertilisation of mouse eggs in vitro. J Reprod Fert 26:197–208.

Iwamatsu T, Chang MC (1972): Sperm penetration in vitro of mouse oocytes at various times during maturation. J Reprod Fert 31:237–247.

Jenderny J, Düls C, Probeck HD, Richter P, Schulz H, Hansmann I (1980): Genetically determined diploidy in mouse oocytes. Eur J Cell Biol 22:23.

Kaufman MH (1973): Analysis of the first cleavage division to determine the sex-ratio and incidence of chromosome anomalies at conception in the mouse. J Reprod Fert 35:67–72.

Kaufman MH (1983): "Early Mammalian Development: Parthenogenetic Studies." Cambridge: Cambridge University Press.

Kaufman MH, Howlett SK (1986): The ovulation and activation of primary and secondary oocytes in LT/Sv strain mice. Gamete Res 9:255–264.

Marston JH, Chang MC (1964): The fertilizable life of ova and their morphology following delayed insemination in mature and immature mice. J Exp Zool 155:237–252.

Martin-DeLeon PA, Boice ML (1983): Spontaneous heteroploidy in one-cell mouse embryos. Cytogenet Cell Genet 35:57–63.

Niwa K, Chang MC (1975): Fertilization of rat eggs in vitro at various times before and after ovulation with special reference to fertilization of ovarian oocytes in culture. J Reprod Fert 43:435–451.

Niwa K, Miyake M, Iritani A, Nishikawa Y (1976): Fertilization of rat oocytes cultured in vitro from various stages of maturation. J Reprod Fert 47:105–106.

Pearson AP, Enders RK (1943): Ovulation, maturation and fertilization in the fox. Anat Rec 85:69–84.

Rodman TC (1971): Chromosomes of the first polar body in mammalian meiosis. Expl Cell Res 68:205–210.

Rodman TC, Barth AH (1979): Chromosomes of mouse oocytes in maturation: Differential trypsin sensitivity and amino acid incorporation. Devl Biol 68:82–95.

Stevens LC (1975): Comparative development of normal and parthenogenetic mouse embryos, early testicular and ovarian teratomas, and embryoid bodies. In Sherman MI, Solter D (eds): "Teratomas and Differentiation." New York: Academic Press, pp 17–32.

Stevens LC, Varnum DS (1974): The development of teratomas from parthenogenetically activated ovarian mouse eggs. Devl Biol 37:369–380.

Szöllösi D (1971): Morphological changes in mouse eggs due to aging in the fallopian tube. Am J Anat 130:209–226.

Tarkowski AK (1966): An air-drying method for chromosome preparations from mouse eggs. Cytogenetics 5:394–400.

Van der Stricht O (1923): Étude comparée des ovules des mammifères aux différentes périodes de l'ovogenèse, d'après les travaux de Laboratoire d'Histologie et d'Embryologie de l'Université de Gand. Arch de Biol, Paris 33:229–300.

Vickers A (1969): Delayed fertilisation and chromosomal anomalies in mouse embryos. J Reprod Fert 20:69–76.

Whittingham DG (1971): Culture of mouse ova. J Reprod Fert Suppl 14:7–21.

Aneuploidy, Part B: Induction and Test Systems, pages 95–122

Aneuploidy Induced by Ethanol

M.H. Kaufman and G.T. O'Neill

EFFECT OF ETHANOL ON EGGS AND EMBRYOS— BACKGROUND INFORMATION

Several epidemiological studies have demonstrated that ethanol consumption during human pregnancy may disrupt the normal development of the conceptus. The most severely affected infants suffer craniofacial anomalies with associated mental retardation and this condition has been termed the fetal alcohol syndrome (FAS) [1–5]. In less severely affected infants growth retardation and neurological disorders have been recognized, and it is now generally accepted that ethanol consumption during pregnancy should be avoided [6]. In animal experiments in which the developing embryo/fetus has been exposed to ethanol either *in vivo* or *in vitro* during the early postimplantation period, similar anomalies have been induced [7–9]. The primary metabolite of ethanol, acetaldehyde, is also known to act as a typical teratogen, effecting the maximum damage to the embryo/fetus during the early organogenetic period [10,11].

More recently, a series of experimental studies have indicated that ethanol is capable of inducing the malsegregation of chromosomes during the first and second meiotic divisions of the mouse egg [12–18]. Malsegregation of chromosomes is believed to result from the direct action of ethanol upon the components of the meiotic spindle apparatus responsible for ensuring the normal segregation of chromosomes [17,18]. The resulting genomic imbalance induced in the fertilized egg is invariably fatal in the mouse, as all autosomal aneuploids are incapable of surviving to term [19,20]. This review will attempt to highlight the recent findings that clearly demonstrate the deleterious effect of ethanol on chromosome segregation in the mouse, propose mechanisms that may be responsible for the induction of aneuploidy in ethanol-exposed gametes and, finally, consider whether it is valid to extrapolate from the observations in the mouse to the clinical situation in man.

Department of Anatomy, University Medical School, Edinburgh EH8 9AG, Scotland

INFLUENCE OF ETHANOL ON MEIOTIC CHROMOSOME SEGREGATION IN MAMMALIAN OOCYTES: PARTHENOGENETIC STUDIES

Several investigators [12,21,22] have demonstrated that exposure of mouse oocytes either *in vivo* or *in vitro* to a dilute solution of ethanol in phosphate-buffered saline (PBS) may induce a high frequency of parthenogenetic activation. Highest frequencies of activation occurred when eggs were allowed to "age" within the oviduct for about 5 h *in vivo* after the expected time of ovulation prior to the activation stimulus. Activation of oocytes at this time produced a high incidence of single pronuclear haploid (1 pron + 2PB) parthenogenones [23]. A wide range of chemical and physical stimuli are capable of inducing the parthenogenetic development of mammalian eggs [23]. Analysis of the chromosome constitution of activated eggs at metaphase of the first cleavage mitosis, however, has consistently shown that parthenogenesis *per se* is not normally associated with errors in chromosome segregation [23]. Nevertheless, cytogenetic analysis of ethanol-activated single pronuclear haploid eggs has revealed that exposure to ethanol is not only capable of inducing high rates of activation but may also disrupt the normal segregation of chromosomes that occurs during the second meiotic division in a significant proportion of the activated eggs [12]. Both the activation frequency and the incidence of chromosome malsegregation were found to be directly related to the dose of ethanol employed and the duration of exposure. The incidence of aneuploidy observed varied from 14% to 19% of the oocytes analyzed, and metaphase spreads with 18, 19, 21, and 22 chromosomes were observed (see Fig. 1; the normal haploid complement of the mouse is 20 chromosomes). It therefore appears that direct exposure to a dilute solution of ethanol in PBS has the ability to induce both the biochemical and morphological changes that occur at activation (which are similar but probably not identical to those that occur at fertilization [23]), but it is also capable of disrupting the normal segregation of chromosomes at the second meiotic division.

EFFECTS OF ETHANOL ON CHROMOSOME SEGREGATION DURING FIRST AND SECOND MEIOTIC DIVISIONS AS DETERMINED FROM ANALYSIS OF FERTILIZED EGGS AT FIRST-CLEAVAGE METAPHASE

Analysis of the chromosome constitution of mouse eggs at metaphase of the second meiotic division [24,25] and at metaphase of the first-cleavage mitosis [26,27] has revealed that the spontaneous incidence of aneuploidy in

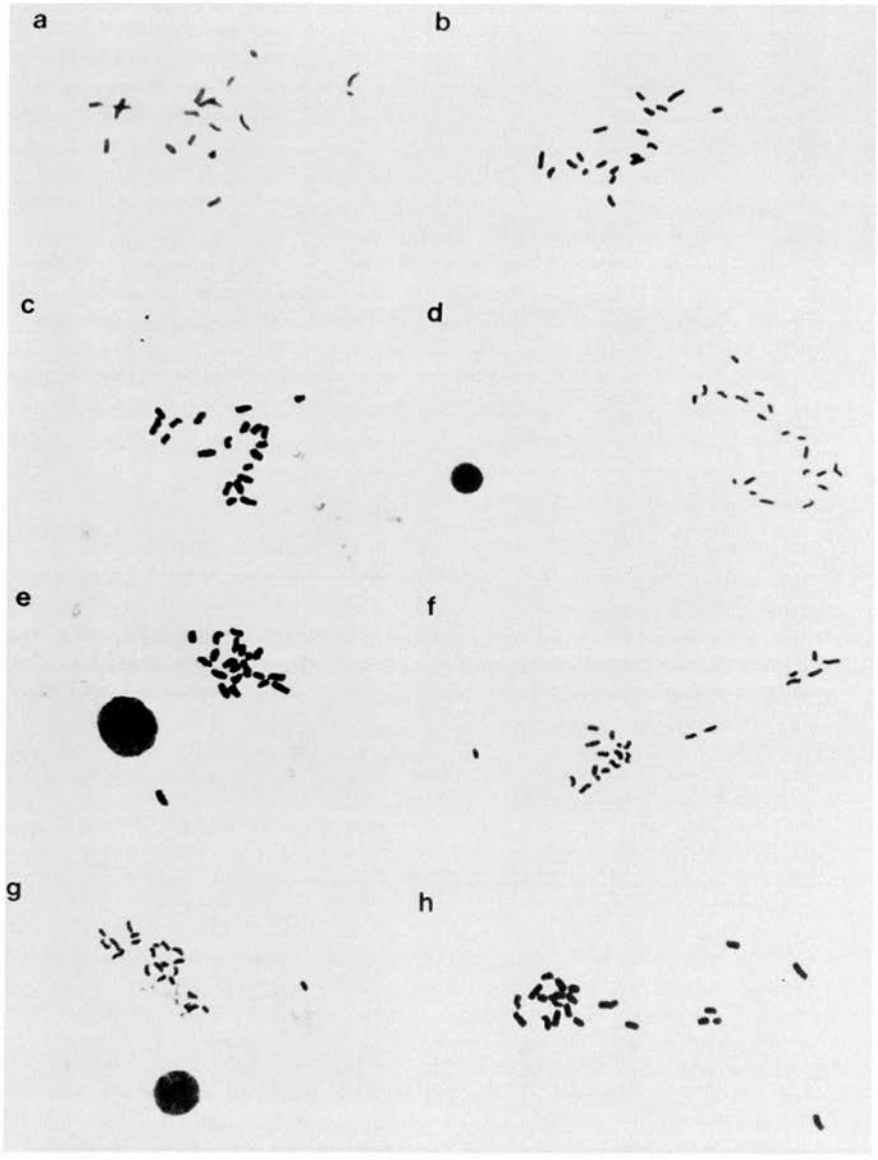


Fig. 1. Representative air-dried chromosome spreads of the first-cleavage metaphase of one-pronuclear haploid parthenogenetic mouse eggs activated *in vitro* following the exposure of ovulated secondary oocytes to a dilute solution of ethanol. All oocytes were incubated for about 12 h in medium containing Colcemid. Metaphase spreads with 18 (**a**), 19 (**b**), 20 (**c,d**) (representing the normal euploid haploid complement in the mouse), 21 (**e,f**), and 22 (**g,h**) chromosomes present are shown. The condensed round object seen in panels d; e, and g is the nucleus of the second polar body. All preparations were stained with Giemsa. (Reproduced from Kaufman [23], with permission of the publisher.)

the mouse is extremely low. As it had been clearly demonstrated that exposure to ethanol induces numerical chromosomal anomalies in single pronuclear haploid embryos, subsequent studies were conducted in order to investigate whether exposure to a dilute solution of ethanol *in vivo* could also induce high rates of chromosome malsegregation in recently fertilized eggs. In a preliminary study [13], a dilute solution of ethanol in distilled water was administered by intragastric injection into the stomach of recently mated females at about the predicted time of fertilization. The intragastric injection of ethanol produced a blood level of about 200–280 mg/100 ml after about 20 min, and this level was maintained for a period of approximately 2 h. The blood alcohol level then dropped rapidly, so that after about 6-h baseline levels were achieved. Exposure to ethanol in these experiments therefore extended over the critical period when the eggs were completing their second meiotic division.

Several hours later, the mice were autopsied and the pronuclear stage fertilized eggs were isolated and subsequently transferred into tissue culture medium containing Colcemid. The presence of this agent in the medium inhibited the fertilized eggs from progressing beyond metaphase of the first cleavage mitosis. Furthermore, the male and female pronuclear chromosome sets remained as two distinct entities because of the inhibitory influence of this agent. Chromosome analysis revealed that the incidence of aneuploidy induced, as in the earlier parthenogenesis studies [12], was also directly related to the ethanol concentration employed. Furthermore, as the chromosomes from each of the two pronuclear groups could be analyzed separately (due to the presence of a "marker" chromosome within the male-derived set), it was established that the ethanol-induced malsegregation event was invariably restricted to the female-derived chromosome set (Fig. 2). As these results had clearly demonstrated that exposure to a dilute solution of ethanol was capable of inducing chromosomal malsegregation when exposure occurred during the second meiotic division, further studies were carried out to determine which other stages of oocyte maturation and zygote development might also be sensitive to the influence of ethanol.

In a subsequent series of experiments [14,15], female mice were given a single similar dose of ethanol at specific times before or at various times after the superovulatory injection of human chorionic gonadotropin (HCG) that induced the resumption of meiotic maturation and ovulation. The female mice were subsequently mated with fertile males. The timing of the ethanol administration was chosen in order to produce high blood alcohol levels either during the first meiotic division (HCG − 1.5 h, HCG + 1.75 h, and HCG + 4 h), at about the time of fertilization (HCG + 13.5 h), and during the period of chromosome condensation when the female pronucleus was

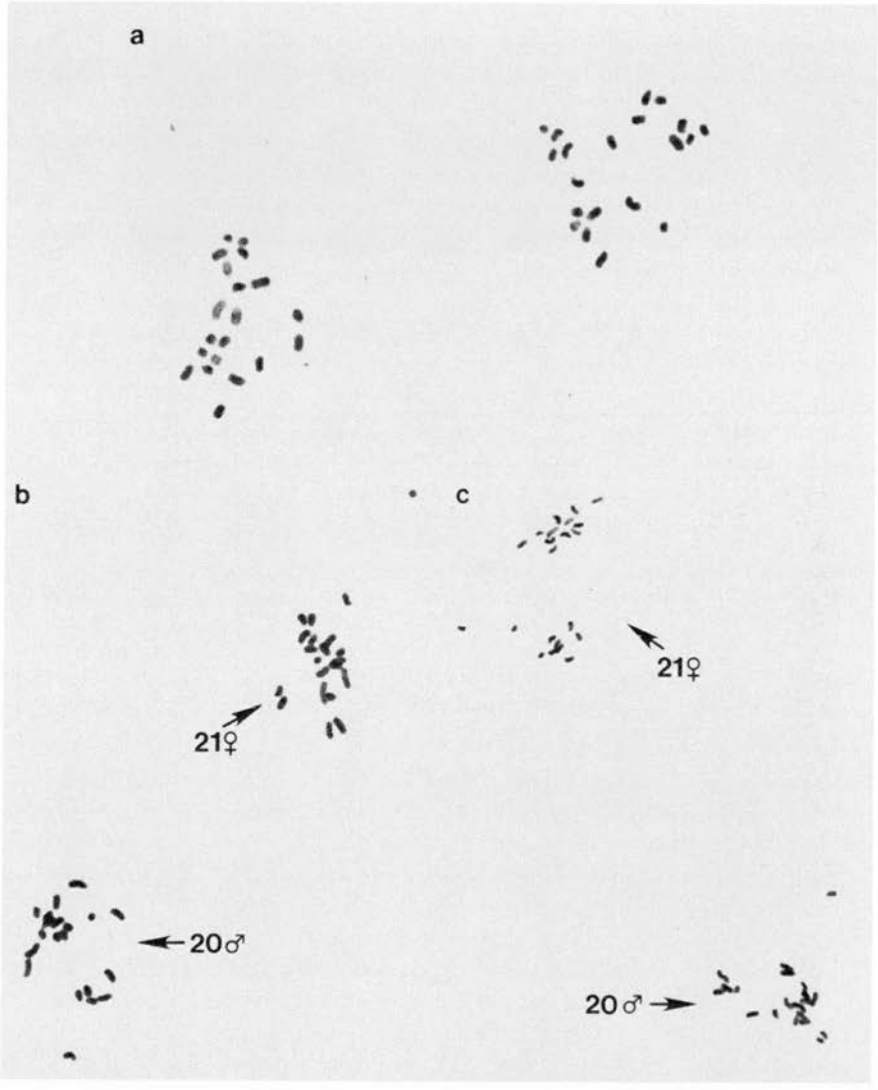


Fig. 2. Maternally and paternally derived chromosome groups are observed as two discrete sets, when one-cell fertilized eggs are incubated in medium containing Colcemid. **a:** "Control" embryo, with two "pronuclear" groups each containing 20 chromosomes. **b,c:** Two "experimental" embryos isolated from female mice previously given an intragastric injection of a dilute solution of ethanol shortly after mating. One of the two groups contains 21 chromosomes (the presumptive oocyte-derived set), whereas the other group contains the normal complement of 20 chromosomes (the presumptive sperm-derived set). All preparations were stained with Giemsa. (Reproduced from Kaufman [23], with permission of the publisher.)

being formed (HCG + 17 h, and HCG + 20 h). The chromosome constitution of the zygotes was determined at metaphase of the first cleavage mitosis, at the morula stage, and on the 10th and 11th days of gestation [14,15].

In this more extensive series of experiments, the chromosome constitution of embryos previously exposed to a dilute solution of ethanol either prior to or shortly after fertilization indicated that chromosome malsegregation could be induced during either the first or the second meiotic division. However, if exposure occurred after the expected time of second polar body extrusion, the incidence of aneuploidy dropped dramatically to control (unexposed female) levels. These findings led the authors to conclude that numerical chromosome anomalies could be induced only when ethanol interfered with the normal function of certain components (e.g., the microtubules) of the meiotic spindle apparatus of the egg. The possible effects of ethanol upon chromosome segregation during the first and second meiotic divisions are represented in Figures 3 and 4, respectively.

POSSIBLE MODE OF ACTION OF ETHANOL IN INDUCING ANEUPLOIDY

The results of the analyses of the chromosome constitution of both ethanol-activated parthenogenones and fertilized eggs exposed to ethanol strongly suggest that it disrupts the normal functioning of the egg's meiotic spindle apparatus. However, although several possible underlying mechanisms have been proposed that might result in meiotic and/or mitotic chromosome malsegregation [28], the exact mechanisms of action of this agent, or of its primary metabolite acetaldehyde, have yet to be established. It is possible, for example, that the action of ethanol may be similar to that of the spindle-active drugs such as Colcemid and colchicine [29–32], and vincristine sulphate [33], when these drugs are administered in low dosages. The incidence of chromosome malsegregation observed following exposure to ethanol during meiotic maturation may result from disruption of the contractile elements of the spindle apparatus. Colchicine, like ethanol, is a weak anesthetic and central nervous system depressant [34,35], and it is possible that both are capable of inhibiting or disrupting meiotic divisions through their influence on the microtubular elements of the meiotic spindle apparatus. It is relevant in this context that Figueroa and Vig [36] have reported that Colcemid does not interfere with the normal process of centromeric separation that occurs during chromosome segregation at early anaphase, or the sequence of events underlying this process. These observations, if correct, would appear to indicate that ethanol-induced chromo-

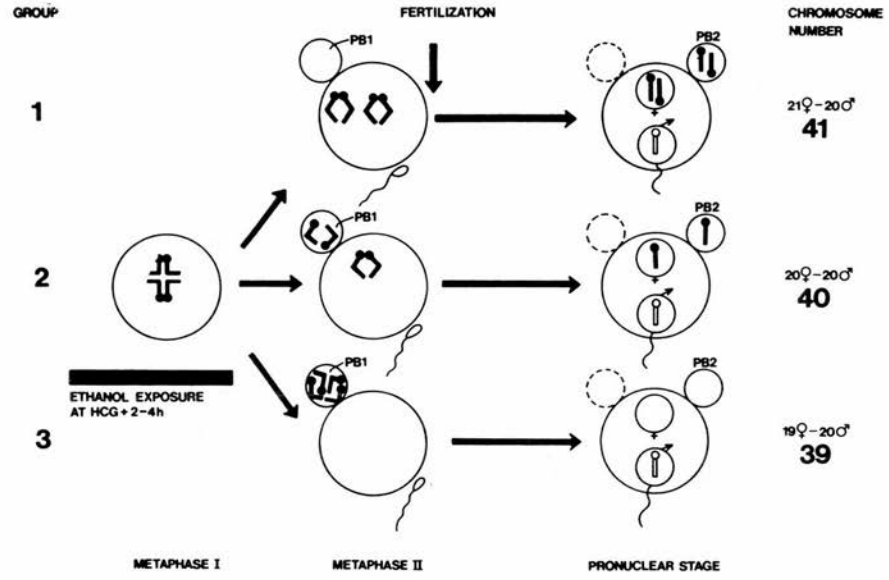


Fig. 3. Possible outcome of chromosome segregation following *in vivo* exposure of ovarian oocytes to a dilute solution of ethanol during the first meiotic division. In group 2, normal segregation of the meiotic chromosomes occurs during both the first and second meiotic divisions, and results in the formation of a normal (euploid) diploid zygote following monospermic fertilization. In groups 1 and 3, exposure to ethanol *in vivo* during the first meiotic division induces the malsegregation of individual chromosomes to occur, and results in the formation of hyperdiploid (group 1) or hypodiploid (group 3) secondary oocytes. Fertilization of the latter results in the development of hyperdiploid (group 1) or hypodiploid (group 3) zygotes, where the source of the aneuploidy is from the female gamete.

Figs. 3, 4, 7, 8. Series illustrates diagramatically the cytogenetic events that occur in ovarian meiotic oocytes associated with normal and abnormal chromosome segregation, and the resultant chromosome constitution of embryos following fertilization (Figs. 3, 4) or parthenogenetic activation (Figs 7, 8).

Abbreviations

HCG	human chorionic gonadotropin
Metaphase I	metaphase of the first meiotic division
Metaphase II	metaphase of the second meiotic division
PB1	first polar body, which degenerates shortly after ovulation has occurred
PB2	second polar body
Pronuclear stage	prometaphase/metaphase of the first cleavage division

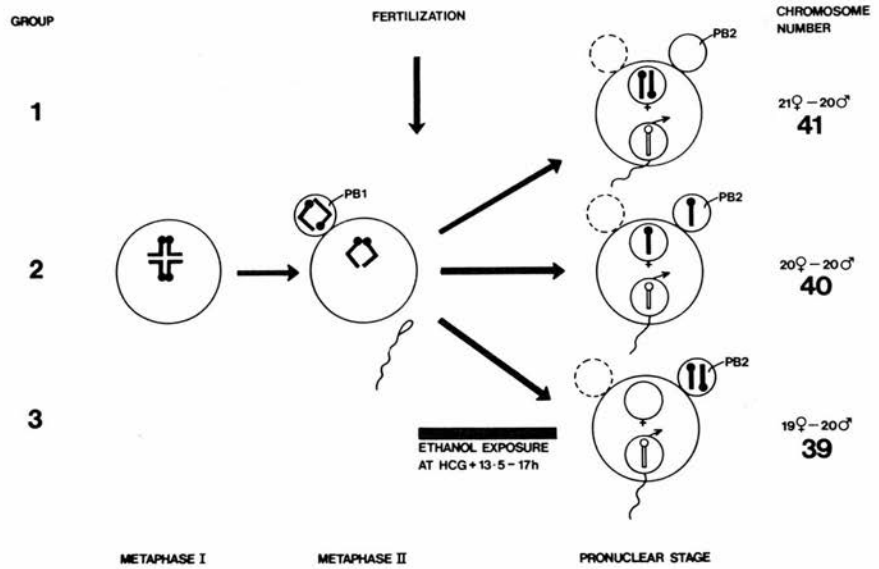


Fig. 4. Possible outcome of chromosome segregation following *in vivo* exposure of ovarian oocytes, or recently ovulated oocytes to a dilute solution of ethanol during the second meiotic division. In all three groups, it has been assumed that normal segregation of the meiotic chromosomes has occurred at the first meiotic division. In group 2, normal segregation of the meiotic chromosomes has also occurred at the second meiotic division, and results in the formation of a normal (euploid) diploid zygote following monospermic fertilization. In groups 1 and 3, exposure to ethanol *in vivo* during the second meiotic division induces the malsegregation of individual chromosomes to occur, and results in the development of hyperdiploid (group 1) or hypodiploid (group 3) zygotes, where the source of the aneuploidy is from the female gamete. (See legend below Fig. 3.)

some malsegregation, which may occur at either the first or the second meiotic division, may not be due to interference with this process.

The incidence of triploidy observed in these studies was not significantly greater than the spontaneous levels previously reported in the literature [14,26,27,37]. Equally, the type of triploidy observed, whether of dispermic or digynic origin, did not appear to be influenced by the high blood alcohol levels encountered in these studies [16]. These observations would seem to indicate that the underlying mechanism of action of ethanol, at the concentrations administered in these studies, was probably on the components of the meiotic spindle apparatus. The fact that no increase in the frequency of triploidy is observed appears to be strong evidence that this agent does not disrupt the process of cytokinesis. This is in marked contrast to the action of the cytochalasins, which are capable of inducing digynic triploid embryos by

disrupting the polymerization of the actin filaments that are thought to be essential for the extrusion of the second polar body [38–40]. As the exposure of oocytes to ethanol did not appear to interfere with the process of cytokinesis, but was clearly responsible for an increase in the frequency of chromosome malsegregation, it would be reasonable to suggest that this agent probably acts directly upon the microtubular components of the meiotic spindle apparatus.

The recent availability of antibodies to the various cytoskeletal components should allow an instructive investigation to be undertaken into the events that occur during normal chromosome segregation. Recent reports have demonstrated the changes that can occur in the meiotic spindle apparatus due to postovulatory aging in mouse eggs [41,42], and investigations of this type may provide a greater degree of insight than previously available into the effects of, for example, maternal aging, and of a variety of environmental factors upon the stability and functioning of the meiotic spindle apparatus. This approach may also shed light on its relationship with the meiotic chromosomes and provide information on the events that occur during chromosome malsegregation. It seems likely, however, that only an ultrastructural study will be capable of resolving the finer details of the dynamics of normal chromosome segregation, as these are in all probability beyond the resolving power of the light microscope. Appropriate modifications of this approach will also have to be employed in order to establish the mechanism of induction of aneuploidy associated with the exposure of eggs (and possibly also male germ cells) to ethanol and related spindle-active agents.

CAN ETHANOL INDUCE ANEUPLOIDY IN MALE GERM CELLS?

Several recent studies in which male rodents were exposed to a dilute solution of ethanol by gastric injection have also reported that this agent may interfere with normal chromosome segregation during spermatogenesis. Barilyak and Kozachuk [43] have observed that 48 h after the oral administration of a single 2-ml dose of 40% ethanol to male rats, the incidence of chromosomal anomalies observed in spermatocyte preparations was higher than in unexposed controls. The results from similar studies [44,45] in which male mice were exposed to cumulative doses of ethanol has also indicated that this agent may disrupt the normal cytogenetic development of both spermatogonia and spermatocytes. More recent studies by Hunt [46] have also shown an effect of ethanol on spermatogenesis in the mouse, though the incidence of chromosomal malsegregation observed was rela-

tively low, and this author concluded that the "aneuploidy-inducing effect of ethanol may be substantially greater in the female than in the male."

The most frequent chromosome anomaly reported by Barilyak and Kozachuk [43] was an increase in the incidence of univalents. However, these findings are particularly difficult to interpret, since many authors have clearly demonstrated that no direct correlation exists between the induction of univalency and the development of aneuploid gametes [47–51].

An increase in the frequency of embryonic mortality in the progeny of male rats previously administered a dose of ethanol 8–13 days before mating has also been reported [43,52,53], which would seem to suggest that chromosome defects could be induced at the spermatid or even later stages of spermatogenesis. However, it should be stated that these positive findings have not been observed by other researchers [54]. Because of these conflicting findings, one can only conclude that there is, as yet, much work to be carried out before it is possible to state unequivocally that ethanol and/or its metabolite acetaldehyde is or is not capable of interfering with the chromosome constitution of male germ cells.

INFLUENCE OF ETHANOL ON SOMATIC CELLS

Numerous reports have been published that have described a wide range of effects that they have attributed to the influence of ethanol, namely, on chromosome structure and function in mitotic cells *in vivo,* and on mammalian cells maintained *in vitro,* as well as on plant cells. For example, analyses of chromosome spreads from bone marrow cells isolated from male rats that had previously been exposed to a dilute solution of ethanol has indicated that it was possible to induce both numerical and structural chromosome anomalies in a small proportion of the exposed cells [43]. The anomalies observed apparently persisted for at least 4 weeks. Chromosomal aberrations have also been reported in alcoholics [55–57] but the underlying mechanism is far from clear, as it is possible that other factors may be of critical importance in this group, such as an impaired DNA repair mechanism, which could account for the mutagenic effects observed.

Studies on the small lymphocytes isolated from peripheral blood samples have also proved to be a particularly instructive system for analyzing the induction of chromosomal aberrations by a variety of agents. An increase in the incidence of chromosomal breakages, for example, has been observed when lymphocyte cultures were exposed to dilute solutions of ethanol for periods of 4–48 h [58]. However, variable results have been reported when cultured Hela cells [59] and Chinese hamster cells were exposed to dilute solutions of ethanol [60]. In the former study, sister chromatid exchanges

were induced, but in the latter series, no chromosomal abnormalities were reported. By contrast, repeated exposure of cultured Chinese hamster cells to a dilute solution of acetaldehyde resulted in an increase in the incidence of sister chromatid exchanges over control values [60], and more recent findings have indicated that the mutagenic effect of ethanol (as determined by the incidence of structural chromosomal aberrations) is more likely to be due to the action of its primary metabolite acetaldehyde rather than to the influence of ethanol itself [61].

It is of interest that similar findings had previously been observed in plant studies in which *Vicia faba* had been exposed to these agents [62]. In subsequent studies ethanol did not exhibit chromosome breaking activity in root tips when roots were grown on bulbs, but aberrations were induced in root tips when they were grown from seed in the presence of ethanol [63,64].

Although these reports would seem to indicate that the mutagenic and possibly carcinogenic effects of ethanol may be due more to the effects of its primary metabolite acetaldehyde, other evidence exists that ethanol may itself act as a spindle-active agent. Ethanol shows many of the physical and chemical characteristics of the inhalational anesthetics that are known to disrupt the process of cell division [65–67], and although it is acknowledged that modern inhalational anesthetics are probably not mutagens, those that contain the vinyl moiety may be potential mutagens or carcinogens.

POSSIBLE SIMILARITY OF ACTION BETWEEN CERTAIN ANESTHETIC AGENTS AND ETHANOL

It has been suggested that most anesthetic agents and some narcotics may affect the M-stage of the cell cycle by depolymerizing microtubules [68,69] and are therefore potentially capable of interfering with normal chromosome segregation.

Several ultrastructural studies have revealed that halothane can interfere with the assembly of mitotic microtubules [68,70,71], induced by conformational changes in the microtubule subunits, resulting in the formation of macrotubules. Other studies have indicated that nitrous oxide may impair the microtubular interaction between the spindle subunits and the centromere necessary for the movement of chromosomes during prometaphase [72,73]. While numerous hypotheses have been proposed to explain the mitotic inhibitory effect of anesthetics, the situation is confused by differences in the susceptibility at specific mitotic cycle stages to anesthetic agents [69]. However, the present, albeit indirect, evidence would seem to indicate that a similar underlying mechanism may be involved when meiotic and mitotic cells are exposed to these agents. Thus anesthetics are clearly capable of inducing

errors in chromosome segregation in mammalian cells grown in tissue culture [74], and of increasing the incidence of chromosomal anomalies in rat spermatogonial cells [75]. When female mice were exposed to tribromoethanol (Avertin) [14,76,77], numerical chromosomal anomalies were induced when exposure occurred prior to and during the resumption of meiotic maturation.

Both ethanol and certain anesthetics are capable of activating rodent eggs and inducing them to develop parthenogenetically [12,76]. As indicated previously, parthenogenetic activation *per se* is not normally associated with the induction of errors in chromosome segregation [23]. The underlying mechanism of activation of ovulated mouse oocytes by tribromoethanol [76] and ethanol [12], not only involves the release of intracellular membrane-bound calcium ions (a prerequisite for activation in all species [23]), but also appears to be capable of interfering with the assembly of the microtubules in the meiotic spindle apparatus. The latter may well be the reason why chromosomal malsegretation is observed following activation by these agents. The indirect evidence indicates that both their physicochemical properties and the effects that they have upon the oocyte are similar, and therefore it appears reasonable to suggest that the interaction that occurs between the microtubules of the meiotic spindle apparatus and these various agents may share a common mechanism. It is of interest that the role of calcium ions in maintaining the stability of the microtubule–microfilament system in the oocyte, and the biochemical trigger for its release, remains to be determined.

CLINICAL IMPLICATIONS OF ETHANOL CONSUMPTION

Recent animal studies have clearly demonstrated that ethanol and other agents with related physicochemical properties (such as anesthetics) are capable of inducing numerical chromosomal anomalies at the first and second meiotic divisions in the mouse egg [17,18], and rather less satisfactory evidence also exists that ethanol may induce similar chromosomal anomalies during spermatogenesis [43–46].

At the present time, there is little direct evidence that implicates ethanol, or the related spindle-active agents, to the induction of aneuploidy in man. However, in two recent large epidemiological studies, the findings indicated an increase in the risk of spontaneous abortion that appeared to be correlated with ethanol consumption, all other factors being equal [78,79]. Until relatively recently, it had long been recognized that the incidence of pregnancy wastage in female operating room personnel was about twice that observed in matched unexposed females [77,80–83]. Unfortunately, owing to the lack of detailed cytogenetic information at the time, it is uncertain whether this higher incidence of pregnancy wastage resulted from an increase

in the incidence of chromosome malsegregation events in the oocyte, or was due to a teratogenic effect of these agents upon the early development of the conceptus. Fortunately, this effect is no longer seen, because of the adequate venting of anesthetic gases from the operating room environment.

Recent studies have indicated that at least 15–20% of all recognized human pregnancies result in spontaneous abortion [28,84]. Cytogenetic analyses of spontaneous abortus products have indicated that between 50% and 60% of these are associated with numerical chromosomal anomalies, of which about 80% result from autosomal trisomic or monosomic states [28,85–94]. These values for the incidence of spontaneous abortions may be a conservative estimate, as these studies are necessarily unable to include the group of unrecognized pregnancies that are terminated spontaneously prior to or shortly after implantation. Numerous studies have indicated that 40–75% of embryos may be lost before clinical pregnancy is recognized [95–100] and it is now believed that the high frequency of human fetal loss may be related directly to the aberrant chromosome constitution of a large proportion of human oocytes [101]. The majority of the chromosomal anomalies were restricted to the E-, G-, and D-groups of the genome. To date, the limited number of cytogenetic analyses of mostly 8-cell-stage preimplantation human embryos obtained following *in vitro* fertilization [102–104] have also indicated that an unexpectedly high proportion of those examined exhibited numerical chromosomal anomalies.

Cytogenetic analyses of spontaneous abortion material [90–92,105] and live-born trisomics [106–109] have indicated that segregation errors in the maternal (rather than the paternal) first (rather than the second) meiotic division are most frequently involved in the development of trisomic conceptuses.

It has also been clearly recognized that a strong etiological association exists between increased maternal age and the frequency of occurrence of aneuploidy, and that a proportion of the chromosomes of the human genome are more commonly involved in this state than others [105]. In man, it has also been suggested that the increase observed in the frequency of trisomic offspring born to aged mothers may be due to an increase in the presence of univalents in the oocyte, as suggested by Henderson and Edwards [110], possibly due to a decrease in chiasma frequency in the eggs of aged females. However, several more recent reports have clearly indicated that the occurrence of univalents at meiosis I observed in the eggs of aged mice and Chinese hamsters is not associated with aneuploidy at metaphase II (mouse: [111,112]; Chinese hamster: [49]). In fact, it has been demonstrated that desynapsis of bivalents may occur as a natural feature of oocyte development in the mouse [48,112]. Furthermore, it has been suggested that a proportion

of the univalents observed may be artifacts of the chromosome-spreading technique [50].

In the mouse it has been demonstrated that postovulatory aged secondary oocytes exhibit abnormalities in spindle structure [41,42,113]. At present we are unable to state unequivocally that similar defects in spindle alignment occur in the primary and secondary oocytes of aged females. However, previous cytogenetic analysis of fertilized postovulatory aged mouse eggs has indicated that postovulatory aging within the oviduct, in contrast to biological aging [114,115], does not predispose the oocyte to errors of chromosome segregation at the second meiotic division [37,116].

It has still to be fully determined why human oocytes, even taking the age-related effects into consideration, are prone to a high incidence of chromosomal malsegregation.

It seems likely, therefore, that a number of factors, both exogenous and endogenous, are probably involved that induce errors in segregation of meiotic chromosomes, but that the maternal and paternal organisms are not equally susceptible to these noxious stimuli, possibly because of fundamental differences between the processes of oogenesis and spermatogenesis.

Possibly an increased duration of exposure to potential hazards occurs during the first meiotic division—because of its prolonged duration. In all probability a combination of factors is at work, with exogenous influences superimposed on the age-related processes.

Exposures to these factors, but particularly to ethanol—because of its greater availability—should therefore be regarded as potential hazards that are certainly capable of interfering with the normal development of the maturing oocyte, and also of interfering with the chromosome segregation events that occur at conception in association with the extrusion of the second polar body. There is in addition the possibility that continuous or chronic exposure of ovarian oocytes to the effects of low levels of ethanol may increase the likelihood of segregation errors occurring at the first meiotic division. This effect of ethanol has yet to be demonstrated unequivocally in the mouse, though it has already been established that tribromoethanol (Avertin) administration prior to the resumption of meiotic maturation may result in fetal death [76].

ARE CERTAIN CHROMOSOMES MORE LIKELY TO BE INVOLVED IN MALSEGREGATION THAN OTHERS?

Human Studies

Various detailed cytogenetic studies have been undertaken to investigate the chromosome constitution of large numbers of spontaneous abortuses

[84,93,94,105]. These have indicated that, even accounting for age-related effects on the frequency of aneuploidy, certain chromosomes, especially the smaller acrocentrics, appear to show an increased probability of being involved in malsegregation events. However, it must be acknowledged that drawing such conclusions from an analysis of available spontaneous abortion material is likely to provide an incomplete picture, as it fails to take into account pre- and early postimplantation losses. The latter probably include all of the autosomal monosomics and those autosomal trisomies with a very limited degree of viability [117,118].

Only an extensive cytogenetic study of possibly thousands of preimplantation human embryos would enable investigators to establish whether *all* of the chromosomes are equally liable to undergo malsegregation events or whether, as appears to be more likely, specific chromosomes are more liable to undergo nondisjunction than others. At present, the results of only a limited cytogenetic analysis of preimplantation human embryos [104] and oocytes [101] are available, and this would seem to indicate that the majority of the numerical chromosome anomalies encountered were in fact confined to the chromosomes of groups D, E, and G.

Mouse Studies Using Fertilized Embryos

The most accurate means of establishing the incidence of aneuploidy at conception requires embryos to be analyzed at metaphase of the first cleavage division. Extensive studies of this type have been carried out only in the mouse, where the incidence of aneuploidy has been found to be extremely low [24–27], and certainly much lower than even the most conservative estimates for the situation at conception in man. Analysis of the chromosome constitution of embryos at later developmental stages provides a less accurate means of assessing the initial incidence of aneuploidy because of the progressive loss of the monosomic and trisomic embryos [19,20]. Very few *autosomal* monosomics survive beyond the very early postimplantation period; the remaining monosomics are invariably *X monosomics,* which if the fetus survives to birth is typically associated with Turner syndrome [87].

Preliminary studies in the mouse have indicated that certain chromosomes may be more frequently involved in ethanol-induced malsegregation events during the first and second meiotic divisions than others. Karyological evidence derived from G-banding analysis of first-cleavage metaphase spreads from fertilized eggs that had previously been exposed to a dilute solution of ethanol *in vivo* during either the first or second meiotic division has indicated that in general terms (with the exception of chromosome 19) the smaller chromosomes of the genome are particularly susceptible to malsegregation [16].

In a similar study in which spontaneous chromosome anomalies resulting from segregation errors that presumably occurred at both the first and second meiotic divisions were analyzed at the same stage of development, Martin-De Leon and Boice [119] reported that chromosome 19 was most commonly involved in malsegregation events. This is in complete contrast to the findings of O'Neill and Kaufman [16], who found no examples of aneuploidy involving this chromosome. These authors also established that the larger chromosomes of the mouse genome were only infrequently associated with errors in chromosome segregation, whereas the smaller heterochromatic chromosomes 15, 16, and 18 were more frequently involved in these events.

Mouse Studies Using Parthenogenetic Material

Additional evidence from a similar study of the chromosome constitution of ethanol-activated single pronuclear haploid parthenogenetic mouse embryos has also clearly demonstrated that the smaller chromosomes were more prone to malsegregation than the other members of the genome [120] (Figs. 5, 6). We believe that parthenogenetic activation of ovulated mouse eggs induced by a brief exposure to a dilute solution of ethanol *in vitro* [12] serves as a valuable model system to study the effects of this agent on chromosome segregation at the second meiotic division. The activation technique employed induces a high frequency of single pronuclear haploid parthenogenones [23], in which up to about 20% have numerical chromosomal anomalies [12].

The chromosome anomalies that are induced can result only from the direct action of ethanol upon the meiotic spindle apparatus or on the centromere region of the chromosomes. Parthenogenetic activation also produces a more synchronized population of embryos, which greatly facilitates the analysis of large populations of embryos [23]. The parthenogenetic activation of human oocytes by a similar technique might provide one means of assessing the effect of exposure to ethanol upon chromosome segregation at the second meiotic division. While human eggs are undoubtedly capable of undergoing parthenogenetic activation *in vivo* within the ovary, giving rise to a proportion of ovarian teratomas [121], only indirect evidence exists to date that they are capable of being induced *in vitro* [102,122,123]. However valuable the long-term results might be, it seems unlikely that a sufficiently large study will ever be undertaken that would be capable of providing any meaningful information in this regard.

Various pathways of development can be induced when mouse eggs are activated parthenogenetically, and this enables hemitetrad analysis of meiotic development to be performed [23,124]. Unfertilized mouse eggs that have been retained within the oviduct for about 5–10 h after ovulation can be

readily activated both *in vivo* and *in vitro* by a wide variety of stimuli [23] to produce a high frequency of "two-pronuclear" and "one-pronuclear" parthenogenones, the former having retained both products of the second meiotic division whereas in the latter case, one product is retained within the egg while the second product is extruded within the second polar body.

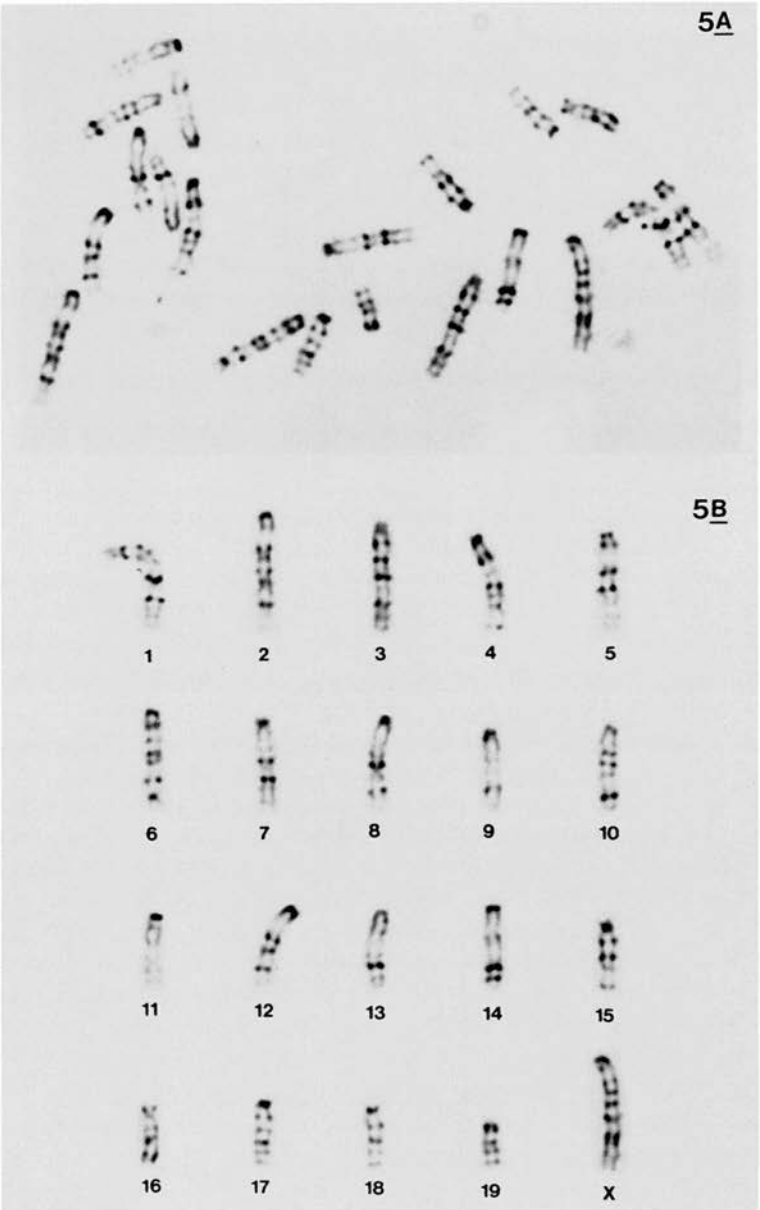
Analysis of the chromosome constitution of both classes of parthenogenone at the first-cleavage mitosis provides a means of studying the effects of a wide variety of agents on chromosome segregation during either the first or the second meiotic division (Figs. 7, 8). Furthermore, these experimental approaches provide additional information on the possible mechanisms underlying meiotic chromosome malsegregation following exposure to these agents.

CONCLUSIONS FROM THE ANALYSIS OF FERTILIZED AND PARTHENOGENETIC MOUSE EMBRYOS

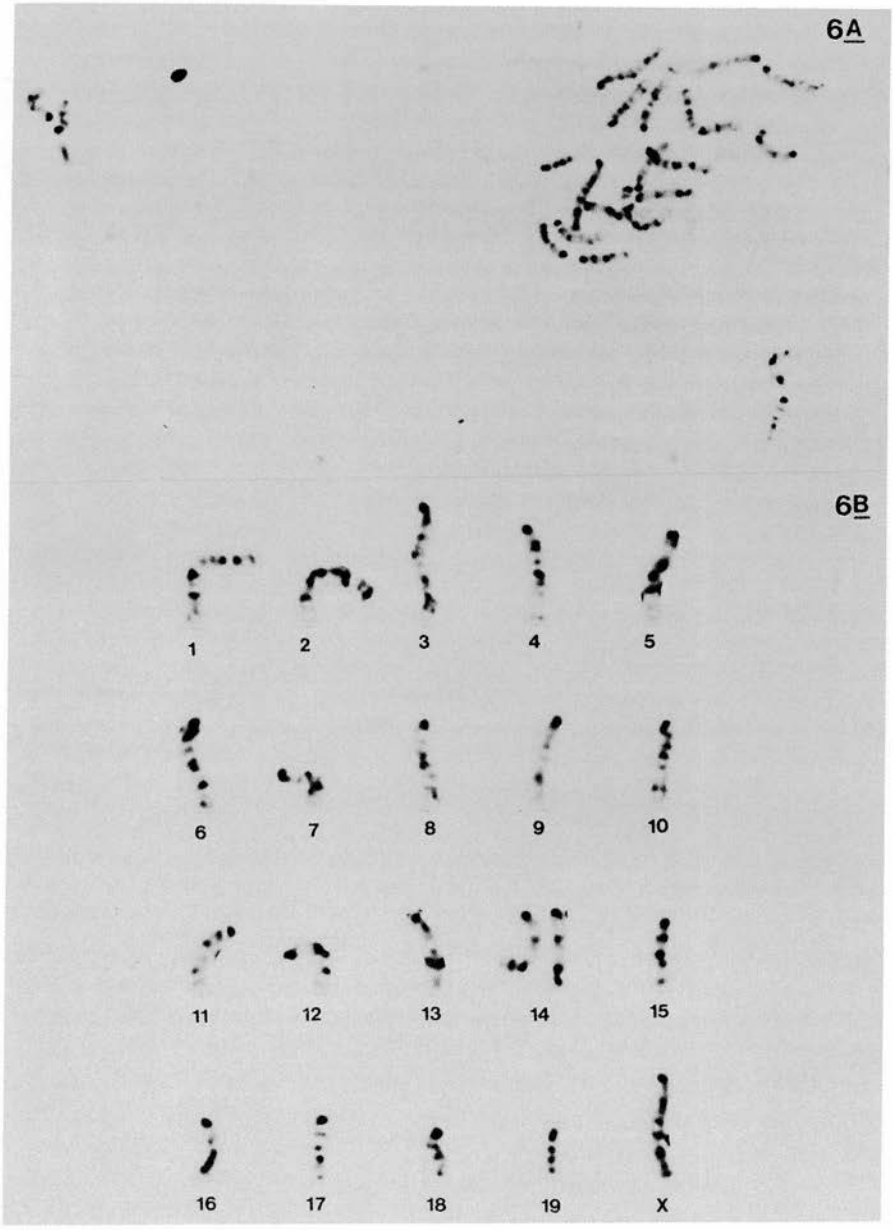
Recent preliminary evidence from first-cleavage analyses in the mouse [16,120] indicates that, as already recognized in the numerous karyological studies of spontaneous human abortions [93,94,105], specific autosomes of the mouse genome appear to be more prone to undergo nondisjunction, both spontaneously and following exposure to spindle-active agents such as ethanol, than other members of the genome. These and earlier studies of the effects of ethanol on chromosomal segregation in the mouse have demonstrated that in the majority of cases only one chromosome is either lost or gained [12–16,120]. This would seem to indicate that the action of ethanol, at the concentrations administered to the female mice in these studies, must have had a quite subtle effect upon the dynamics of the spindle apparatus, which principally manifested itself by tending to increase the baseline or "spontaneous" level of malsegregation of the smaller chromosomes. As the interference by ethanol and its related spindle-active agents on normal spindle function is in all probability a rather nonspecific form of interaction with one or more of the spindle subunits, it appears likely that the increased susceptibility of a few of the chromosomes to malsegregation when exposed to these agents may be a reflection more of the individual characteristics of these chromosomes than the agents to which they were exposed.

CONCLUSIONS

Displacement of chromosomes within or from the meiotic spindle apparatus may occur as a primary step [125,126] leading to the development of aneuploid gametes, and it appears likely that during meiosis some of the



Figs. 5,6. Panels **5A** and **6A**, respectively, illustrate air-dried first-cleavage metaphase Giemsa-banded chromosome spreads from ethanol-induced single-pronuclear haploid parthenogenones, which display either a normal chromosome constitution (n = 20, **5A**) or a hyperhaploid chromosome constitution (n = 21, **6A**). Panels **5B** and **6B** illustrate karyograms



of the chromosome spreads illustrated in panels **5A** and **6A**, respectively. Whereas the karyogram illustrated in panel **5B** is that of a normal euploid haploid parthenogenone in which each of the 19 autosomes and an X chromosome are seen to be present, that illustrated in panel **6B** is of an aneuploid haploid parthenogenone that is disomic for chromosome 14.

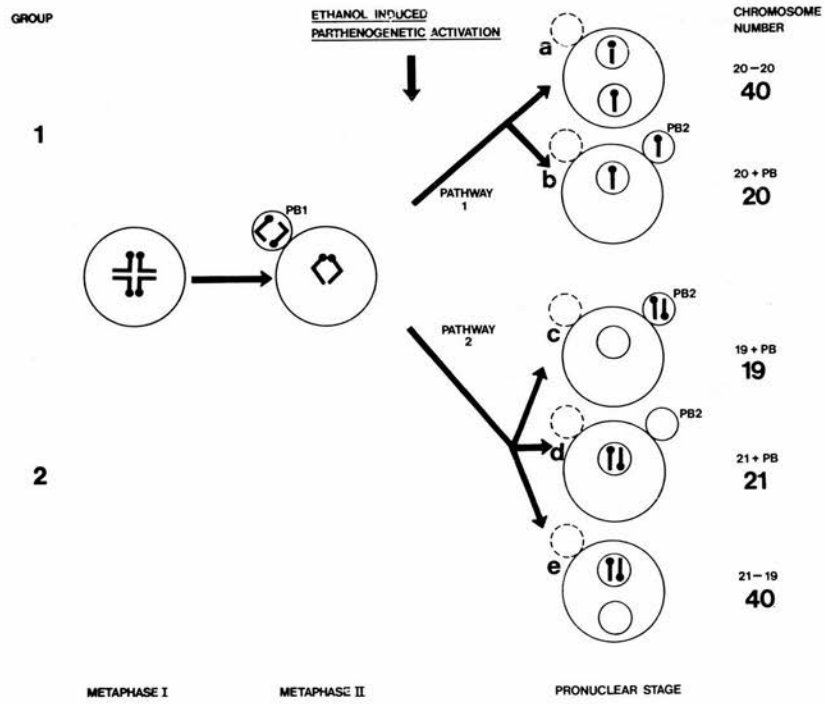


Fig. 7. Development of ethanol-induced two-pronuclear and one-pronuclear parthenogenones following *in vitro* exposure of normal secondary oocytes to a dilute solution of ethanol. Pathways 1a and 1b illustrate the situations observed when normal segregation of chromosomes occurs, with the formation of euploid diploid and haploid parthenogenones, respectively. In the eggs that develop along pathways 2c and 2d, exposure to ethanol has induced chromosomal nondisjunction to occur during the second meiotic division, and results in the formation of aneuploid haploid parthenogenones with complements of 19 and 21 chromosomes, respectively. The chromosomes in the egg that had developed along pathway 2e have also undergone nondisjunction. However, since both products of the second meiotic division have been retained within the egg, its resultant chromosome constitution is diploid and euploid. One pronucleus is hypohaploid, and the other is hyperhaploid, the two complementing each other. Its subsequent development would be as a normal euploid diploid embryo. (See legend below Fig. 3.)

smaller chromosomes of the genome may be displaced more readily than their longer partners. Equally, the increased susceptibility of certain chromosomes to displacement from the spindle may be related to the proportion of heterochromatin present [127]. Ford and Lester [127] have therefore proposed that under normal circumstances any tendency towards displacement may be counterbalanced by the "stabilizing" activity of the microtu-

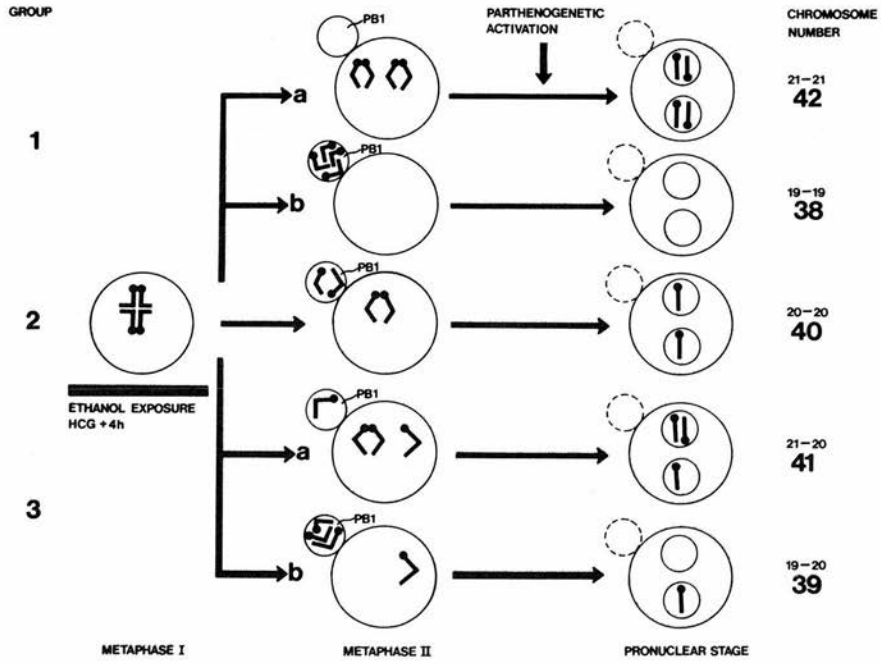


Fig. 8. Illustration of one technique that can be used to analyze the various events that may occur following exposure to ethanol during the first meiotic division. In this approach, first-cleavage chromosome preparations of two-pronuclear parthenogenones are analyzed following *in vitro* exposure to hyaluronidase, an activating stimulus that does not induce chromosomal malsegregation to occur. The parthenogenones shown in group 2 develop as normal euploid diploids, having in each nucleus one euploid haploid set of chromosomes. In the parthenogenones that develop along pathways 1a and 1b, nondisjunction induced by *in vivo* exposure to ethanol during the first meiotic division results in the formation of activated eggs with complements of 42 and 38 chromosomes, respectively. Alternatively, the secondary oocyte may be hyperdiploid or hypodiploid (pathways 3a and 3b, respectively) if premature centromere division has been induced during the first meiotic division, with the formation of parthenogenones with complements of 41 and 39 chromosomes, respectively. (See legend below Fig. 3.)

bular components of the meiotic spindle apparatus. The possibility exists, therefore, that the disruption of microtubule polymerization that almost certainly occurs in the presence of spindle-active agents such as ethanol and certain anesthetics tends to decrease the chances that stabilization will occur, and consequently increases the incidence of malsegregation of those chromosomes that are already prone to displacement. This hypothesis provides one possible explanation for the nonrandom involvement of certain chromosomes

associated with aneuploidy induced by the administration of ethanol. We believe that the information presented here goes some way towards justifying Ford's [117] depressing observation that "the uneasy suspicion remains that the very high incidence of chromosomal abnormality, particularly non-disjunctional abnormality, in human zygotes may be another 'disease of civilization'". Of the many environmental hazards to which man is exposed, alcohol may turn out to be one of the most insidious and the most dangerous, because of its potential effects on all stages of development from the developing germ cells to the adult organism.

ACKNOWLEDGMENTS

G.T. O'Neill is supported by a Bonnar Scholarship from the Faculty of Medicine, University of Edinburgh. The work is supported by a grant (to M.H.K.) from the National Fund for Research Into Crippling Diseases (Action Research for the Crippled Child), and an equipment grant (to M.H.K.) from the Scottish Home and Health Department (Ref. no K/MRS/50/C866). We would like to thank Dr. E.P. Evans for his advice and assistance with the interpretation of the G-banded chromosome preparations.

REFERENCES

1. Colangelo W, Jones DG: The fetal alcohol syndrome: A review and assessment of the syndrome and its neurological sequelae. Prog Neurobiol 19:271, 1982.
2. Lemoine P, Harrousseau M, Borteyru JP, Menuet JC: Les enfants de parents alcoöliques. Anomalies observées. A propos de 127 cas. Quest Med 21:476, 1968.
3. Newman NM, Correy JF: Effects of alcohol in pregnancy. Med J Aust 2:5, 1980.
4. Pratt OE: Alcohol and the developing fetus. Br Med Bull 38:48, 1982.
5. Ulleland CN: The offspring of alcoholic mothers. Ann NY Acad Sci 197:167, 1972.
6. Editorial: Alcohol and the fetus—Is zero the only option? Lancet 1:682, 1983.
7. Brown NA, Goulding EH, Fabro S: Ethanol embryotoxicity: Direct effects on mammalian embryos in vitro. Science 206:573, 1979.
8. Randall CL, Riley EP: Prenatal alcohol exposure: Current issues and the status of animal research. Neurobehav Toxicol Teratol 3:11, 1981.
9. Sulik KK, Johnston MC: Sequence of developmental alterations following acute ethanol exposure in mice: Craniofacial features of the fetal alcohol syndrome. Am J Anat 166:257, 1983.
10. O'Shea KS, Kaufman MH: The teratogenic effect of acetaldehyde: Implications for the study of the fetal alcohol syndrome. J Anat 128:65, 1979.
11. O'Shea KS, Kaufman MH: Effect of acetaldehyde on the neuroepithelium of early mouse embryos. J Anat 132:107, 1981.
12. Kaufman MH: The chromosome complement of single-pronuclear haploid mouse embryos following activation by ethanol treatment. J Embryol Exp Morphol 71:139, 1982.

13. Kaufman MH: Ethanol-induced chromosomal abnormalities at conception. Nature, Lond 302:258, 1983.
14. Kaufman MH, Bain IM: Influence of ethanol on chromosome segregation during the first and second meiotic divisions in the mouse egg. J Exp Zool 230:315, 1984.
15. Kaufman MH, Bain IM: The development potential of ethanol-induced monosomic and trisomic conceptuses in the mouse. J Exp Zool 231:149, 1984.
16. O'Neill GT, Kaufman MH: Cytogenetic analysis of first cleavage fertilized mouse eggs following *in vivo* exposure to ethanol shortly before and at the time of conception. Development 100:441, 1987.
17. Kaufman MH: The influence of alcohol on female germ cells. BioEssays 1:117, 1984.
18. Kaufman MH: An hypothesis regarding the origin of aneuploidy in man: Indirect evidence from an experimental model. J Med Genet 22:171, 1985.
19. Magnuson T: Genetic abnormalities and early mammalian development. In Johnson MH (ed): "Development in Mammals," Vol 5. Amsterdam: Elsevier, 1983, p 209.
20. Epstein CJ: The mouse trisomies. Experimental systems for the study of aneuploidy. In H Kalter (ed): "Issues and Reviews in Teratology," Vol. 3, New York: Plenum, 1985, p 171.
21. Dyban AP, Khozhai LI: Parthenogenetic development of ovulated mouse ova under the influence of ethyl alcohol. Bull Exp Biol Med 89:528, 1980.
22. Cuthbertson KSR: Parthenogenetic activation of mouse oocytes *in vitro* with ethanol and benzyl alcohol. J Exp Zool 226:311, 1983.
23. Kaufman MH: "Early Mammalian Development: Parthenogenetic Studies." Cambridge: Cambridge University Press, 1983.
24. Röhrborn G: Frequencies of spontaneous non-disjunction in metaphase II oocytes of mice. Hum Genet 16:123, 1972.
25. Hansmann I, El-Nahaas E: Incidence of nondisjunction in mouse oocytes. Cytogenet Cell Genet 24:115, 1979.
26. Donahue RP: Cytogenetic analysis of the first cleavage division in mouse embryos. Proc Natl Acad Sci USA 69:74, 1972.
27. Kaufman MH: Analysis of the first cleavage division to determine the sex-ratio and incidence of chromosome anomalies at conception in the mouse. J Reprod Fertil 35:67, 1973.
28. Bond DJ, Chandley AC: "Aneuploidy." Oxford: Oxford University Press, 1983.
29. McGaughey RW, Chang MC: Inhibition of fertilization and production of heteroploidy in eggs of mice treated with colchicine. J Exp Zool 171:465, 1969.
30. Sugawara S, Mikamo K: An experimental approach to the analysis of mechanisms of meiotic nondisjunction and anaphase lagging in primary oocytes. Cytogenet Cell Genet 28:251, 1980.
31. Hummler E, Hansmann I: Preferential nondisjunction of specific bivalents in oocytes from Djungarian hamsters (Phodopus sungorus) following colchicine treatment. Cytogenet Cell Genet 39:161, 1985.
32. Tease C, Fisher G: Oocytes from young and old female mice respond differently to colchicine. Mutat Res 173:31, 1986.
33. Danford N, Parry JM: Induction of sex chromosome aneuploidy in mice by vincristine sulphate. IRCS Med Sci 14:15, 1986.
34. Ferguson FC Jr: Colchicine: I. General pharmacology. J Pharmacol Exp Ther 106:261, 1952.
35. Balek RW, Kocsis JJ, Geiling EM: Potentiation of several hynotic and anaesthetic agents by colchicine. Arch Int Pharmacodynam Ther 111:182, 1957.

36. Figueroa ML, Vig BK: Sequence of centromere separation: Lack of Colcemid effect on the Chinese hamster genome. Cytogenet Cell Genet 36:627, 1983.
37. Vickers AD: Delayed fertilization and chromosomal anomalies in mouse embryos. J Reprod Fertil 20:69, 1969.
38. Niemierko A: Induction of triploidy in the mouse by cytochalasin B. J Embryol Exp Morphol 34:279, 1975.
39. Maro B, Johnson MH, Pickering SJ, Flach G: Changes in actin distribution during fertilization of the mouse egg. J Embryol Exp Morphol 81:211, 1984.
40. Karasiewicz J, Soltynska MS: Effects of cytochalasin B on the cleavage furrow in mouse blastomeres. Roux's Arch Dev Biol 195:137, 1986.
41. Webb M, Howlett SK, Maro B: Parthenogenesis and cytoskeletal organization in ageing mouse eggs. J Embryol Exp Morphol 95:131, 1986.
42. Eichenlaub-Ritter U, Chandley AC, Gosden RG: Alterations to the microtubular cytoskeleton and increased disorder of chromosome alignment in spontaneously ovulated mouse eggs aged in vivo: An immunofluorescence study. Chromosoma 94:337, 1986.
43. Barilyak IR, Kozachuk SY: Effects of ethanol on the genetic apparatus of mammalian germ cells. Cytol Genet 15:24, 1981.
44. Alvarez MR: Numerical chromosome variation in mouse spermatogonia resulting from alcohol consumption. J Hered 74:58, 1983.
45. Alvarez MR: Aneuploidy in male germ cells induced by alcohol ingestion. Gamete Res 12:165, 1985.
46. Hunt P: Ethanol-induced aneuploidy in male germ cells of the mouse. Cytogenet Cell Genet 47:7, 1987.
47. Speed RM: The effects of ageing on the meiotic chromosomes of male and female mice. Chromosoma 64:241, 1977.
48. Speed RM, Chandley AC: Meiosis in the foetal mouse ovary II, Oocyte development and age related aneuploidy. Does a production line exist? Chromosoma 88:184, 1983.
49. Sugawara S, Mikamo K: Absence of correlation between univalent formation and meiotic nondisjunction in aged female Chinese hamsters. Cytogenet Cell Genet 35:34, 1983.
50. Sugawara S, Mikamo K: Maternal ageing and nondisjunction: A comparative study of two chromosomal techniques on the formation of univalents in the first meiotic metaphase oocytes of the mouse. Chromosoma 93:321, 1986.
51. Liang JC, Sherron DA, Johnston D: Lack of correlation between mutagen-induced chromosomal univalency and aneuploidy in mouse spermatocytes. Mutat Res 163:285, 1986.
52. Badr FM, Badr RS: Induction of dominant lethal mutation in male mice by ethyl alcohol. Nature Lond 253:134, 1975.
53. Badr FM, Badr RS, Asker RL, Hussain FH. Evaluation of the mutagenic effects of ethyl alcohol by different techniques. In Gross MM (ed): "Alcohol Intoxication and Withdrawal, Vol IIIa: Biological Aspects of Ethanol." New York: Plenum, 1977, p 25.
54. Obe G, Ristow H, Herha J: Effect of ethanol on chromosomal structure and function. In Majchrowicz E, Noble EP (eds): "Biochemistry and Pharmacology of Ethanol," 1979, p 659.
55. de Torok D: Chromosomal irregularities in alcoholics. Ann NY Acad Sci 197:90, 1972.
56. Obe G, Ristow H, Herha J: Chromosomal damage by alcohol *in vitro* and *in vivo*. In Gross MM (ed): "Alcohol Intoxication and Withdrawal, Vol IIIa: Biological Aspects of Ethanol." New York: Plenum, 1977, p 47.
57. Obe G, Ristow H: Mutagenic, cancerogenic and teratogenic effects of alcohol. Mutat Res 65:229, 1979.

58. Bregman AA: Cytogenetic effects of ethanol in human leukocyte cultures. Environ Mutat Soc Newsl 4:35, 1971.
59. Alvarez MR, Cimino LE, Cory MJ, Gordon RE: Ethanol induction of sister chromatid exchanges in human cells in vitro. Cytogenet Cell Genet 27:66, 1980.
60. Obe G, Ristow H: Acetaldehyde, but not ethanol, induces sister chromatid exchanges in Chinese hamster cells *in vitro*. Mutat Res 56:211, 1977.
61. Obe G, Jonas R, Schmidt S: Metabolism of ethanol *in vitro* produces a compound which induces sister chromatid exchanges in human peripheral lymphocytes *in vitro*. Acetaldehyde not ethanol is mutagenic. Mutat Res 174:47, 1986.
62. Rieger R, Michaelis A: Chromosomenaberrationen nach Einwirkung von Acetaldehyd auf Primärwurzeln von *Vicia faba*. Biol Zbl 79:1, 1960.
63. Sax K, Sax HJ: Radiomimetic beverages, drugs and mutagens. Proc Natl Acad Sci USA 55:1431, 1966.
64. Sax K, Sax JH: Radiomimetic beverages and drugs. Science 152:676, 1966.
65. Andersen NB: The effect of CNS depressants on mitosis. Acta Anaesth Scand Suppl 22:1, 1966.
66. Fink BR: Effect of anaesthesia on cell division. In Gray TC, Nunn JF (eds): "General Anaesthesia, Vol 1: Basic Sciences." London: Butterworths, 1971, pp 42.
67. Östergren G: An efficient chemical for the induction of sticky chromosomes. Hereditas 30:213, 1944.
68. Allison AC, Nunn JF: Effects of general anaesthetics on microtubules. A possible mechanism of anaesthesia. Lancet 2:1326, 1968.
69. Jackson SH: Anesthetics and cell multiplication. Clin Anesth 11:75, 1975.
70. Hinkley RE, Samson FE: Anesthetic-induced transformation of axonal microtubules. J Cell Biol 53:258, 1972.
71. Hinkley RE, Telser AG: The effects of halothane on cultured mouse neuroblastoma cells. I. Inhibition of morphological differentiation. J Cell Biol 63:531, 1974.
72. Brinkley BR, Rao PN: Nitrous oxide: Effects on the mitotic apparatus and chromosome movement in HeLa cells. J Cell Biol 58:96, 1973.
73. Cox SM, Rao PN, Brinkley BR: Differential effects of nitrous oxide on the spindle and cytoplasmic microtubule complex. J Cell Biol 75:291a, 1977.
74. Kusyk CJ, Hsu TC: Mitotic anomalies induced by three inhalation halogenated anesthetics. Environ Res 12:366, 1976.
75. Coate WB, Kapp RW, Lewis TR: Chronic exposure to low concentrations of halothane–nitrous oxide: Reproductive and cytogenetic effects in the rat. Anesthesiology 50:310, 1979.
76. Kaufman MH: Parthenogenetic activation of mouse oocytes following avertin anaesthesia. J Embryol Exp Morphol 33:941, 1975.
77. Kaufman MH: Effect of anaesthetic agents on eggs and embryos. In Johnson MH (ed): "Development in Mammals, Vol 1." Amsterdam: North-Holland, 1977, p 137.
78. Harlap S, Shiono PH: Alcohol, smoking, and incidence of spontaneous abortions in the first and second trimester. Lancet 2:173, 1980.
79. Kline J, Shrout P, Stein Z, Susser M, Warburton D: Drinking during pregnancy and spontaneous abortion. Lancet 2:176, 1980.
80. American Society of Anesthesiologists: Occupational disease among operating room personnel: A national survey. Anesthesiology 41:321, 1974.
81. Askrog V, Harvald B: Teratogen effekt af inhalationsanaestetika. Nord Med 83:498, 1970.
82. Knill-Jones RP, Rodrigues LV, Moir DD, Spence AA: Anaesthetic practice and

pregnancy. Controlled survey of women anaesthetists in the United Kingdom. Lancet 1:1326, 1972.
83. Vaisman AI: Working conditions in surgery and their effect on the health of anesthesiologists. Eksp Khir Anesteziol 12:44, 1967 [English translation].
84. Hassold TJ: Chromosome abnormalities in human reproductive wastage. Trends Genet 2:105, 1986.
85. Carr DH: Chromosome abnormalities and spontaneous abortions. In Jacobs PA, Price WH, Law P (eds): "Human Population Cytogenetics." Edinburgh: Edinburgh University Press, 1970, p 103.
86. Carr DH: Chromosomes and abortion. Adv Hum Genet 2:201, 1971.
87. Boué J, Boué A: Anomalies chromosomiques dans les avortements spontanés. In Boué A, Thibault C (eds): "Les accidents chromosomiques de la reproduction." Paris: Institut National de la Santé et de la Recherche Médicale, 1973, p 29.
88. Boué JG, Boué A: Chromosomal anomalies in early spontaneous abortion. Their consequences on early embryogenesis and in vitro growth of embryonic cells. Curr Top Pathol 62:193, 1976.
89. Therkelsen AJ, Grunnet N, Hjort T, Jensen OM, Jonasson J, Lauritsen JG, Lindsten J, Petersen GB: Studies on spontaneous abortions. In Boué A, Thibault C (eds): "Les accidents chromosomiques de la reproduction." Paris: Institut National de la Santé et de la Recherche Médicale, 1973, p 81.
90. Hassold TJ, Matsuyama A: Origin of trisomies in human spontaneous abortions. Hum Genet 46:285, 1979.
91. Hassold TJ, Chen N, Funkhouser J, Jooss T, Manuel B, Matsuura J, Matsuyama A, Wilson C, Yamane JA, Jacobs PA: A cytogenetic study of 1,000 spontaneous abortions. Ann Hum Genet 44:151, 1980.
92. Hassold TJ, Jacobs P, Kline J, Stein Z, Warburton D: Effect of maternal age on autosomal trisomies. Ann Hum Genet 44:29, 1980.
93. Chandley AC: The origin of aneuploidy. In Bonné-Tamir B, (ed), Cohen T, Goodman RM (assoc eds): "Human Genetics, Part B: Medical Aspects." New York: Alan R. Liss, 1982, p 337.
94. Lin CC, De Braekeleer M, Jamro H: Cytogenetic studies in spontaneous abortion: The Calgary experience. Can J Genet Cytol 27:565, 1985.
95. Roberts CJ, Lowe CR: Where have all the conceptions gone? Lancet 1:498, 1975.
96. Miller JF, Williamson E, Glue J, Gorden YB, Grudzinskas JG, Sykes A: Fetal loss after implantation. A prospective study. Lancet 2:554, 1980.
97. Edmonds DK, Lindsay KS, Miller JF, Williamson E, Wood PJ: Early embryonic mortality in women. Fertil Steril 38:447, 1982.
98. Whittaker PG, Taylor A, Lind T: Unsuspected pregnancy loss in healthy women. Lancet 1:1126, 1983.
99. Grudzinskas JG, Nysenbaum AM: Failure of human pregnancy after implantation. In Seppälä M, Edwards RG (eds): "*In vitro* Fertilization and Embryo Transfer." New York: New York Academy of Sciences, 1985, p 38.
100. Rudak E, Jehoshua D, Mashiach S, Nevel L, Goldman B: Chromosome analysis of human oocytes and embryos fertilized *in vitro*. In Seppälä M, Edwards RG (eds): "*In Vitro* Fertilization and Embryo Transfer." New York: New York Academy of Sciences, 1985, p 476.
101. Wramsby H, Fredga K, Liedholm P: Chromosome analysis of human oocytes recovered from preovulatory follicles in stimulated cycles. N Engl J Med 316:121, 1987.

102. Angell RR, Aitken RJ, van Look PFA, Lumsden MA, Templeton AA: Chromosome abnormalities in human embryos after *in vitro* fertilization. Nature Lond 303:336, 1983.
103. Angell RR, Aitken RJ, van Look PFA, Lumsden MA, Templeton AA: Chromosome anomalies in human preimplantation embryos. J Embryol Exp Morphol 82(suppl:193), 1984 (Abstract).
104. Angell RR, Templeton AA, Aitken, RJ: Chromosome studies in human *in vitro* fertilization. Hum Genet 72:333, 1986.
105. Hassold T, Chiu D: Maternal age-specific rates of numerical chromosome abnormalities with special reference to trisomy. Hum Genet 70:11, 1985.
106. Jacobs PA, Hassold TJ: The origin of chromosome abnormalities in spontaneous abortions. In Porter IH, Hook EB (eds): "Human Embryonic and Fetal Death." New York: Academic, 1980, pp 289.
107. Magenis RE, Overton KM, Chamberlin J, Brady T, Lovrien E: Parental origin of the extra chromosome in Down's syndrome. Hum Genet 37:7, 1977.
108. Mikkelsen M, Hallberg A, Poulsen H: Maternal and paternal origin of extra chromosome in trisomy 21. Hum Genet 32:17, 1976.
109. Mikkelsen M, Poulsen H, Grinsted J, Lange A: Non-disjunction in trisomy 21. Study of chromosomal heteromorphisms in 110 families. Ann Hum Genet 44:17, 1980.
110. Henderson SA, Edwards RG: Chiasma frequency and maternal age in mammals. Nature Lond 218:22, 1968.
111. Polani PE, Jagiello GM: Chiasmata, meiotic univalents, and age in relation to aneuploid imbalance in mice. Cytogenet Cell Genet 16:505, 1976.
112. Speed RM: Meiosis in the foetal mouse ovary. I. An analysis at the light microscope level using surface spreading. Chromosoma 85:427, 1982.
113. Szöllösi D: Morphological changes in mouse eggs due to aging in the fallopian tube. Am J Anat 130:209, 1971.
114. Gosden RG: Chromosome anomalies of pre-implantation mouse embryos in relation to maternal age. J Reprod Fertil 35:351, 1973.
115. Brook JD, Gosden RG, Chandley AC: Maternal ageing and aneuploid embryos—Evidence from the mouse that biological and not chronological age is the important influence. Hum Genet 66:41, 1984.
116. Donahue RP, Karp LE: Chromosomal anomalies after fertilization of aged, post-ovulatory mouse oocytes. Am J Hum Genet 25:24a, 1983.
117. Ford CE: The time in development at which gross genome unbalance is expressed. In Balls M, Wild AE (eds): "The Early Development of Mammals." Cambridge: Cambridge University Press, 1975, pp 285.
118. Phillipe E, Boué JF: Placenta et aberrations chromosomiques au cours des avortements spontanés. Présse Méd 78:641, 1970.
119. Martin-De Leon PA, Boice ML: Spontaneous heteroploidy in one-cell mouse embryos. Cytogenet Cell Genet 35:57, 1983.
120. O'Neill GT, Kaufman MH: Cytogenetic analysis of first cleavage parthenogenetic mouse eggs following *in vivo* and in vitro exposure to ethanol. Manuscript in preparation.
121. Linder D, McCaw BK, Hecht F: Parthenogenetic origin of benign ovarian teratomas. N Engl J Med 292:63, 1975.
122. Kaufman MH: Chromosome abnormalities. Nature Lond 304:482, 1983.
123. Plachot M, Mandelbaum J, de Grouchy J: La parthénogenèse in vitro dans l'espèce humaine. Ann Genet (Paris) 27:158, 1984.
124. Phillips RJS, Kaufman MH: Bare-patches, a new sex-linked gene in the mouse associated

with a high production of XO females. II. Investigations into the nature and mechanisms of the XO production. Genet Res 24:27, 1974.
125. Ford JH, Roberts C: Displacement of chromosomes in mitosis: A technique for assessing differential chromosome error. Cytogenet Cell Genet 36:537, 1983.
126. Ford JH, Correll AT, Llewelyn M, Stirling JW: A perspective of chromosome displacement as a primary step in the induction of aneuploidy. Cytobios 43:213, 1985.
127. Ford JH, Lester P: Factors affecting the displacement of human chromosomes from the metaphase plate. Cytogenet Cell Genet 33:327, 1982.

THE JOURNAL OF EXPERIMENTAL ZOOLOGY 248:125-131 (1988)

RAPID COMMUNICATION

Influence of Postovulatory Aging on Chromosome Segregation During the Second Meiotic Division in Mouse Oocytes: A Parthenogenetic Analysis

G.T. O'NEILL AND M.H. KAUFMAN
Department of Anatomy, University Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland

ABSTRACT A high incidence of parthenogenetic activation was observed when postovulatory aged mouse oocytes were exposed briefly to hyaluronidase in culture medium at 18–26 h after the human chorionic gonadotropin injection for inducing superovulation. The majority of the activated oocytes extruded a second polar body and developed a single haploid pronucleus. Cytogenetic analysis of this class of parthenogenone at metaphase of the first-cleavage mitosis has clearly demonstrated that the completion of the second meiotic division in activated aged oocytes is not associated with a significant increase in the incidence of chromosome segregation errors. The increasing postovulatory age of oocytes prior to activation was observed to significantly decrease the capacity of activated oocytes to extrude the second polar body.

In young mature female mice maintained under controlled environmental conditions, the spontaneous ovulation of oocytes occurs at about the midpoint of the dark cycle during proestrus (Braden, '57). When female mice at this stage of the estrous cycle are caged with proven fertile males it has been observed that mating approximately coincides with the expected time of ovulation. This behaviour therefore ensures that freshly ovulated oocytes are fertilized by recently capacitated sperm.

Numerous studies involving a variety of mammalian species have demonstrated that postovulatory aging of oocytes prior to fertilization, termed delayed mating, was responsible for a significant decrease in the number of live-born offspring (reviewed Austin, '70). Delayed fertilization in vivo increases the incidence of dispermic and digynic triploidy (Vickers, '69; reviewed Austin, '70) and facilitates spontaneous parthenogenesis in a small proportion of aging oocytes (Marston and Chang, '64; reviewed Austin, '70; Kaufman, '83). Studies involving in vitro fertilization have also shown that an increase in the postovulatory age of an oocyte increases the incidence of polyspermy and digynic triploidy, and it is also associated with a reduction in embryonic development beyond the pronuclear stage of the first cell cycle (Juetten and Bavister, '83; Smith and Lodge, '87).

Ultrastructural analyses of ovulated mouse oocytes have indicated that postovulatory aging induces changes in the tangential orientation and cortical location of the second meiotic spindle (Szöllösi, '71, '75). Degenerative changes in the organization of both the cytoskeleton and the second meiotic spindle apparatus have also been observed in aged ovulated oocytes by a variety of immunocytochemical techniques (Webb et al., '86; Eichenlaub-Ritter et al., '87). It has been proposed that these degenerative changes may be associated with an increase in the incidence of chromosome segregation errors during the completion of the second meiotic division and that the limited embryonic potential of a proportion of fertilized aged oocytes may be due, in part, to their aneuploid chromosome constitution (Eichenlaub-Ritter et al., '87).

A preliminary analysis of the chromosome constitution of one-cell mouse embryos that developed in vivo following delayed mating (Donahue and Karp, '73) indicated that postovulatory aging prior to fertilization did not appear to be associated with a significant increase in the incidence of aneuploidy. However, an increase in the incidence of parthenogenetic activation was observed.

A high proportion of ovulated oocytes that have aged in vivo for several hours may also initiate parthenogenetic development when briefly incubated in tissue culture medium containing hyal-

TABLE 1. Chromosome constitution of single pronuclear haploid parthenogenones (1PN) at metaphase of the first cleavage division

Group	Postovulatory age (h)[1]	No. of chromosome spreads	No. not analysable	Chromosome constitution					Frequency of aneuploidy (%)[2]
				19	20	21	22	24	
1	18	83	10	0	71	1	1	0	2.7
2	20	81	6	2	72	1	0	0	4.0
3	22	94	12	0	79	2	0	1	3.7
4	24	75	4	1	69	1	0	0	2.8
5	26	80	8	2	70	1	0	0	4.1

[1]Time of isolation of oocytes after HCG injection. Ovulation occurs at about HCG + 11–12 h.
[2]Group 1: the incidence of aneuploidy is not significantly different from group 5: $\chi^2 = 0.2$, $P = .7–.5$.

TABLE 2. Chromosome constitution of fertilized freshly ovulated oocytes from (C57BL × CBA)F1 hybrid mice analysed at metaphase of the first cleavage mitosis

Group	No. of chromosome spreads	No. not analysable	Chromosome No.			Triploid	Adjusted percentage of aneuploidy excluding triploidy[1]
			19:20	20:20	21:20		
1	364	51	8	298	2	5	1.3

[1]The adjusted incidence of aneuploidy in Table 2 group 1 does not differ significantly from the total incidence of aneuploidy in Table 3 groups 1–5; $\chi^2 = 3.2$, $P = .1–.05$.

uronidase (Kaufman, '73, '83). A high proportion of these parthenogenones extrude a second polar body and subsequently develop a single haploid pronucleus. In the present study, this group of haploid parthenogenones has been analysed at metaphase of the first cleavage division in order to establish whether increasing postovulatory age predisposes the completion of the second meiotic division to errors in chromosome segregation. The experimental approach employed in the present study enables the precise postovulatory age of the oocyte at the time of activation to be controlled, it excludes the possibility of recording sperm-derived aneuploidy, and furthermore it removes the necessity of separately accounting for both sperm-mediated and parthenogenetic pathways of development.

MATERIALS AND METHODS

Hyaluronidase-induced parthenogenesis

Ten- to 12-week old (C57BL × CBA) F1 hybrid female mice were superovulated with 5 IU of pregnant mares' serum gonadotrophin (PMSG) followed 48 h later by 5 IU of human chorionic gonadotropin (HCG). At 18, 20, 22, 24, and 26 h after the HCG administration the mice were killed by cervical dislocation. Their oviducts were dissected out and placed in M16 embryo culture medium supplemented with 4 mg/ml of bovine serum albumin (BSA) (Whittingham, '71) previously equilibrated overnight in an atmosphere of 5% CO_2 in air at 37°C. The cumulus masses containing the ovulated oocytes were released from the ampullary region of the oviduct and transferred to microdrops of the culture medium containing 3 mg/ml of hyaluronidase (Sigma: sheep testis type II). The oocytes were incubated in this medium for 15 min, washed three times in hyaluronidase-free medium, and then left in culture for 6 h. At the end of this period, most of the activated oocytes had extruded a second polar body and developed a single haploid pronucleus (1PN + 2PB). Other parthenogenetically activated oocytes failed to extrude a second polar body and developed either two haploid pronuclei (2PN) or a single diploid pronucleus (1PND). Yet others underwent immediate cleavage (IC) in which two equal-sized blastomeres developed, each of which contained a single haploid pronucleus (see Kaufman, '83). Only the single pronuclear haploid parthenogenones were studied further: they were isolated and transferred to fresh microdrops of culture medium. Ten hours after activation the single pronuclear haploid eggs were transferred to microdrops of culture medium containing colcemid (1 μg/ml) in order to arrest their development at metaphase of the first cleavage division. Chromosome spreads were prepared by the air-drying technique described by Tarkowski ('66) and stained in 3% Giemsa at pH 6.8. Only one preparation was made on each slide, and the chromosome constitution of each preparation was determined under the oil immersion objective of a Leitz Orthoplan microscope.

Fertilized oocytes

An additional group of F1 hybrid female mice were superovulated as described above and caged individually with F1 hybrid males of proven fertility. Early the next morning the females were examined for the presence of a vaginal plug, and this was taken as evidence that mating had occurred. At 6–8 h after the expected time of fertilization, the females were killed, their oviducts were isolated, and their cumulus masses were released and transferred to microdrops of culture medium containing 0.5 mg/ml of hyaluronidase for 2–4 min. The cumulus-cell-free fertilized oocytes had generally extruded a second polar body and possessed two pronuclei. The eggs were washed in three changes of hyaluronidase-free culture medium and then left in culture for a further 5–6 h. The fertilized oocytes were then transferred to culture medium containing 1 μg/ml of colcemid in order to arrest them at metaphase of the first cleavage division. Chromosome spreads of the fertilized oocytes were prepared and analysed as described earlier.

RESULTS

Chromosome constitution of single pronuclear haploid parthenogenones

The cytogenetic analysis of the single-pronuclear haploid parthenogenones that developed following exposure to hyaluronidase at 18, 20, 22, 24, and 26 h after the HCG injection is presented in Table 1. The incidence of aneuploidy observed in these various groups was 2.7, 4.0, 3.7, 2.8, and 4.1% respectively. These values did not differ significantly from each other and clearly demonstrate that no increase is the incidence of aneuploidy occurs in association with the increasing postovulatory age of the oocyte at the time of activation. In the majority of the aneuploid chromosome preparations (84.6%) this involved the loss or gain of only one chromosome. The overall ratio of hypohaploidy to hyperhaploidy was 1:1.6. Representative euploid and aneuploid chromosome spreads from this study are illustrated in Figure 1.

The chromosome constitution of in vivo fertilized oocytes at metaphase of the first cleavage division

The chromosome constitution of freshly superovulated one-cell embryos that developed following fertilization in vivo is presented in Table 2. The relatively higher incidence of hypodiploidy over hyperdiploidy encountered in the present study would seem to indicate that a proportion of these chromosome preparations may have resulted from mechanical loss incurred during the fixation procedure. As it is generally believed that the incidence of hyperdiploid spreads is a more reliable indicator of malsegregation events, the incidence of aneuploidy has been recalculated by using the following formula:

$$\frac{\text{No. of hyperdiploid spreads}}{\text{Total No. of chromosome spreads analysed (excluding triploids)}} \times 2 = \text{Incidence of aneuploidy (adjusted)}$$

According to this formula, 1.3% of one-cell fertilized embryos exhibited an aneuploid chromosome constitution. This value did not differ significantly from the incidence of aneuploidy observed in the hyaluronidase-activated parthenogenones ($P = .1$–$.05$); 1.5% of the fertilized oocytes were found to be triploid.

Effects of postovulatory aging on the incidence of single pronuclear haploid development

The influence of increasing postovulatory age on the activation frequency following exposure to hyaluronidase in vitro is presented in Table 3. The lowest incidence of activation (34.9%) was observed when oocytes were exposed to hyaluronidase at HCG+18 h. A significant increase in the proportion of oocytes that developed parthenogenetically (87.6%) was observed at HCG+20 h, and the highest incidence of activation (95.4%) was achieved when oocytes were exposed to hyaluronidase at HCG+24 h. A slight decrease in the activation frequency was, however, observed when oocytes were isolated at HCG+26 h.

Of the four principal pathways of parthenogenetic development which eggs may take following activation (see Fig. 2), the single pronuclear haploids were the most commonly encountered class in all of the groups studied. A decrease in the incidence of this developmental pathway (from 91.9 to 64.3% of the activated oocytes examined) was observed as the postovulatory age of the oocytes increased from HCG+18 h to HCG+26 h. The decreased incidence of this class was associated with a proportionate increase in the incidence of parthenogenones in which polar body extrusion failed to occur (see Fig. 2). The proportionate incidence of the four main pathways of parthenogenetic development encountered in the different time groups was found to be significant.

DISCUSSION

This study has clearly demonstrated that the exposure of aged oocytes to culture medium containing hyaluronidase induces a high incidence of

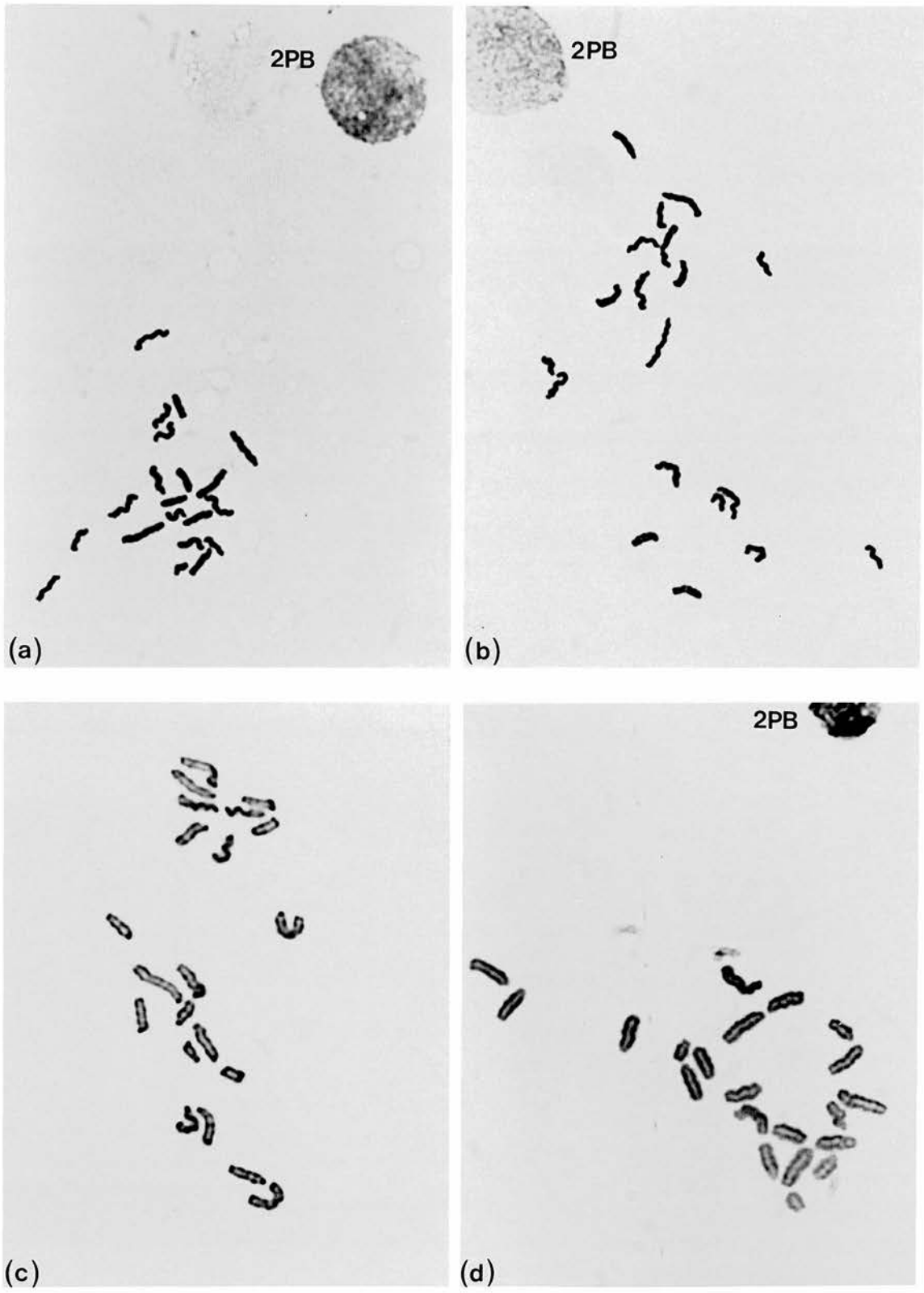


Fig. 1. Representative air-dried chromosome spreads of hyaluronidase-activated single pronuclear haploid parthenogenones at metaphase of the first cleavage mitosis that exhibit **(a)** 20, **(b)** 19, and **(c,d)** 21 chromosomes. The nucleus of the second polar body is visible in (a), (b) and (d).

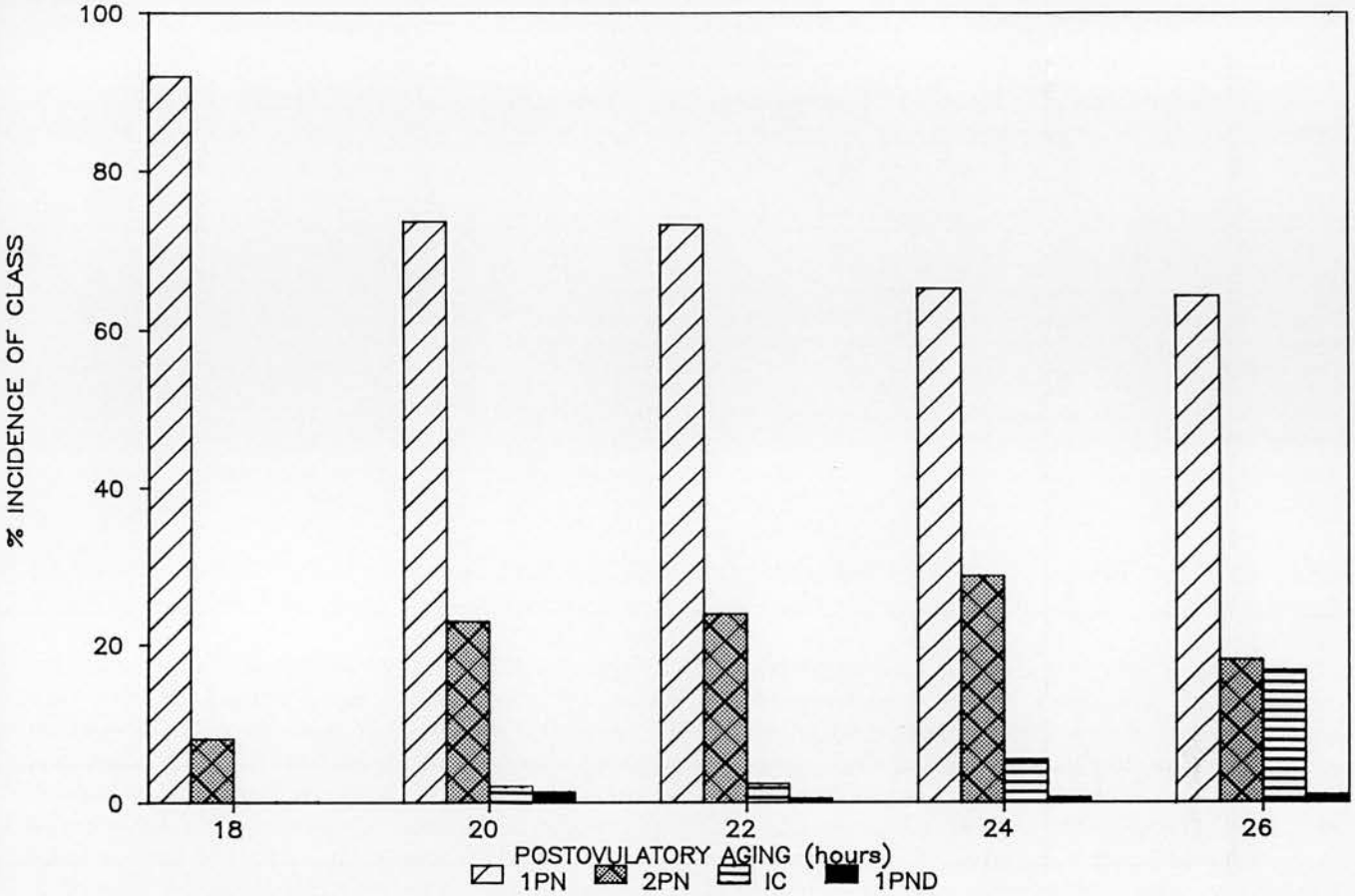


Fig. 2. The proportionate incidence of the four pathways of parthenogenetic development following the hyaluronidase activation of ovulated oocytes at 18–26 h after HCG injection.

parthenogenetic activation. Furthermore, the majority of the activated oocytes develop as single pronuclear haploid parthenogenones. The proportionate incidence of this developmental pathway decreased significantly as the postovulatory age of activated oocytes increased from HCG+18 h to HCG+26 h. This relationship between postovulatory age, parthenogenetic activation, and the subsequent development of several distinct classes of parthenogenone has previously been reported (Kaufman, '73, '83). Ultrastructural analysis of ovulated mouse oocytes (Szöllösi, '71, '75) has revealed that postovulatory aging induces changes in the cortical and tangential location of the second meiotic spindle. It has been suggested that these changes observed in aging oocytes directly influence both the activation frequency and the developmental pathways undertaken by these eggs (Kaufman, '75). Recent immunocytochemical studies of mouse oocytes have also revealed that degenerative changes in the organization of the second meiotic spindle are associated with increased postovulatory age (Eichenlaub-Ritter et al., '87; Webb et al., '86).

Cytogenetic studies of unfertilized ovulated oocytes and one-cell fertilized embryos from several strains of mice (reviewed by Dyban and Baranov, '87) have revealed that the incidence of spontaneous aneuploidy is in the order of 0.2–2.6%. In the present study, the cytogenetic analysis of fertilized one-cell embryos has revealed that the "baseline" incidence of spontaneous aneuploidy was about 1.3% and that the majority of the aneuploid embryos had either lost or gained only one chromosome. The analysis of the single pronuclear haploid parthenogenones revealed that this group had a slightly higher overall incidence of aneuploidy and that aneuploids with a complement of 22 and 24 chromosomes were also occasionally observed. The difference in the incidence of aneu-

TABLE 3. The effect of increasing postovulatory age on the incidence of hyaluronidase-induced parthenogenetic development

Group[1]	Postovulatory age (h)[2]	No. of oocytes	No. of activated oocytes	Activation rate(%)	Class of parthenogenone			
					1PN	2PN	IC	1PND
1	18	284	99	34.9	91	8	0	0
2	20	169	148	87.6	109	34	3	2
3	22	254	209	82.3	153	50	5	1
4	24	175	167	95.4	109	48	9	1
5	26	280	216	77.1	139	39	36	2

[1]Group 1: the incidence of 1PN development is significantly different from group 3 : $\chi^2 = 14.2$, $P > .01$, and group 5 : $\chi^2 = 16.1$, $P > .01$.
Group 3: the incidence of 1PN development is significantly different from group 5 : $\chi^2 = 3.8$, $P > .05$.
Group 4: the frequency of parthenogenetic activation is significantly different from group 2 : $\chi^2 = 6.8$, $P > .01$, and group 3 : $\chi^2 = 16.5$, $P > .01$.
Group 3: the frequency of parthenogenetic activation is not significantly different from group 2 : $\chi^2 = 2.1$, $P = .2–.1$.
[2]See note, Table 1.

ploidy observed between the fertilized and parthenogenetically activated embryos was not significant.

It has been suggested that the degenerative changes that have been observed in the position and alignment of the second meiotic spindle may account for the decrease in the incidence of polar body extrusion in the more aged eggs (Webb et al., '86). Furthermore, Eichenlaub-Ritter et al. ('87) have suggested that delayed fertilization may predispose the aged eggs to an increased incidence of aneuploidy. Such a finding was not observed in the present study, though the developmental pathway undertaken by a proportion of these aged oocytes meant that this group failed to extrude a second polar body.

Chromosome polymorphism studies of live-born trisomy 21 patients (Mikkelsen et al., '80; Juberg and Mowry, '83) have revealed that most aneuploid conceptuses develop as a result of chromosome segregation errors that occur during the first meiotic division in the oocyte. However, 10–20% of aneuploid conceptuses develop following chromosome nondisjunction that occurs in the oocyte during the completion of the second meiotic division. Increased maternal age is thought to be one of the most significant factors underlying the chromosome segregation errors that occur at the first meiotic division of the human oocyte (Hassold and Jacobs, '84), and animal studies have also indicated that an increased incidence of aneuploidy may be encountered in association with advanced *maternal* age (Maudlin and Fraser, '78; Brook et al., '84). It has recently been proposed that exposure to spindle-active agents such as ethanol may also increase the incidence of segregation errors at both the first and, to a lesser extent, the second meiotic division (Kaufman, '85; O'Neill and Kaufman, '87).

Various authors have proposed that the fertilization of *postovulatory aged* human oocytes may increase the incidence of chromosome segregation errors at the second meiotic division and that this may be a significant factor in the origin of human aneuploidy (German, '68; Juberg, '83; Juberg and Mowry, '83). This suggestion does not appear to be consistent with the present findings, however, which have clearly demonstrated that the incidence of aneuploidy in activated *postovulatory aged* mouse oocytes did not differ significantly from that observed in fertilized one-cell mouse embryos. Our results would seem to indicate that delayed fertilization of oocytes probably plays no significant role, or at most only a minor role, in the pathogenesis of human aneuploidy. Postovulatory aging is, however, associated with an increase in the incidence of triploid conceptuses (Kaufman, '88).

ACKNOWLEDGMENTS

G.T. O'Neill is supported by a Bonnar Scholarship from the Faculty of Medicine, University of Edinburgh. The work is supported by a grant (to M.H.K.) from the National Fund for Research into Crippling Diseases (Action Research for the Crippled Child) and an equipment grant (to M.H.K.) from the Scottish Home and Health Department ref. No. K/MRS/50/C866.

LITERATURE CITED

Austin, C.R. (1970) Ageing and reproduction: Postovulatory deterioration of the egg. J. Reprod. Fertil. [Suppl.], *12*:39–53.

Braden, A.W.H. (1957) The relationship between the diurnal light cycle and the time of ovulation in mice. J. Exp. Biol., *34:*177–188.

Brook, J.D., R.G. Gosden, and A.C. Chandley (1984) Maternal ageing in aneuploid embryos—Evidence from the mouse that biological and not chronological age is the important influence. Hum. Genet., *66:*41–45.

Donahue, R.P., and L.E. Karp (1973) Chromosomal anomalies after fertilization of aged, postovulatory mouse eggs. Am. J. Hum. Genet., *25:*24a.

Dyban, A.P., and V.S. Baranov (1987) Cytogenetics of Mammalian Embryonic Development. Oxford University Press, Oxford, pp. 68–91.

Eichenlaub-Ritter, U., A.C. Chandley, and R.G. Gosden (1987) Alterations to the microtubular cytoskeleton and increased disorder of chromosome alignment in spontaneously ovulated mouse oocytes in vivo: An immunofluorescence study. Chromosoma, *94:*337–345.

German, G. (1968) Mongolism, delayed fertilization and human sexual behaviour. Nature, *217:*516–518.

Hassold, T.J., and P.A. Jacobs (1984) Trisomy in man. Annu. Rev. Genet., *18:*69–97.

Juberg, R.C. (1983) Origin of chromosomal anomalies: Evidence for delayed fertilization in meiotic nondisjunction. Hum. Genet., *64:*122–127.

Juberg, R.C., and P.N. Mowry (1983) Origin of nondisjunction in trisomy 21 syndrome; All studies compiled, parental age analysis, and international comparisons. Am. J. Med. Genet., *16:*111–116.

Juetten, J., and B.D. Bavister (1983) Effects of egg aging on *in vitro* fertilization and first cleavage division in the hamster. Gamete Res., *8:*219–230.

Kaufman, M.H. (1973) Parthenogenesis in the mouse. Nature, *242:*475–476.

Kaufman, M.H. (1975) The experimental induction of parthenogenesis in the mouse. In: The Early Development of Mammals. M. Balls and A.E. Wild, eds. Cambridge University Press, Cambridge, pp. 25–44.

Kaufman, M.H. (1983) Early Mammalian Development: Parthenogenetic Studies. Cambridge University Press, Cambridge.

Kaufman, M.H. (1985) An hypothesis regarding the origin of aneuploidy in man: Indirect evidence from an experimental model. J. Med Genet., *22:*171–178.

Kaufman, M.H. (1988) Hydatidiform mole: Genetic and practical implications. Hosp. Update, *14:*1415–1420.

Marston, J.H., and M.C. Chang (1964) The fertilizable life of ova and their morphology following delayed insemination in mature and immature mice. J. Exp. Zool., *155:*237–252.

Maudlin, I., and L.R. Fraser (1978) Maternal age and the incidence of aneuploidy in first-cleavage mouse embryos. J. Reprod. Fertil., *54:*423–426.

Mikkelsen, M., H. Poulsen, J. Grinsted, and A. Lange (1980) Non-disjunction in trisomy 21: Study of chromosomal heteromorphisms in 110 families. Ann. Hum. Genet. (Lond.), *44:*17–27.

O'Neill, G.T., and M.H. Kaufman (1987) Cytogenetic analysis of first cleavage fertilized mouse eggs following *in vivo* exposure to ethanol shortly before and at the time of conception. Development, *100:*441–448.

Smith, A.L., and J.R. Lodge (1987) Interactions of aged gametes: *In vitro* fertilization using *in vitro*-aged sperm and *in vivo*-aged ova in the mouse. Gamete Res., *16:47–56.*

Szöllösi, D. (1971) Morphological changes in mouse eggs due to aging in the fallopian tube. Am. J. Anat., *130:*209–226.

Szöllösi, D. (1975) Mammalian eggs aging in the fallopian tubes. In: Aging Gametes. R.J. Blandau, ed. Karger, Basel, pp. 98–121.

Tarkowski, A.K. (1966) An air-drying method for chromosome preparations from mouse eggs. Cytogenetics, *5:*394–400.

Vickers, A.D. (1969) Delayed fertilization and chromosomal anomalies in mouse embryos. J. Reprod. Fertil., *20:*69–76.

Webb, M., S.K. Howlett, and B. Maro (1986) Parthenogenesis and cytoskeletal organization in ageing mouse eggs. J. Embryol. Exp. Morphol., *95:*131–145.

Whittingham, D.G. (1971) Culture of mouse ova. J. Reprod. Fertil. [Suppl.], *14:*7–21.

THE JOURNAL OF EXPERIMENTAL ZOOLOGY 249:182-192 (1989)

Cytogenetic Analysis of Ethanol-Induced Parthenogenesis

G.T. O'NEILL AND M.H. KAUFMAN
Department of Anatomy, University Medical School, Edinburgh, EH8 9AG, Scotland

ABSTRACT The brief exposure of recently ovulated mouse oocytes to a dilute solution of ethanol in vitro for 1, 3, or 5 min induced a uniform high incidence of parthenogenetic activation. The majority of parthenogenones developed a single haploid pronucleus after the extrusion of a second polar body. The proportionate incidence of this parthenogenetic class was significantly reduced as the duration of ethanol exposure increased from 1 min to 5 min. There was a concomitant increase in the incidence of parthenogenones that developed two haploid pronuclei following failure of extrusion of the second polar body. Cytogenetic analysis of the ethanol-induced single-pronuclear haploid parthenogenones at metaphase of the first cleavage division clearly demonstrated that a significant proportion were aneuploid. The incidence of aneuploidy observed was directly related to the duration of ethanol exposure. G-band analysis of the aneuploid metaphases revealed that the chromosomes were not randomly involved in the malsegregation events. This observation may be a reflection of the relationship of particular chromosomes to the meiotic spindle apparatus rather than on any specific property of the agent to which they were exposed. It is believed that ethanol disrupts the organisation of cytoskeletal elements and, in particular, interferes with the processes of chromosome segregation at the second meiotic division.

Ovulated mouse oocytes exhibit a high incidence of parthenogenetic activation after in vitro exposure to a dilute solution of ethanol. Several distinct classes of parthenogenone may be identified several hours after the completion of the second meiotic division (Kaufman, '82). The incidence of spontaneous nondisjunction, which may occur at either the first or second meiotic divisions of mouse oocytes, is very low (Rohrborn, '72; Donahue, '72; Kaufman, '73a; Hansmann and El-Nahaas, '79; reviewed Dyban and Baranov, '87). However, analysis of the chromosome constitution of ethanol-activated single-pronuclear haploid parthenogenones (1PN) at metaphase of the first cleavage division revealed that a significant number had an abnormal chromosome constitution (Kaufman, '82, '83a). Previous cytogenetic studies have demonstrated that parthenogenetic activation per se does not induce chromosome malsegregation during the second meiotic division (Kaufman, '83a; O'Neill and Kaufman, '88).

Intragastric administration of a dilute solution of ethanol to female mice also may induce a significant increase in the incidence of chromosome malsegregation when exposure to this agent occurs during either the first or second meiotic divisions (Kaufman, '83a,b, '85; Kaufman and Bain, '84; O'Neill and Kaufman, '87; Kaufman and O'Neill, '88). Preliminary analyses of karyotyped G-banded aneuploid first cleavage mitoses have indicated that the smaller chromosomes of the maternally derived genome were more frequently involved in nondisjunction than were other members of the genome (O'Neill and Kaufman, '87).

It has been postulated that ethanol disorganises the balanced segregation of the meiotic chromosomes by interfering with the normal functioning of certain components of the spindle apparatus. Although there is no direct evidence that ethanol is a spindle-active agent, similar results have been observed when colchicine (McGaughey and Chang, '69; Sugawara and Mikamo, '80; Hummler and Hansmann, '85; Tease and Fisher, '86) and vincristine sulphate (Danford and Parry, '86) were administered to female rodents in low doses. Anaesthetics, which share a number of the physiological properties of ethanol, also disrupt the integrity of microtubules. This is seen when cultured cells and isolated neural tissue are exposed to these agents (Allison et al., '70; Hinkley and Samson, '72; Brinkley and Rao, '73; Hinkley and Telser, '74; Jackson, '75; Cox et al., '77); it has been reported that Avertin (tribromoethanol) induces chromosome malsegregation when mouse oocytes are exposed to

Received June 28, 1988; accepted September 27, 1988.
Address reprint requests to Prof. M.H. Kaufman, Department of Anatomy, University Medical School, Teviot Place, Edinburgh EH89AG, Scotland.

this agent prior to and during meiotic maturation (Kaufman, '77a,b).

The present study has confirmed that both the pathways of parthenogenesis and the incidence of ethanol-induced aneuploidy observed in chromosome spreads prepared from single-pronuclear haploid parthenogenones are directly related to the duration of exposure to this agent. Analysis of the karyotype of G-banded first-cleavage metaphase spreads has demonstrated that the majority of malsegregation events involved the smaller chromosomes of the mouse genome. This investigation of the developmental pathways and cytogenetics of activated oocytes provides a valuable model for the analysis of the processes involved in the induction of chromosome segregation errors.

MATERIALS AND METHODS

Ethanol-induced activation

Ten- to twelve-week old (C57BL × CBA) F_1 hybrid female mice were superovulated with 5 iu of pregnant mares' serum gonadotrophin (PMSG), followed 48 hr later with 5 iu of human chorionic gonadotrophin (HCG). The females were killed by cervical dislocation 17 hr after the HCG injection; their oviducts were then removed and transferred into drops of Dulbecco's phosphate-buffered saline (PBS) maintained at 37°C. The cumulus masses containing the ovulated oocytes were released from the ampullary region of the oviduct, transferred by pasteur pipette into 1 ml of freshly prepared 7% Analar quality ethyl ethanol in PBS at 37°C, and incubated in this solution for either 1,3 or 5 min. The cumulus masses were washed three times in ethanol-free PBS, followed by two washes in M16 culture medium (Whittingham, '71) containing 4 mg/ml of BSA maintained at 37°C in an atmosphere of 5% CO_2 in air.

Individual cumulus masses were then transferred to separate microdrops of culture medium under paraffin oil maintained at 37°C in an atmosphere of 5% CO_2 in air. The cumulus masses were cultured under these conditions for 6 hr before the adhering cumulus cells were removed after a brief exposure to medium containing hyaluronidase (0.5 mg/ml). The cumulus cell-free oocytes were separated into fresh microdrops of culture medium, and the activation frequency was determined.

The four main classes of parthenogenetic development were observed at this stage. The majority of activated oocytes had extruded a second polar body, developed a single haploid pronucleus, and were classified as single-pronuclear haploid parthenogenones (1PN). Parthenogenones in which the extrusion of the second polar body had failed to occur were observed at a lower frequency and represented two of the three remaining possible pathways of development. These parthenogenones retained both sets of chromosomes after the completion of the second meiotic division and exhibited either two haploid pronuclei (2PN) or a single diploid pronucleus (1PND). The remaining oocytes had undergone immediate cleavage (IC) and possessed two blastomeres of equal size, each containing a single haploid pronucleus. Activated oocytes of the same developmental class were transferred to separate fresh microdrops of culture medium. Only the 1PN class were retained in culture for chromosome analysis.

Hyaluronidase activation: Control

An additional group of F_1 hybrid female mice were superovulated, as described above, and were killed at either 18 hr or 20 hr after the HCG injection by cervical dislocation. The oviducts from these females were removed and transferred into drops of Dulbecco's PBS maintained at 37°C. The cumulus masses containing the ovulated oocytes were released from the ampullary region and were transferred for 15 min to microdrops of culture medium under paraffin oil containing 3 mg/ml of hyaluronidase. After this incubation period the cumulus-free oocytes were washed in three changes of hyaluronidase-free culture medium and transferred to microdrops of culture medium at 37°C in an atmosphere of 5% CO_2 in air for 6 hr. After this period, the oocytes were examined for the presence of pronuclei to determine the incidence of parthenogenetic activation. Activated oocytes of the various developmental classes were separated and transferred into fresh microdrops of culture medium. Exposure of oocytes at HCG + 20 hr to hyaluronidase induces parthenogenetic activation at a similar frequency to that observed after ethanol exposure (Kaufman, '73b; Kaufman, '83a).

Chromosome preparations

The 1PN parthenogenones were left in culture until they had entered metaphase of the first cleavage division. This stage of the cell cycle was signalled by the dissolution of the pronuclear membrane and occurred about 15–18 hr after ethanol activation and 13–15 hr after hyaluronidase activation. Parthenogenones at this stage of the cell cycle were harvested, and air-dried chromosome preparations were made according to the method described by Tarkowski ('66). The chromosome preparations were left for at least 14 days before they were stained, using a modification of the G-banding technique described by Gal-

TABLE 1a. The proportionate incidence of the pathways of parthenogenetic development observed after the exposure of ovulated oocytes from (C57BL × CBA)F_1 female mice to a 7% solution of ethanol in PBS at HCG + 17h

Group	Duration of activation stimulus (min)	Total no. of ovulated oocytes	Total no. of activated oocytes	Activation frequency (%)	Pathway of parthenogenetic development at the pronuclear stage			
					1PN	2PN	IC	1PND
1	1	497	321	64.6	285	24	8	4
2	3	969	673	69.5	574	83	4	12
3	5	1,864	1,188	63.7	801	325	37	25

Group 1 is not significantly different from Group 2; $\chi^2 = 2.3$, $P = 0.2–0.1$.
PBS, Dulbecco's phosphate-buffered saline; HCG, human chorionic gonadotrophin; 1PN, single-pronuclear haploid parthenogenones; 2PN, parthenogenones with two haploid pronuclei; IC, immediate cleavage; 1PND, parthenogenones with a single diploid pronucleus.

TABLE 1b. The chromosome constitution of single-pronuclear haploid parthenogenones at metaphase of the first cleavage division following activation in a 7% solution of ethanol in PBS at HCG + 17 hr

Group	Duration of activation stimulus (min)	Total no. of chromosome preparations	No. of preparations not analysable	Chromosome constitution							Aneuploidy (%)
				18	19	20	21	22	23	24	
1	1	100	7	1	9	79	4	0	0	0	15.1
2	3	275	17	5	17	218	15	2	1	0	15.5
3	5	506	46	25	41	342	37	8	5	2	25.7

Group 1 is not significantly different from Group 2; $\chi^2 = 7.7 \times 10^{-3}$; $P = 0.99–0.98$.
Group 2 is significantly different from Group 3; $\chi^2 = 6.5$, $P = 0.2–0.1$.
PBS, Dulbecco's phosphate-buffered saline; HCG, human chorionic gonadotrophin.

limore and Richardson ('73). Karyograms of banded chromosome spreads were prepared according to the Committee on Standardized Nomenclature for Mice (1972) and Nesbitt and Francke ('73).

RESULTS

Observations on the proportionate incidence of the main pathways of parthenogenetic development

The incidence of the various pathways of parthenogenetic development that were observed after the isolation of ovulated oocytes at HCG + 17 hr and their incubation for either 1, 3, or 5 min in a 7% solution of ethanol are presented in Table 1a. Comparable values for ovulated oocytes isolated at HCG + 18 hr and HCG + 20 hr and exposed to culture medium containing hyaluronidase are presented in Table 2a. The highest overall frequency of parthenogenetic activation in the present study was observed after hyaluronidase activation at HCG + 20 hr (78.9%). A lower frequency of activation, in the range of 63.7–69.3%, was observed following ethanol-induced activation, but these values are higher than those observed after hyaluronidase activation at HCG + 18 hr.

The majority of parthenogenones that developed after exposure to ethanol or hyaluronidase were 1PN

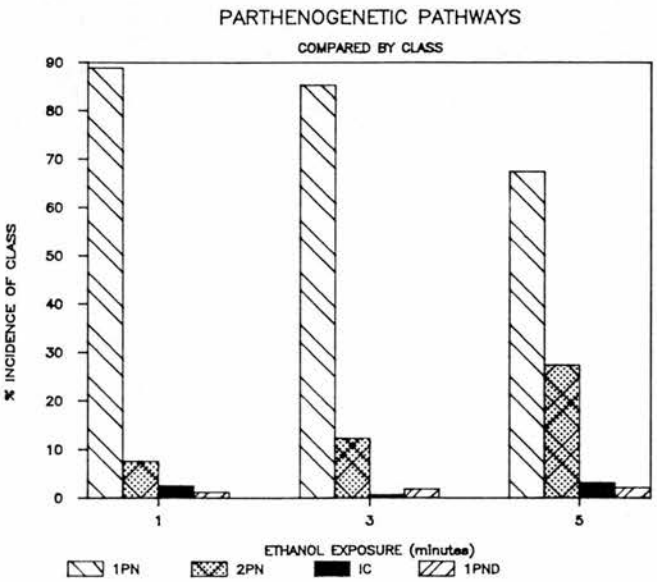


Fig. 1. The proportionate incidence of the four pathways of parthenogenetic development after in vitro exposure of ovulated mouse oocytes at HCG + 17 hr to a dilute solution of ethanol for 1, 3, or 5 min. HCG, human chorionic gonadotrophin.

TABLE 2a. The proportionate incidence of the pathways of parthenogenetic development observed when ovulated oocytes from (C57BL × CBA)F_1 female mice were exposed to hyaluronidase

Group	Postovulatory age (hr)[1]	Total no. of ovulated oocytes	Total no. of activated oocytes	Activation frequency (%)	Pathway of parthenogenetic development at the pronuclear stage			
					1PN	2PN	IC	1PND
1	18	401	155	38.7	146	9	0	0
2	20	692	546	78.9	434	100	9	3

[1]Time of isolation after the HCG injection. Ovulation occurs at HCG + 11–12 hr.
HCG, human chorionic gonadotrophin; 1PN, single-pronuclear haploid parthenogenones; 2PN, parthenogenones with two haploid pronuclei; IC, immediate cleavage; 1PND, parthenogenones with a single diploid pronucleus.

TABLE 2b. The chromosome constitution of single-pronuclear haploid parthenogenones at metaphase of the first cleavage division following hyaluronidase activation at HCG + 20 hr

Group	Postovulatory age (h)[1]	Total no. of chromosome preparations	No. of preparations not analysable	Chromosome constitution					Aneuploidy (%)
				18	19	20	21	22	
2	20	328	27	2	6	288	4	1	4.3

[1]see Table 2a.
Table 1b Group 1 is significantly different from Table 2b Group 2; × = 10.64, $P > .01$.

parthenogenones (Figs. 1 and 2). An increase in the duration of ethanol exposure from 1 min to 3 min was not associated with a significant decrease in the proportionate incidence of the 1PN class (Table 1a). However, the proportionate incidence of 1PN development was significantly reduced from 85.3% to 67.4% as the period of ethanol exposure increased from 3 min to 5 min. This decrease in the incidence of 1PN development was related directly to an increase in the incidence of 2PN, 1PND, and IC parthenogenones. An increase in the postovulatory age of oocytes at the time of their activation from HCG + 18 hr to HCG + 20 hr was also associated with a decrease in the proportionate incidence of the 1PN class.

Chromosome constitution of single-pronuclear haploids at metaphase of the first cleavage division

The chromosome constitution of ethanol- and hyaluronidase-activated 1PN parthenogenones is presented in Tables 1b and 2b, respectively. The chromosome spreads that are recorded as non-analysable represent those in which the presence of overlapping chromosomes prevented an accurate analysis of the number of chromosomes present and those that were obviously hypohaploid due to chromosome scattering

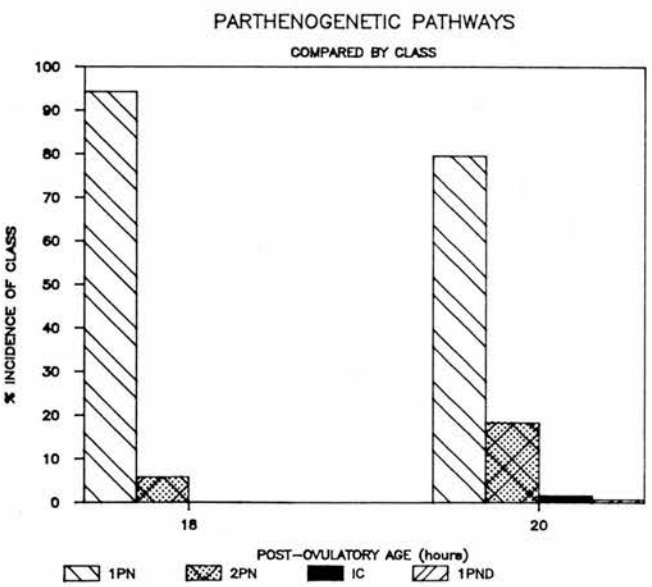


Fig. 2. The proportionate incidence of the four pathways of parthenogenetic development after in vitro exposure of ovulated mouse oocytes to hyaluronidase at HCG + 18 hr and HCG + 20 hr. HCG, human chorionic gonadotrophin.

incurred during the chromosome spreading procedure.

The overall ratio of hypohaploidy to hyperhaploidy observed after ethanol-induced activation was 1.0 : 0.8. As it is possible that a small proportion of hypohaploid spreads arise from the mechanical loss of chromosomes during the spreading technique and that hyperhaploid counts represent a more reliable indicator of errors in chromosome segregation, an adjusted value for the incidence of aneuploidy has been determined from the following equation:

$$\frac{\text{Total number of hyperhaploid chromosome spreads}}{\text{Total number of chromosome spreads}} \times 2.$$

The cytogenetic analysis of hyaluronidase-induced 1PN parthenogenones indicates that the control or "spontaneous" incidence of aneuploidy is low (total: 4.3%, adjusted: 3.3%). A significantly higher incidence of aneuploidy (total: 15.1%, adjusted: 8.6%) was observed when oocytes were activated after 1 min exposure to ethanol. When oocytes were exposed to ethanol for either 3 min or 5 min, the incidence of aneuploidy was 15.5% (adjusted: 13.9%) and 25.7% (adjusted: 22.6%), respectively; both of these values were significantly different from those observed after hyaluronidase activation. Although the incidence of aneuploidy does not significantly rise when the duration of ethanol exposure increases from 1 min to 3 min, the incidence of malsegregation events that involved more than one chromosome increased from 7.1% to 20.0%. When the duration of exposure was increased from 3 min to 5 min, the percentage of nondisjunctional events that involved more than one chromosome was increased to 36.0%. Thus an increase in the duration of ethanol exposure clearly increases the probability that more than one chromosome is involved in each nondisjunctional event.

Karyogram analysis of single-pronuclear haploid parthenogenones

The karyogram analysis of G-banded chromosome preparations from ethanol-induced 1PN parthenogenones has indicated that not all chromosomes of the mouse genome are equally predisposed to malsegregation at the second meiotic division (Figs. 3 and 4). The results presented in Table 3 indicate that several chromosomes, notably 5, 6, 7, 8, 9, 14, and 19, were less commonly encountered in metaphase preparations than expected if chromosome segregation errors were completely random events. However, a complementary number of hypohaploid metaphase preparations nullisomic for chromosome 14 were not observed. In contrast, chromosome 4 and the majority of the smaller chromosomes of the mouse genome (10, 11, 12, 15, 17, and 18) were observed in aneuploid metaphase preparations at a higher frequency than expected. All malsegregation events that involved chromosome 17 were observed in hyperhaploid metaphase preparations, whereas chromosome 11 was more frequently associated with hypohaploidy. The remaining chromosomes 1, 2, 3, 13, 16, and X were involved in malsegregation at a frequency equivalent to that expected for random chromosome error. Chromosomes 1 and X were more frequently associated with hyperhaploidy.

TABLE 3. The incidence of specific chromosome malsegregation observed in ethanol-induced single-pronuclear haploid parthenogenones at metaphase of the first cleavage division

	Chromosome constitution		
Chromosome	Hyperhaploid	Hypohaploid	Total incidence
1	4	0	4
2	2	2	4
3	1	3	4
4	4	2	6
5	0	1	1
6	1	1	2
7	0	0	0
8	0	3	3
9	1	1	2
10	3	3	6
11	1	4	5
12	3	4	7
13	2	2	4
14	3	0	3
15	6	2	8
16	2	2	4
17	6	0	6
18	5	5	10
19	0	1	1
X	3	1	4

Expected incidence (E) of malsegregation for each chromosome is 4.

$$E = \frac{\text{No. of hypohaploid + hyperhaploid events}}{\text{No. of chromosomes in euploid 1PN parthenogenone}}$$

$$E = \frac{(37 + 47)}{20}$$

1PN, single-pronuclear haploid parthenogenones.

Karyogram analysis of 57 randomly chosen metaphase spreads that contained the euploid number of chromosomes has established that all were genetically balanced (Fig. 5). This indicates that double malsegregation events, in which one chromosome pair migrate to one pole while another pair migrate to the opposite pole to provide an apparently euploid complement, either did not occur or were very rare events. These observations also indicate that hyper-

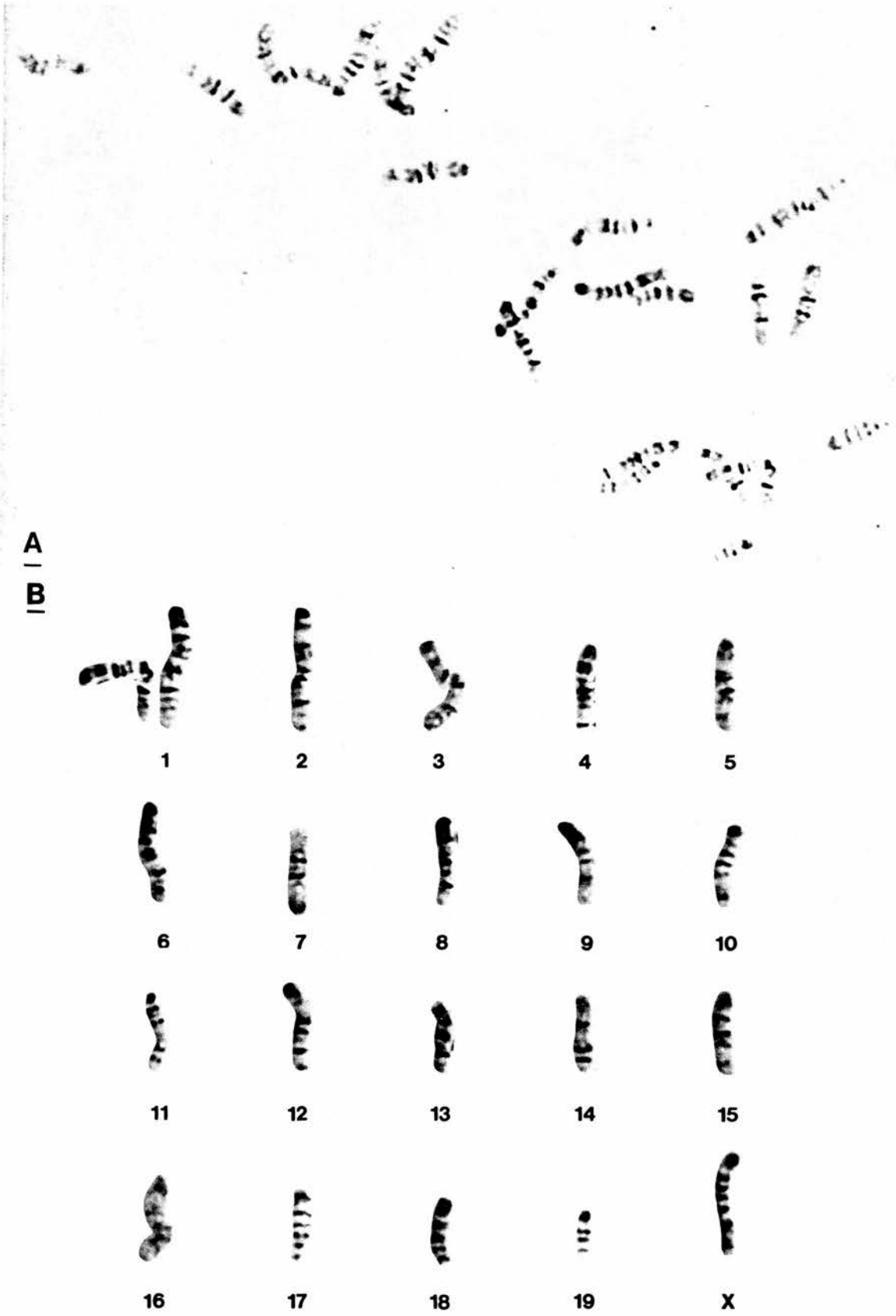


Fig. 3. **A:** Air-dried chromosome spread from a hyperhaploid (n = 21) ethanol-activated 1PN parthenogenone. Giemsa-banded preparation. **B:** Karyogram analysis illustrates that this preparation is disomic for chromosome 1.

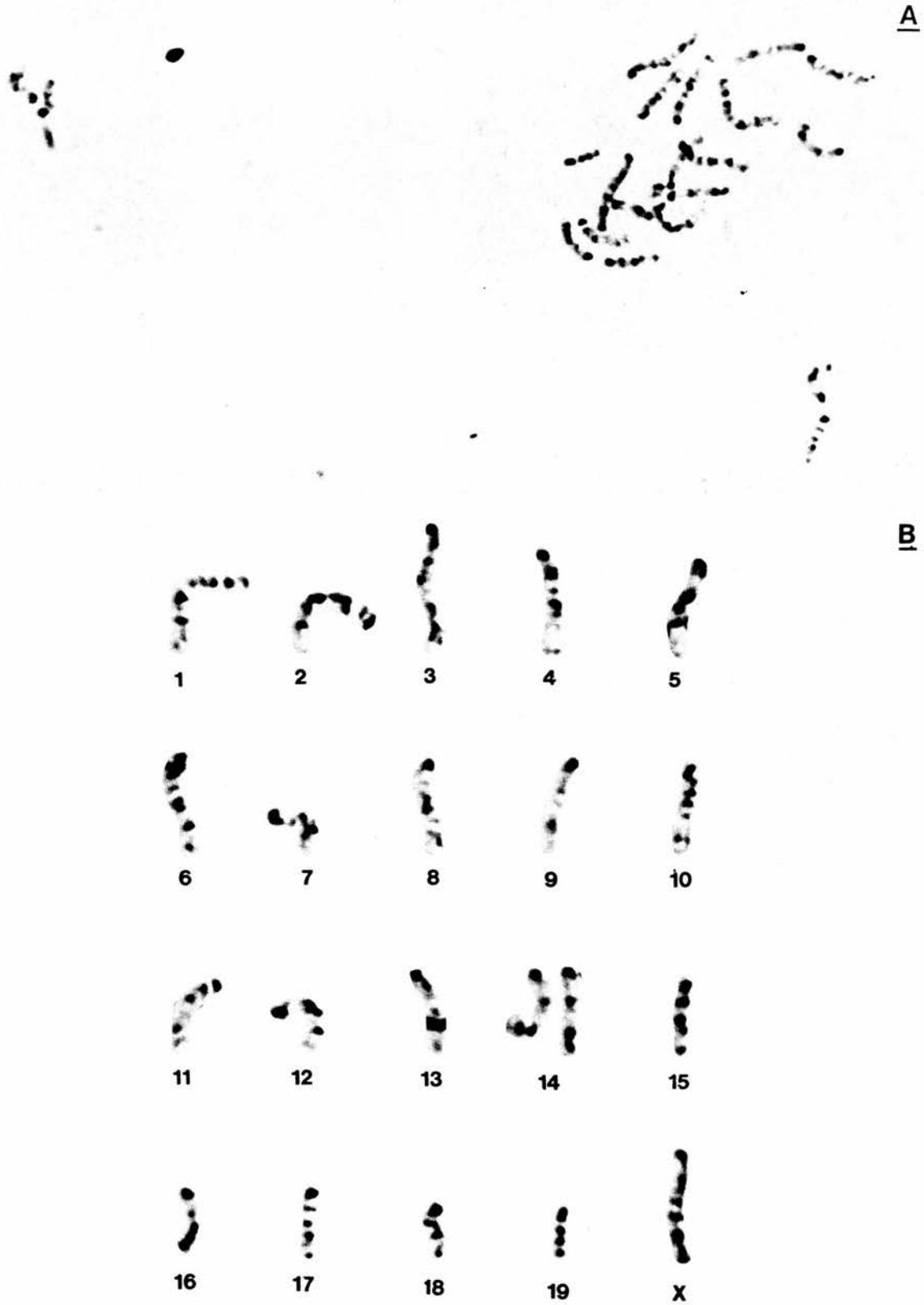


Fig. 4. **A:** Air-dried chromosome spread from a hyperhaploid (n = 21) ethanol-activated 1PN parthenogenone. Giemsa-banded preparation. **B:** Karyogram analysis illustrated that this preparation is disomic for chromosome 14.

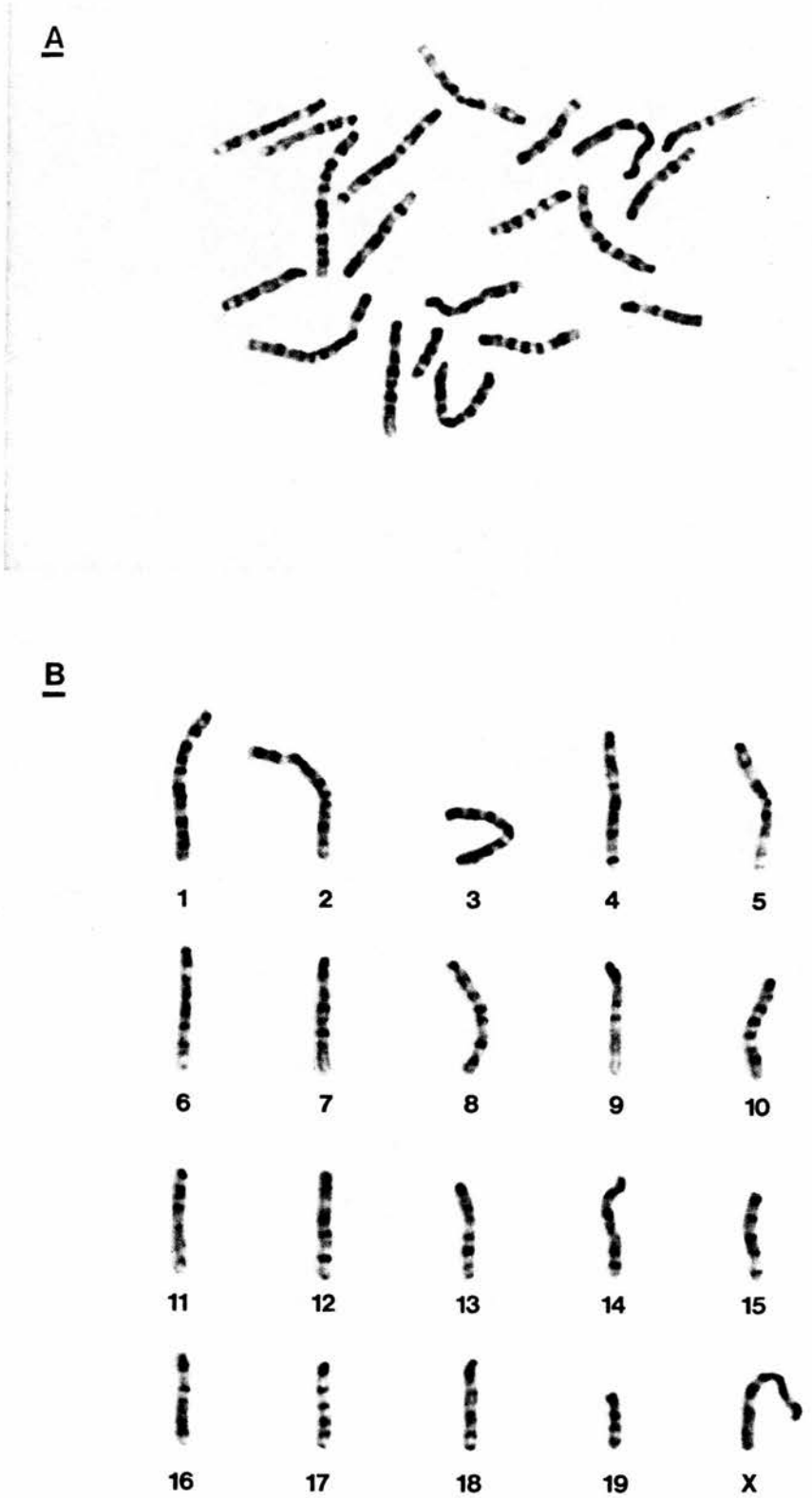


Fig. 5. **A:** Air-dried chromosome spread from a euploid (n = 20) ethanol-activated 1PN parthenogenone. Giemsa-banded preparation. **B:** Karyogram analysis illustrates that this preparation has a normal euploid chromosome constitution.

haploid parthenogenones are not reduced to the euploid number by the mechanical loss of chromosomes during the spreading procedure.

DISCUSSION

This investigation has demonstrated that the exposure of recently ovulated mouse oocytes to a dilute solution of ethanol, for either 1, 3, or 5 min, induced a high incidence of parthenogenetic activation. A significant alteration to the frequency of activation was not influenced by the duration of ethanol exposure. The majority of ethanol-activated oocytes developed as 1PN parthenogenones, but the proportionate incidence of this developmental class decreased as the duration of exposure to the activation stimulus increased from 1 min to 5 min. This decrease in the incidence of 1PN development was associated with an increase in the proportionate incidence of 2PN, 1PND, and IC parthenogenones. These findings have clearly demonstrated that the duration of ethanol exposure is capable of regulating the proportionate incidence of the four main developmental pathways of parthenogenesis.

A similar decrease in the proportionate incidence of the 1PN class was observed in the present study when oocytes of increasing postovulatory age were exposed to hyaluronidase (Kaufman, '73b, '83a; O'Neill and Kaufman, '88). The proportion of ethanol-activated oocytes that failed to extrude a second polar body was significantly greater when the exposure duration to ethanol was for 5 min at HCG + 17 hr than that observed after hyaluronidase activation at HCG + 18 hr. This would indicate that the normal cytokinetic events associated with the extrusion of the second polar body are disrupted, or at least interfered with, after exposure to this agent for this period of time.

Numerous studies have clearly demonstrated that the extrusion of the second polar body in mouse and hamster oocytes is dependent on the close alignment of the second meiotic spindle apparatus with an overlying actin-rich and microvillus-free zone of the plasma membrane (mouse: Szöllösi, '71; Longo and Chen, '85; Wasserman and Fujiwara, '78; Sato and Blandau, '79; Maro et al., 86; Webb et al., '86; Eichenlaub-Ritter et al., '86; hamster: Okada et al., '86). Immunocytochemical analyses of the postovulatory age-related changes that occur in the organisation of actin filaments in mouse oocytes have demonstrated that in an increasing proportion of oocytes the actin-rich cortical zone becomes disorganised and may facilitate the migration of the meiotic spindle to a more central location in the oocyte (Webb et al., '86). In postovulatory aged oocytes, the latter consequently favours the development of parthenogenones in which both products of the second meiotic division are retained within the oocyte. Second polar body extrusion is inhibited when activated oocytes are cultured in the presence of cytochalasin B (Balakier and Tarkowski, '76) or cytochalasin D (authors' unpublished observations). The actin filaments within the cytoskeleton of neural crest cells also become disorganised when these cells are exposed in vitro to very low concentrations of ethanol (Hassler and Moran, '86). It appears likely that under certain experimental conditions ethanol activation may interfere with the stability of actin filaments and consequently reproduce premature postovulatory age-related changes in relatively recently ovulated oocytes.

Analysis of the chromosome constitution of 1PN parthenogenones has consistently shown that the induction of parthenogenesis per se is not associated with errors in chromosome segregation during the completion of the second meiotic division (Kaufman, '83a; O'Neill and Kaufman, '88). However, a significant proportion of the ethanol-activated 1PN parthenogenones exhibited an aneuploid chromosome constitution. The incidence of aneuploidy and the number of chromosomes involved in each malsegregation event was clearly directly related to the duration of exposure to this agent.

All G-banded preparations with a euploid chromosome constitution were found to be genetically balanced. The absence of homologous associations between disomic chromosome pairs in hyperhaploid metaphase preparations from ethanol-induced parthenogenones indicates that the segregation errors were not the result of classical nondisjunction but may have been the product of nonconjunctional segregation or anaphase lagging (Bond and Chandley, '83).

The analysis of both hyperhaploid and hypohaploid metaphase preparations also has established that not all chromosomes were equally predisposed to undergo malsegregation. This is consistent with the recent finding that the smaller chromosomes of the mouse genome were more frequently involved in malsegregation when mouse oocytes were exposed in vivo to a dilute solution of ethanol at the first or second meiotic divisions (O'Neill and Kaufman, '87). The fact that the meiotic chromosomes of the mouse were not equally involved in aneuploidy may relate more to their individual characteristics and their relationship to the meiotic spindle, rather than reflecting on any specific property of the agent to which they are exposed. The majority of the smaller chromosomes of the mouse genome were more frequently

involved in segregation errors. However, chromosome 19 was underrepresented in malsegregation events. A continued cytogenetic analysis of ethanol-induced aneuploid 1PN parthogenones will establish the genomic factors that influence nondisjunction.

The preferential involvement of chromosomes in segregation errors was not related to their constitutive heterochromatin content (Yoshida and Kodama, '83) or restricted to those possessing nucleolar organising regions (Dev et al., '77). Cytogenetic analyses of human spontaneous abortions (Chandley, '82; Linn et al., '85), human oocytes and preimplantation embryos (Wramsby et al., '87; Plachot et al., '87), and human sperm (Brandriff et al., '85; Jenderny and Rohrborn, '87) have demonstrated also that the majority of numerical anomalies encountered resulted from both the loss or gain of the smaller human chromosomes.

It has been proposed that one of the primary steps in the induction of segregation errors may be the displacement of chromosomes from the meiotic spindle (Ford and Roberts, '83a). Cytogenetic analyses of human lymphocyte cultures has indicated that this phenomenon more frequently involved the smaller chromosomes (Ford and Lester, '82) and that the incidence of these events increased when the pH (Ford and Roberts, '83b) or the calcium concentration of the culture medium was altered prior to analysis (Ford and Roberts, '84). Under normal conditions, the tendency toward displacement is reduced by the "stabilizing" activity of the microtubular components of the spindle. Disruption of microtubular dynamics during the completion of the second meiotic division, as a result of ethanol exposure, may, however, decrease the chances of the latter occurring.

ACKNOWLEDGMENTS

G.T. O'Neill is supported by a Bonnar Scholarship from the Faculty of Medicine, University of Edinburgh. The work is supported by a grant (to M.H.K.) from the National Fund for Research into Crippling Diseases (Action Research for the Crippled Child), and an equipment grant (to M.H.K.) from the Scottish Home and Health Department, Ref. No. K/MRS/50/C866. We would like to thank Dr. E.P. Evans for his advice and assistance with the interpretation of the G-banded preparations.

LITERATURE CITED

Allison, A.C., G.H. Hulands, J.F. Nunn, J.A. Kitching, and A.C. MacDonald (1970) The effects of inhalational anaesthetics on the microtubular system of *Actinosphaerium nucleofilium*. J. Cell Sci. *7*:483–499.

Balakier, H., and A.K. Tarkowski (1976) Diploid parthenogenetic mouse embryos produced by heat shock and cytochalasin B. J. Embryol. Exp. Morphol., *35*:25–39.

Bond, J.D., and A.C. Chandley (1983) Aneuploidy. Oxford University Press, Oxford.

Brandriff, B., L. Gordon, L. Ashworth, G. Watchmaker, I.I. Moore, A.J. Wyrobek, and A.V. Carrano (1985) Chromosomes of human sperm: Variability among normal individuals. Hum. Genet., *70*:18–24.

Brinkley, B.R., and P.N. Rao (1973) Nitrous oxide: Effects on the mitotic apparatus and chromosome movement in Hela cells. J. Cell. Biol., *58*:96–106.

Chandley, A.C. (1982) The origin of aneuploidy. In: Human Genetics. Part B: Medical Aspects. B. Bonné-Tamir, ed. Alan R. Liss, Inc., New York, pp. 337–347.

Committee on Standardized Genetic Nomenclature for Mice (1972) Standard karyotype of the mouse, *Mus musculus*. J. Hered., *63*:69–72.

Cox, S.M., P.N. Rao, B.R. Brinkley (1977) Differential effects of nitrous oxide on the spindle and cytoplasmic microtubule complex. J. Cell Biol., *75*:291a.

Danford, N., and J.M. Parry (1986) Induction of sex chromosome aneuploidy in mice by vincristine sulphate. I.R.C.S. Med. Sci., *14*:15–16.

Dev, V.G., R. Tantravahi, D.A. Miller, and O.J. Miller (1977) Nucleolus organizers in *Mus musculus* subspecies and in the Rag mouse cell line. Genetics, *86*:389–398.

Donahue, R.P. (1972) Cytogenetic analysis of the first cleavage division in mouse embryos. Proc. Natl. Acad. Sci. U.S.A., *69*:74–77.

Dyban, A.P., and V.S. Baranov (1987) Cytogenetic Analysis of Mammalian Embryonic Development. Oxford University Press, Oxford.

Eichenlaub-Ritter, U., A.C. Chandley, and R.G. Gosden (1986) Alterations in the microtubular cytoskeleton and increased disorder of chromosome alignment in spontaneously ovulated mouse oocytes aged in vivo: An immunofluorescence study. Chromosoma, *94*:337–345.

Ford, J.H., and P. Lester (1982) Factors affecting the displacement of human chromosomes from the metaphase plate. Cytogenet. Cell Genet., *33*:327–332.

Ford, J.H., and C. Roberts (1983a) Displacement of chromosomes in mitosis: A technique for assessing differential chromosome error. Cytogenet. Cell Genet., *36*:537–541.

Ford, J.H., and C.G. Roberts (1983b) Chromosome displacement and spindle tubule polymerization. 1. The effect of alterations in pH on displacement frequency. Cytobios, *37*:163–169.

Ford, J.H., and C.G. Roberts (1984) Chromosome displacement and spindle tubule polymerization. 2. Effect of alterations of extracellular calcium on displacement parameters. Cytobios, *39*:81–88.

Gallimore, P.H., and C.R. Richardson (1973) An improved banding technique exemplified in the karyotype of two strains of the rat. Chromosoma, *41*:259–263.

Hansmann, I., and E. El-Nahaas (1979) Incidence of nondisjunction in mouse oocytes. Cytogenet. Cell Genet., *24*:115–121.

Hassler, J.A., and D.J. Moran (1986) The effects of ethanol on embryonic actin: A possible role in teratogenesis. Experientia, *42*:575–577.

Hinkley, R.E., and F.E. Samson (1972) Anaesthetic induced transformation of axonal microtubules. J. Cell Biol., *53*:258–263.

Hinkley, R.E., and A.G. Telser (1974) The effects of halothane on cultured mouse neuroblastoma cells: Inhibi-

tion of morphological differentiation. J. Cell. Biol., *63:*531–540.

Hummler, E., and I. Hansmann (1985) Preferential nondisjunction of specific bivalents in oocytes from Djungarian hamsters (*Phodopus sungorus*) following colchicine treatment. Cytogenet. Cell Genet., *39:*161–167.

Jackson, S.H. (1975) Anaesthetics and cell multiplication. Clin. Anesth., *11:*75–92.

Jenderny, J., and G. Rohrborn (1987) Chromosome analysis of human sperm. I. First results with a modified method. Hum. Genet., *76:*385–388.

Kaufman, M.H. (1973a) Analysis of the first cleavage division to determine the sex-ratio and the incidence of chromosome anomalies at conception in the mouse. J. Reprod. Fertil., *35:*67–72.

Kaufman, M.H. (1973b) Parthenogenesis in the mouse. Nature, *242:*475–476.

Kaufman, M.H. (1977a) Effect of anaesthesia on the outcome of pregnancy in female mice. J. Reprod. Fertil., *49:*167–168.

Kaufman, M.H. (1977b) Effect of anaesthetic agents on eggs and embryos. In: Development in Mammals. M.H. Johnson, ed. North Holland, Amsterdam, Vol. 1, pp. 137–163.

Kaufman, M.H. (1982) The chromosome complement of single-pronuclear haploid mouse embryos following activation by ethanol treatment. J. Embryol. Exp. Morphol., 71:139–154.

Kaufman, M.H. (1983a) Early Mammalian Development: Parthenogenetic Studies. Cambridge University Press, Cambridge.

Kaufman, M.H. (1983b) Ethanol-induced chromosomal abnormalities at conception. Nature, *302:*258–260.

Kaufman, M.H. (1985) An hypothysis regarding the origin of aneuploidy in man: Indirect evidence from an experimental model. J. Med. Genet., *22:*171–178.

Kaufman, M.H., and I.M. Bain (1984) Influence of ethanol on chromosome segregation during the first and second meiotic divisions in the mouse egg. J. Exp. Zool., *230:*315–320.

Kaufman, M.H., and G.T. O'Neill (1988) A neuploidy induced by ethanol. In: Aneuploidy: Induction and Model Systems. A.A. Sandberg and B.K. Vig, eds. Alan R. Liss, Inc., New York, Vol. 2. 95–122.

Linn, C.C., M. De Braekeleer, and H. Jamro (1985) Cytogenetic studies in spontaneous abortion: The Calgary experience. Can. J. Genet. Cytol., *27:*565–570.

Longo, F.J., and D.Y. Chen (1985) Development of cortical polarity in mouse eggs: Involvement of the meiotic apparatus. Dev. Biol., *107:*382–394.

Maro, B., M.H. Johnson, M. Webb, and G. Flach (1986) Mechanism of polar body formation in the mouse oocyte: An interaction between the chromosomes, the cytoskeleton and the plasma membrane. J. Embryol. Exp. Morphol., *92:*11–32.

McGaughey, R.W., and M.C. Chang, (1969) Inhibition of fertilization and production of heteroploidy in eggs of mice treated with colchicine. J. Exp. Zool., *171:*465–480.

Nesbitt, M.N., and U. Francke, (1973) A system of nomenclature for band patterns of mouse chromosomes. Chromosoma, *41:*145–158.

Okada, K., R. Yanagimachi, and H. Yanagimachi (1986) Development of a cortical granule-free area of cortex and the perivitelline space in the hamster oocyte during maturation and following ovulation. J. Submicrosc. Cytol., *18:*233–247.

O'Neill, G.T., and M.H. Kaufman (1987) Cytogenetic analysis of first cleavage fertilised mouse eggs following in vivo exposure to ethanol shortly before and at the time of conception. Development, *100:*441–448.

O'Neill, G.T., and M.H. Kaufman (1988) Influence of postovulatory aging on chromosome segregation during the second meiotic division in mouse oocytes: A parthenogenetic analysis. J. Exp. Zool. *248:*125–131.

Plachot, M., J. de Grouchy, A. Junca, J. Mandelbaum, C. Turleau, P. Couillin, J. Cohen, and J. Salat-Baroux (1987) From oocyte to embryo: A model, deduced from in vitro fertilization, for natural selection against chromosome abnormalities. Ann. Genet. (Paris), *30:*22–32.

Rohrborn, G. (1972) Frequencies of spontaneous nondisjunction in metaphase II oocytes of mice. Hum. Genet., *16:*123–125.

Sato, K., and R.J. Blandau (1979) Second meiotic division and polar body formation in mouse eggs fertilized in vitro. Gamete Res., *2:*283–293.

Sugawara, S., and K. Mikamo (1980) An experimental approach to the analysis of mechanisms of meiotic nondisjunction and anaphase lagging in primary oocytes. Cytogenet. Cell Genet., *28:*251–264.

Szöllösi, D. (1971) Morphological changes in mouse eggs due to aging in the fallopian tube. Am. J. Anat., *130:*209–226.

Tarkowski, A.K. (1966) An air-drying method for chromosome preparations from mouse eggs. Cytogenetics, *5:*394–400.

Tease, C., and G. Fisher (1986) Oocytes from young and old female mice respond differently to colchicine. Mutat. Res., *173:*31–34.

Wasserman, P.M., and K. Fujiwara (1978) Immunofluorescent anti-tubulin staining of spindles during meiotic maturation of mouse eggs in vitro. J. Cell Sci., *29:*171–188.

Webb, M., S.K. Howlett, and B. Maro (1986) Parthenogenesis and the cytoskeletal organisation in ageing mouse eggs. J. Embryol. Exp. Morphol., *95:*131–145.

Whittingham, D.G. (1971) Culture of mouse ova. J. Reprod. Fertil. Suppl., *14:*7–21.

Wramsby, H., K. Fredga, and P. Liedholm (1987) Chromosome analysis of human oocytes recovered from preovulatory follicles in stimulated oocytes. N. Engl. J. Med., *316:*121–124.

Yoshida, M.C., and Y. Kodama (1983) C-band patterns of 17 strains of mice. Cytogenet. Cell Genet., *35:*51–56.

Gamete Research 22:285–299 (1989)

Ultrastructural Analysis of Abnormalities in the Morphology of the Second Meiotic Spindle in Ethanol-Induced Parthenogenones

G.T. O'Neill, R.D. McDougall, and M.H. Kaufman

Department of Anatomy, University Medical School, Edinburgh, Scotland

A high frequency of parthenogenetic activation occurs when ovulated mouse oocytes are briefly exposed to a dilute solution of ethanol in vitro. Cytogenetic analyses of parthenogenones at metaphase of the first cleavage division have confirmed that parthenogenetic activation, per se, does not increase the incidence of chromosome segregation errors during the completion of the second meiotic division. Ethanol-induced activation, however, significantly increases the incidence of aneuploidy. The ultrastructural changes that occur in the morphology and organization of the second meiotic spindle apparatus in ethanol- and hyaluronidase-activated oocytes is reported here. Abnormalities in the arrangement of microtubule arrays and chromosome position were principally observed in ethanol-activated oocytes at anaphase and telophase of the second meiotic division, but were only rarely observed in hyaluronidase-activated oocytes. It is proposed that the abnormalities in spindle morphology and chromosome displacement observed in ethanol-activated oocytes represent the initial events that lead to chromosome segregation errors following exposure to this agent.

Key words: parthenogenesis, ethanol-induced, electron microscopy

INTRODUCTION

Numerous cytogenetic analyses have clearly demonstrated that the spontaneous incidence of chromosome segregation errors that occur during the first and second meiotic divisions in mouse oocytes is in the order of 1–2% [reviewed in Nijhoff and de Boer, 1981; Dyban and Baranov, 1987]. An increase in the incidence of chromosome malsegregation during these meiotic divisions can be produced experimentally following the exposure of oocytes to low doses of spindle-active drugs such as colchicine [Edwards, 1958; McGaughey and Chang, 1969; Sugawara and

Received July 5, 1988; accepted September 23, 1988.

Address reprint requests to Professor M.H. Kaufman, Department of Anatomy, University Medical School, Tevior Place, Edinburgh EH8 9AG, Scotland.

Mikamo, 1980; Hummler and Hansmann, 1985; Tease and Fisher, 1986; Mailhes and Yuan, 1987].

It is of interest that the intragastric administration of a dilute solution of ethanol to female mice can also induce an increased incidence of chromosome malsegregation during the first and second meiotic divisions [Kaufman, 1983a; Kaufman and Bain, 1984; O'Neill and Kaufman, 1987; reviewed in Kaufman and O'Neill, 1988].

Ovulated mouse oocytes may also be induced to initiate parthenogenetic development at a high frequency following their in vivo [Dyban and Khozhai, 1980] or in vitro [Kaufman, 1982] exposure to a dilute solution of ethanol. Parthenogenesis, per se, is not usually associated with the induction of chromosome segregation errors [Kaufman, 1983b; O'Neill and Kaufman, 1988a]. However, analysis of the chromosome constitution of ethanol-induced single-pronuclear haploid parthenogenones at metaphase of the first cleavage division has clearly demonstrated that the exposure to this particular activation stimulus significantly increases the incidence of chromosome segregation errors that occur as the egg is completing the second meiotic division [Kaufman, 1982].

It has been proposed that ethanol probably disrupts chromosome segregation by interfering with components of the meiotic spindle apparatus [Kaufman, 1985; Kaufman and O'Neill, 1988]. Anesthetics, which share a number of physiological properties with those of ethanol, are also capable of interfering with microtubular function when cells in tissue culture are exposed to these agents [Nitrous oxide: Brinkley and Rao, 1973; Cox et al., 1977; Halothane: Allison et al., 1970; Hinkley and Samson, 1972; Hinkley and Telser, 1974]. The exposure of female mice to the general anesthetic tribromoethanol (Avertin) is also capable of inducing both meiotic chromosome errors and the parthenogenetic activation of ovulated oocytes [Kaufman, 1977]. The present study has been conducted to analyse the influence of ethanol on the dynamics of the second meiotic spindle apparatus in ethanol-induced parthenogenones.

MATERIALS AND METHODS

Ethanol-Induced Activation

Eight- to 10-week-old (C57BL × CBA) F1 hybrid female mice were injected with 5 IU of pregnant mares' serum gonadotrophin (PMSG) followed 46 h later by 5 IU of human chorionic gonadotrophin (HCG) to induce superovulation. At HCG + 17 h the mice were killed by cervical dislocation and their oviducts were removed. The cumulus masses, containing the ovulated oocytes, were isolated and exposed to a 7% solution of ethanol in Dulbecco's phosphate buffered saline (PBS) for 3 min. The cumulus masses were then isolated and then washed in three changes of ethanol-free PBS and two changes of M16 embryo culture medium [Whittingham, 1971]. They were incubated for either 15, 30, 60, or 75 min in microdrops of M16 medium under light paraffin oil at 37°C in an atmosphere of 5% CO_2 in air prior to fixation for electron microscopy. This method of activation has previously been described and induces a high incidence of parthenogenetic activation and of chromosome segregation errors [Kaufman, 1982; O'Neill and Kaufman, 1988b].

Hyaluronidase Activation

An additional group of young mature mice were superovulated as described above. Their cumulus masses were exposed at HCG + 20 h to M16 medium

containing 3 mg ml of hyaluronidase for 10–15 min. This induces a frequency of activation equivalent to that observed following ethanol-induced activation at HCG + 17 h [Kaufman, 1982; O'Neill and Kaufman, 1988a]. These oocytes were incubated in microdrops of M16 medium for 30 or 75 min prior to fixation.

Non-Activated Ovulated Oocytes

F1 hybrid female mice were superovulated as described above. The cumulus masses were isolated at HCG + 17 h and washed briefly in M16 medium containing 0.5% hyaluronidase to remove the adhering cumulus cells. The cumulus-free oocytes were then fixed and processed as described below.

Electron Microscopy

The nonactivated oocytes and those that had been exposed to the activating stimuli were transferred from the M16 medium to an embryological watchglass containing 1 ml of 3% glutaraldehyde at pH 7.2 and were left to fix in this solution for 1 h. The oocytes were then washed in 0.1 M phosphate buffer containing 0.5% sucrose and subsequently postfixed in 2% osmium tetroxide. The oocytes were dehydrated through a graded alcohol series and then removed to BEEM capsules containing a 1:1 solution of 1, 2, epoxypropane and Araldite for 1 h. The oocytes were transferred to Araldite and retained in this medium at 20°C for 24–48 h before they were finally embedded in fresh Araldite. Three oocytes were embedded in each capsule.

Semithin sections were cut at a nominal thickness of 0.7 μm and were stained with 1% toluidine blue/1% pyronin B in a 0.8% borax solution [Ito and Winchester, 1963]. Ultrathin sections were stained with lead citrate and uranyl acetate. Sections were viewed on a Philips EM301 transmission electron microscope at 60 kV.

RESULTS

Parthenogenetic Activation

The frequency of ethanol- and hyaluronidase-induced parthenogenetic activation was determined from the proportion of sectioned oocytes that exhibited evidence of chromosome disjunction, spindle rotation, and second polar body formation. These criteria confirmed that the nonactivated ovulated oocytes fixed at HCG + 17 h were arrested at metaphase of the second meiotic division. Complete chromatid separation was not observed in all of the sections from individual oocytes that had been retained in culture for 15 min following ethanol exposure, and therefore it was not possible to establish unequivocally the proportion of oocytes in which activation had occurred. It was possible, however, to identify the early events associated with the resumption of meiosis in the activated oocytes that had been retained in culture for periods of 30–75 min (Table 1). These initial changes associated with activation were observed in 56.5% of the ethanol-exposed oocytes ($\bar{X}$ of groups 2–4, Table 1) and 65.7% of the hyaluronidase-activated oocytes ($\bar{X}$ of groups 5-6).

Ultrastructural Observations

Ovulated nonactivated oocytes. The meiotic spindle apparatus of 19 nonactivated oocytes isolated at HCG + 17 h was consistently found closely aligned with the avillous zone of the plasma membrane (Fig. 1), and the polar axes of the spindle were orientated in a tangential plane with respect to the plasma membrane. Electron-dense material was observed to be located at the spindle poles in this group

TABLE 1. The Incidence of Ethanol- and Hyaluronidase-Induced Parthenogenetic Activation as Determined From Semi-Thin and Ultra-Thin Sections of (C57BL × CBA)F1 Hybrid Oocytes

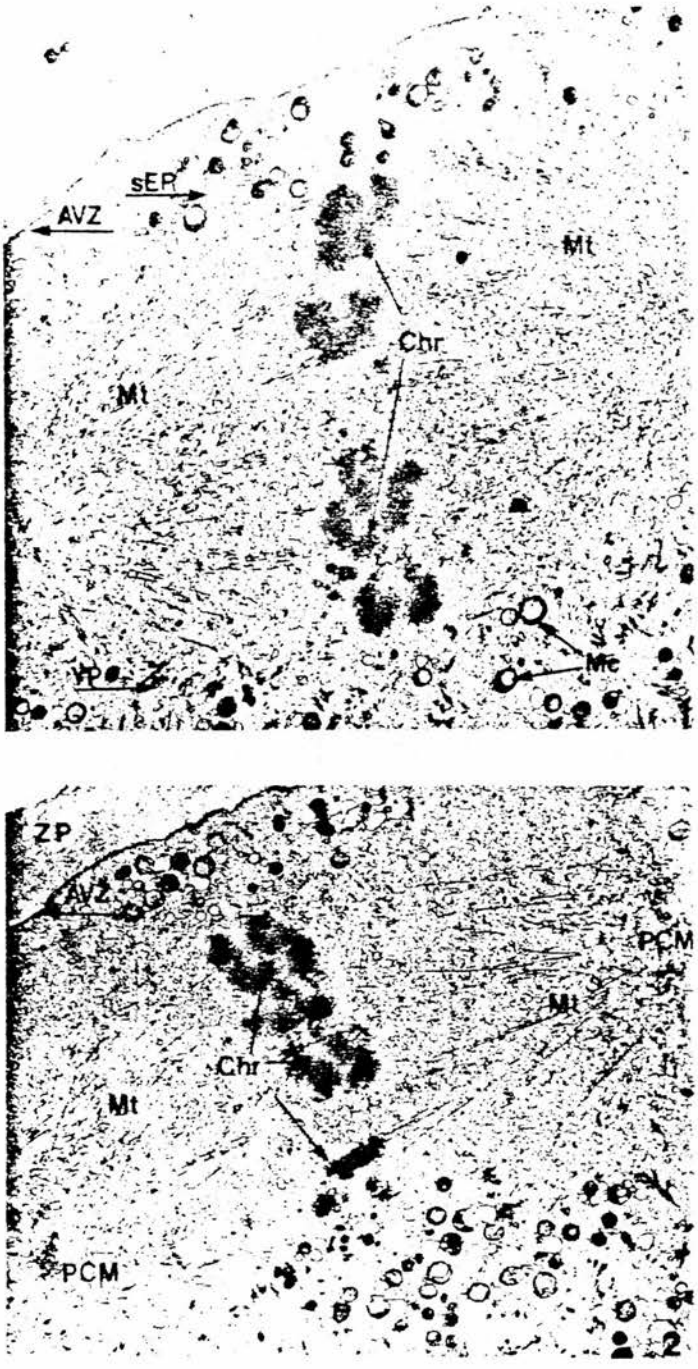
Group	Period of culture (min)	Total no. of oocytes analysed	No. of activated oocytes	Overall activation frequency (%)
1[b]	15	(9)[a]		
2	30	36	21	
3	60	10	5	56.5
4	75	16	9	
5[c]	30	17	10	
6	75	18	13	65.7
7[d]	0	19	0	

[a]At this developmental stage it was not possible to determine unequivocally whether ethanol exposure had induced activation or not.
[b]Groups (1–4): Oocytes were exposed to a 7% ethanol solution for 3 min at HCG + 17 h.
[c]Groups (5, 6): Oocytes were exposed to 3 mg/ml of hyaluronidase for 10–15 min at HCG + 20 h.
[d]Group 7: Ovulated oocytes were fixed at HCG + 17 h.

(Fig. 2), and these have recently been identified as foci of microtubule polymerization [Maro et al., 1985] and termed the pericentriolar material (PCM). Microtubules extend from the spindle poles to the chromosomes located at the equatorial region of the spindle apparatus. The majority of mitochondria, smooth endoplasmic reticulum (sER), and lipid vesicles were positioned in the cytoplasm subjacent to the spindle apparatus.

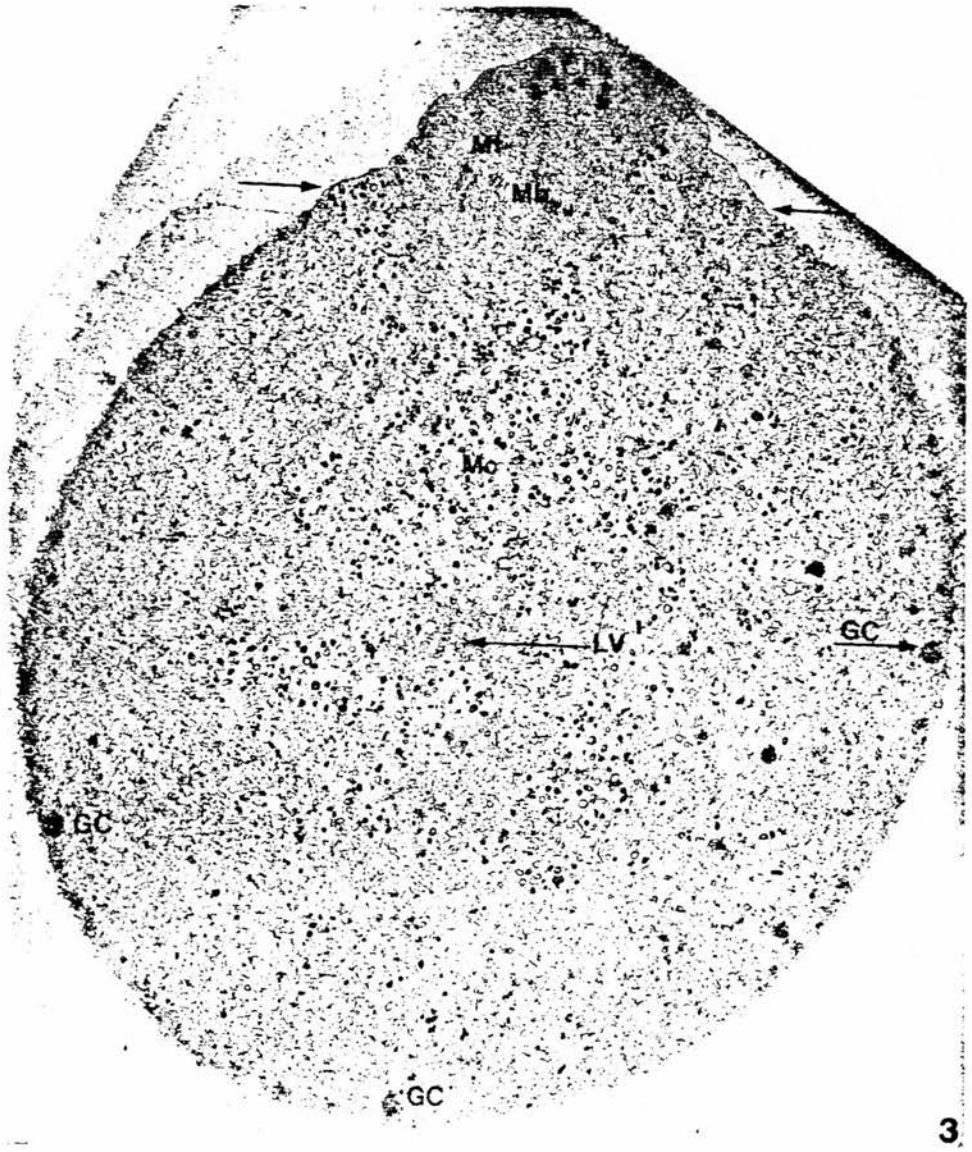
Hyaluronidase activation + 30-min incubation in culture medium. The activated oocytes in this group are observed at an early stage of telophase of the second meiotic division (Fig. 3). The avillous zone of the plasma membrane overlying the spindle apparatus protrudes into the perivitelline space, and a close relationship between this region and the meiotic spindle persists. Electron-dense midbody material is present in the central region of the spindle, and groups of microtubules are organized into distinct arrays at this location. Numerous mitochondria and sER are associated with the spindle pole that is destined to be retained within the oocyte. The site of polar body formation and extrusion is restricted to the avillous zone of the plasma membrane. In this control group, only one of the ten oocytes analysed exhibited an abnormal spindle morphology (Fig. 4). In the latter, disordered arrays of microtubules deviated from the periphery of the main spindle structure and did not appear to be attached to laggard chromosomes.

Ethanol activation + 30-min incubation in culture medium. Ethanol-exposed oocytes at this stage of activation were observed at both anaphase (Figs. 5, 6) and telophase (Fig. 7) stages of the second meiotic division and abnormalities of spindle morphology were observed in several of the oocytes examined. Three of the 21 activated oocytes exhibited multipolar spindles and in these chromatid segregation appeared to be disordered (Fig. 8). Two additional oocytes at telophase possessed a lagging chromosome within the midbody region (Fig. 9), while in two others the segregation of chromatids to the poles of the spindle appeared to be disordered (Fig. 5). Furthermore, in ethanol-exposed oocytes fixed after 15- or 30-min incubation in culture, the lipid vesicles that surround the spindle apparatus were more electron dense and stellate in appearance than those observed in both nonactivated and hyaluronidase-activated oocytes.



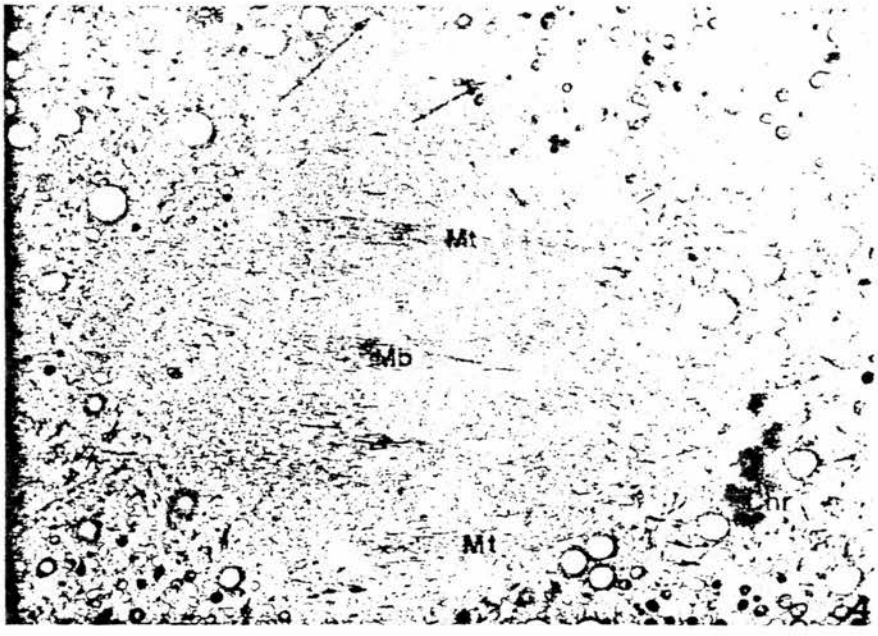
Figs. 1–11. Electron micrographs of ovulated nonactivated, ethanol- and hyaluronidase-activated oocytes. AVZ, avillous zone of the plasma membrane; Chr, chromosomes; Chr A, chromatin "body"; GC, golgi complex; LV, lipid vesicles; MB, midbodies; Mt, microtubules; Mc, mitochondria; PCM, pericentriolar material; sER, vesicles of smooth endoplasmic reticulum; YP, yolk platelets; ZP, zona pellucida.

Figs. 1, 2. The spindle apparatus of two nonactivated ovulated oocytes, arrested at metaphase of the second meiotic division and fixed at HCG + 17 h + 4,30. The spindle apparatus lies in a tangential plane with respect to the avillous zone of the plasma membrane and the zona pellucida. In Figure 2, several foci of pericentriolar material are situated at the spindle poles.



3

Fig. 3. [illegible] second meiotic division following 30-min [illegible] second polar body. The [illegible] second polar body. The chromatin-associated microtubules has rotated into a perpendicular alignment with respect to the [illegible]



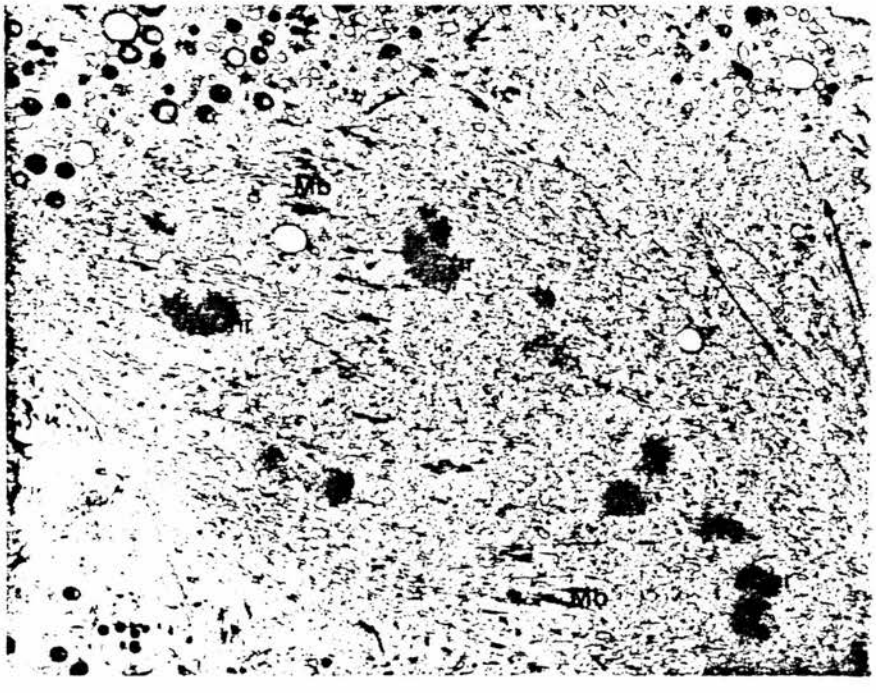


Fig. 4. Hyaluronidase-activated oocyte at early telophase of the second meiotic division following 30-min incubation in culture medium. ×3,600. Some of the microtubules (arrows) at the periphery of the spindle are displaced from the main body of the spindle apparatus.

Fig. 5. Meiotic spindle apparatus of an ethanol-activated oocyte at anaphase of the second meiotic division following 30-min incubation in culture medium. ×4,300. At one pole of the spindle, microtubules (arrows) radiate from the spindle apparatus. The location of the midbodies is irregular and the pole-midbody-pole length is not uniform.

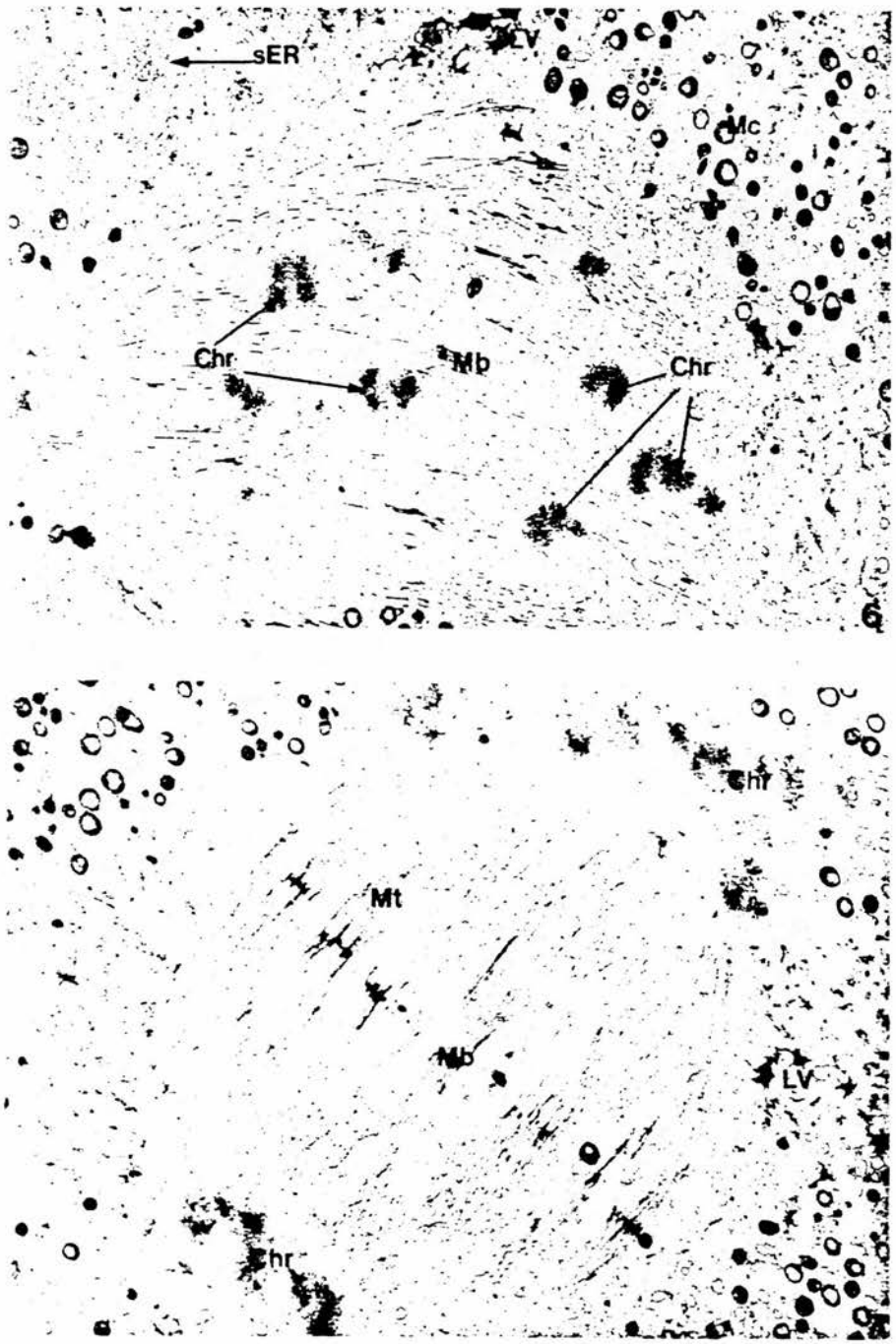


Fig. 6. Meiotic spindle apparatus of an ethanol-activated oocyte at anaphase of the second meiotic division following 30-min incubation in culture medium. ×4,300. The lipid vesicles exhibit a stellate morphology. The mitochondria and vesicles of smooth endoplasmic reticulum are located subjacent to the chromosomes that are to be retained within the oocyte following the completion of the second meiotic division.

Fig. 7. Meiotic spindle apparatus of an ethanol-activated oocyte at telophase of the second meiotic division following 30-min incubation in culture medium. ×4,300. Numerous stellate lipid vesicles are present.

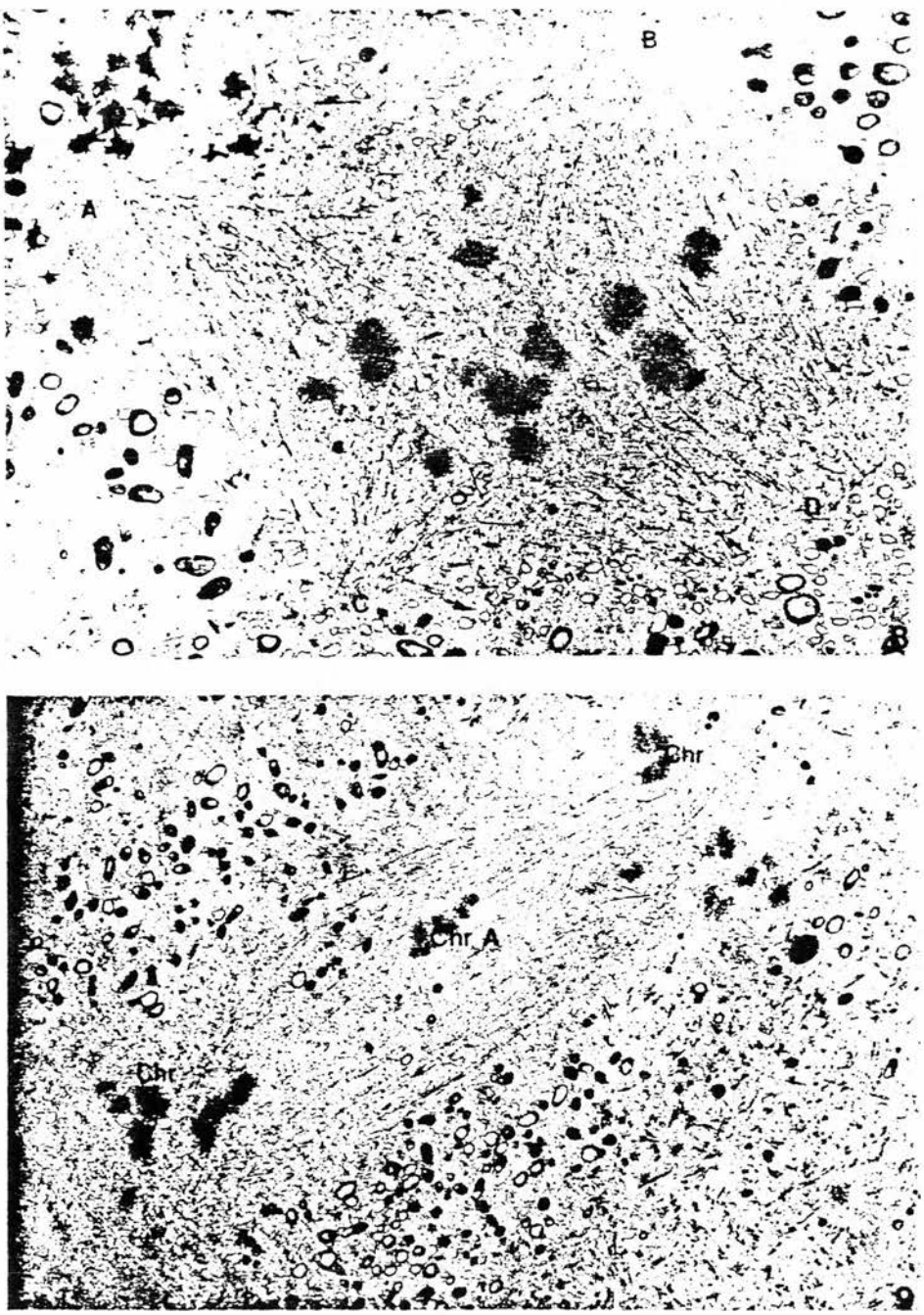


Fig. 8. Meiotic spindle apparatus of an ethanol-activated oocyte at anaphase of the second meiotic division following 30-min. incubation in culture medium. × 4,300. The alignment of the chromosomes is disorganized and arrays of microtubules extend to the chromosomes from four distinct spindle poles (A–D). Stellate lipid vesicles are present.

Fig. 9. Meiotic spindle apparatus of an ethanol-activated oocyte at telophase of the second meiotic division following 30-min incubation in culture medium. × 3,600. One chromatin "body" appears to be "lagging" behind and is located at the equator of the spindle.

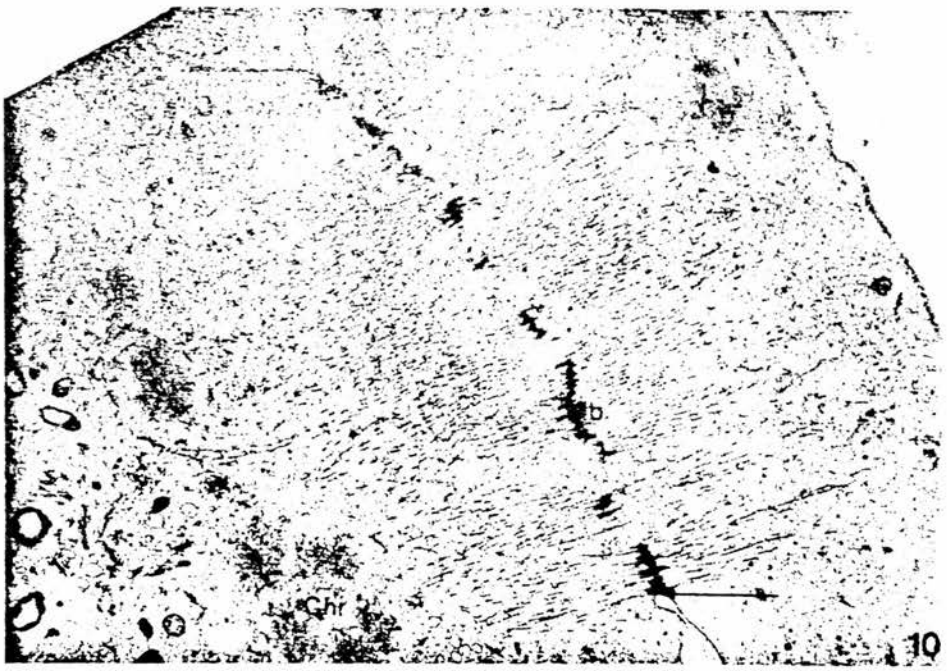
Hyaluronidase activation + 75-min incubation in culture medium. Activated oocytes were at late telophase of the second meiotic division and all oocytes possessed a discrete polar body, the plasma membrane of which was avillous. The main constituents of the polar body were microtubules and chromatin. The latter was located at the pole furthest from the oocyte, while the midbody region was situated at the narrow junction between the oocyte and the polar body. Spindle abnormalities were not observed in any of the 13 activated oocytes in this group.

Ethanol activation + 60-min. + 75-min incubation in culture medium. The activated oocytes in these two experimental groups were at the same developmental stage as the oocytes isolated 75 min after hyaluronidase activation (Fig. 10). Spindle abnormalities were not observed in the five oocytes fixed after 60-min incubation in culture, though abnormal polar body formation was observed in two of the nine oocytes that had been fixed after 75-min incubation in culture. In both of these specimens the protruding avillous region was larger than normal and included numerous cytoplasmic organelles (Fig. 11).

DISCUSSION

The relationship among the plasma membrane, meiotic spindle apparatus, and cytoplasmic organelles in ovulated oocytes has been investigated using immunofluorescence, phase contrast optics, and scanning and transmission electron microscopy [Wasserman and Fujiwara, 1979; Van Blerkom and Runner 1984; Longo and Chen, 1985; Maro et al., 1984; Webb et al., 1986; Longo, 1987]. Similar findings were observed in the nonactivated oocytes isolated at HCG + 17 h in the present study. Szöllösi [1971] reported that in unfertilized eggs the meiotic spindle migrates away from its initial position subjacent to the plasma membrane as the postovulatory age of the oocyte exceeds HCG + 18 h. However, in this study, evidence for this postovulatory age-related change was not observed in the nonactivated ovulated oocytes isolated at HCG + 17 h. Activated oocytes were identified as being those that exhibited evidence of chromosome segregation, spindle rotation, and extrusion of the avillous region of the plasma membrane. The mitochondria were ovoid in appearance and possessed a large and characteristic vesicle that displaced the cristae to the periphery of these organelles [Thibault et al., 1987]. The changes in the cytoskeletal organization of ethanol- and hyaluronidase-activated oocytes has also been observed in fertilized oocytes by using phase contrast optics and immunofluorescence techniques [Sato and Blandau, 1979; Maro et al., 1984; Howlett et al., 1985]. Numerous studies have demonstrated that the frequency of parthenogenetic activation induced by ethanol [Kaufman, 1982; O'Neill and Kaufman, 1988b] or hyaluronidase [Kaufman, 1973; O'Neill and Kaufman, 1988a] is usually in the range of 70–90% and that the majority of the parthenogenones induced by these stimuli develop a single haploid pronucleus following the extrusion of the second polar body. A slightly lower frequency of oocyte activation was observed in this study, possibly because an assessment of the activation frequency was made within a limited period of time after exposure to the activating stimuli.

Cytogenetic analysis of hyaluronidase-induced parthenogenones at metaphase of the first cleavage division has revealed that this stimulus does not induce a significant increase in the incidence of aneuploidy [O'Neill and Kaufman, 1988a].



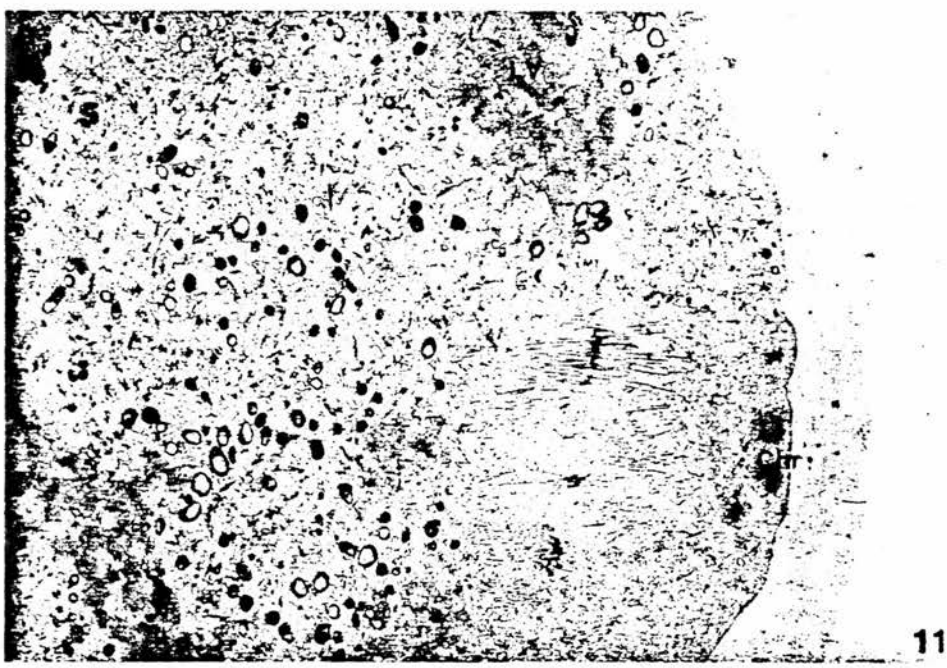


Fig. 10. Meiotic spindle apparatus of a hyaluronidase-activated oocyte at telophase of the second meiotic division following 75-min incubation in culture medium. × 5,900. One set of chromatids is retained within the oocyte, and the other is located subjacent to the avillous zone and will be extruded in the second polar body. The midbodies are positioned at the site of excision between the oocyte and the developing second polar body (arrows).

Fig. 11. The meiotic spindle apparatus of an ethanol-activated oocyte at telophase of the second meiotic division following 75-min incubation in culture medium. × 3,600. The chromosomes to be expelled with the second polar body are seen in this micrograph. The avillous zone of the plasma membrane in this oocyte is abnormally extensive. The numerous vesicles that are present within this region appear to have displaced the spindle apparatus towards the periphery of the egg from its normal location at the junction between the oocyte and the developing second polar body. The lipid vesicles do not exhibit a pronounced stellate morphology.

Other cytogenetic studies have found that a significant proportion of ethanol-induced parthenogenones are aneuploid and that the incidence of aneuploidy is directly related to the duration of exposure to ethanol [Kaufman, 1982; O'Neill and Kaufman, 1988b]. In this ultrastructural study of hyaluronidase- and ethanol-induced oocytes isolated at specific intervals after parthenogenetic stimulation, the arrangement of the cytoskeletal elements in the majority of anaphase and telophase spindles appeared to be normal. However, abnormalities in the alignment of the spindle fibers and/or chromosome segregation at anaphase-telophase were almost invariably confined to the ethanol-activated oocytes. Multipolar spindles, and the persistence of chromatin in the central region of late anaphase and telophase spindles were the two principal forms of spindle anomaly observed. Similar spindle disorders have also been induced by Colcemid [La Fontaine, 1985; Selletto and Kuriyama, 1988] or colchicine [Sugawara and Mikamo, 1980] and may well represent one of the initial events in the sequence that leads to the development of aneuploid parthenogenones. Individual lagging chromosomes were also observed in association with the developing midbodies in telophase spindles, and this would seem to indicate that this phenomenon was not an example of the sequential separation of meiotic chromosomes that is believed to occur normally [Vig, 1983]. The occurrence of spindle abnormalities observed in the present study was higher than the incidence of aneuploidy commonly encountered in ethanol-induced parthenogenones, and would indicate that spindle "abnormalities" are not invariably associated with chromosome segregation errors [Ford and Roberts, 1983].

Ethanol exposure did not induce a direct structural change in the morphology of individual microtubules. The ethanol-induced spindle abnormalities were not obvious until at least 30 min after activation. This would seem to suggest that a minimum period must elapse before a change in the normal ultrastructural morphology of the spindle apparatus is apparent. An increase in the concentration of free intracellular calcium ions is recognised to be one of the earliest physiological changes that occurs following fertilization [Cuthbertson et al., 1981; Miyazaki et al., 1986]. It has also been reported that the potential of numerous stimuli to initiate parthenogenetic activation is directly related to their ability to evoke Ca^{2+}-mediated electrophysiological changes which are related to (but are not necessarily homologous with) those changes that occur at fertilization [Eusebi and Siracusa, 1983]. An increase in the concentration of free intracellular calcium ions is also believed to stimulate the onset of anaphase [Izant, 1983] and the mitochondria, associated sER, and plasma membrane have been identified as sites from which calcium ions are released and sequestered [Kiehart, 1981; Eisen and Reynolds, 1985]. Furthermore, ethanol alters the fluidity of lipid membranes [Goldstein and Chin, 1981] and reduces the capacity for muscle sarcoplasmic reticulum to regulate the sequestration of calcium ions [Ohnishi et al., 1984].

The findings reported here indicate that the ethanol-induced spindle disorders observed in a proportion of ethanol-activated oocytes may have been mediated through the capacity of this agent to disrupt the regulation of changes in the concentration of calcium ions in the proximity of the meiotic spindle apparatus. The regulation of actin filament dynamics is also a calcium-dependent process [Schliwa, 1981; Mooseker et al., 1986], and the finding that two of the ethanol-activated oocytes exhibited abnormal polar body morphology indicates that this process may also be sensitive to the influence of ethanol. The incidence of polar body extrusion is

in any case significantly reduced when the duration of the ethanol exposure exceeds 5 min [Kaufman, 1982; O'Neill and Kaufman, 1988b].

This ultrastructural analysis of recently activated oocytes has identified, in the ethanol-exposed group, the presence of both laggard chromosomes, as well as abnormalities in the arrangement of microtubule arrays at anaphase of the second meiotic division. These deformities in the ultrastructural morphology of the spindle structure may induce chromosome malsegregation if microtubular disorder persists throughout the completion of the second meiotic division. This report represents the first direct ultrastructural analysis of induced abnormalities in the spindle apparatus of oocytes, and strongly suggests that exposure to ethanol and other spindle-active agents in vivo may lead to the production of aneuploid gametes. If similar events occur in human oocytes, this may give rise to genetically unbalanced conceptuses, many of which are destined to be spontaneously aborted during the first trimester of pregnancy.

ACKNOWLEDGMENTS

G.T. O'Neill is supported by a Bonnar Scholarship from the Faculty of Medicine, University of Edinburgh. The work is supported by grants (to M.H.K.) from the National Fund for Research into Crippling Diseases (Action Research for the Crippled Child) and an equipment grant (to M.H.K.) from the Scottish Home and Health Department Ref. No. K/MRS/50/C886.

REFERENCES

Allison AC, Hulands, GH, Nunn JF, Kitching JA, MacDonald AC (1970): The effect of inhalational anaesthetics on the microtubular system in *Actinosphaerium nucleofilum*. J Cell Sci 7:483–499.

Brinkley BR, Rao PN (1973): Nitrous oxide: Effects on the mitotic apparatus and chromosome movement in Hela cells. J Cell Biol 58:96–106.

Cox SM, Rao PN, Brinkley BR (1977): Differential effects of nitrous oxide on the spindle and cytoplasmic microtubule complex. J Cell Biol 75:291a.

Cuthbertson KSR, Whittingham DG, Cobbold PH (1981): Free Ca^{2+} increases in exponential phases during mouse oocyte activation. Nature 294:754–756.

Dyban AP, Khozhai LI (1980): Parthenogenetic development of ovulated mouse ova under the influence of ethyl alcohol. Bull Exp Biol Med 89:528–530.

Dyban AP, Baranov VS (1987): "Cytogenetics of Mammalian Embryonic Development." Oxford: Oxford University Press.

Edwards RG (1958): Colchicine induced heteroploidy in the mouse. I The induction of triploidy by treatment of the gametes. J Exp Zool 137:317–348.

Eisen A, Reynolds GT (1985): Source and sinks for the calcium released during fertilization of single sea urchin eggs. J Cell Biol 100:1522–1527.

Eusebi F, Siracusa G (1983): An electrophysiological study of parthenogenetic activation in mammalian eggs. Dev Biol 96:386–395.

Ford JH, Roberts C (1983): Displacement of chromosomes in mitosis: A technique for assessing differential chromosome error. Cytogenet Cell Genet 36:537–541.

Goldstein DB, Chin JH (1981): Interaction of ethanol with biological membranes. Fed Proc 40:2073–2076.

Hinkley RE, Samson FE (1972): Anaesthetic induced transformation of axonal microtubules. J Cell Biol 53:258–263.

Hinkley RE, Telser AG (1974): The effects of halothane on cultured neuroblastoma cells. Inhibition of morphological differentiation. J Cell Biol 63:531–540.

Howlett SK, Webb M, Maro B, Johnson MH (1985): Meiosis II, meiosis I and the interlinking interphase: A study of the cytoskeleton in the fertilised mouse egg. Cytobios 43:295–305.

Hummler E, Hansmann I (1985): Preferential nondisjunction of specific bivalents in oocytes from Djungarian hamsters (*Phodopus sungorus*) following colchicine treatment. Cytogenet Cell Genet 39:161–167.

Ito S, Winchester RJ (1963): The fine structure of the gastric mucosa in the bat. J Cell Biol 16:541–577.

Izant JG (1983): The role of calcium ions during mitosis. Calcium participates in the anaphase trigger. Chromosoma 88:1–10.

Kaufman MH (1973): Parthenogenesis in the mouse. Nature 242:475–476.

Kaufman MH (1977): Effect of anaesthetic agents on eggs and embryos. In MH Johnson (ed): "Development in Mammals, Vol 1, Amsterdam: North Holland, pp 137–163.

Kaufman MH (1982): The chromosome complement of single-pronuclear haploid mouse embryos following activation by ethanol treatment. J Embryol Exp Morphol 71:139–154.

Kaufman MH (1983a) Ethanol-induced chromosomal abnormalities at conception. Nature 302:258–260.

Kaufman MH (1983b): "Early Mammalian Development: Parthenogenetic Studies." Cambridge: Cambridge University Press.

Kaufman MH (1985): An hypothesis regarding the origin of aneuploidy in man: Indirect evidence from an animal model. J Med Genet 22:171–178.

Kaufman MH, Bain IM (1984): Influence of ethanol on chromosome segregation during the first and second meiotic divisions in the mouse egg. J Exp Zool 230:315–320.

Kaufman MH, O'Neill GT (1988): Aneuploidy induced by ethanol. In Sandberg AA, Vig BK (eds): "Aneuploidy, Induction and Model Systems. Series, Progress and Topics in Cytogenetics." New York: Alan R. Liss, Inc., pp 95–122.

Kiehart DP (1981): Studies on the in vivo sensitivity of spindle microtubules to calcium ions and evidence for a vesicular calcium sequestering system. J Cell Biol 88:604–617.

La Fontaine, Jr. JR (1985): Chromosome segregation and spindle structure in crane fly spermatocytes following colcemid treatment. Chromosoma 91:329–336.

Longo FJ (1987): Actin-plasma membrane association in mouse eggs and oocytes. J Exp Zool 243:299–311.

Longo FJ, Chen DY (1985): Development of cortical polarity in mouse eggs: Involvement of the meiotic apparatus. Dev Biol 107:382–394.

Mailhes JB, Yuan ZP (1987): Differential sensitivity of mouse oocytes to colchicine-induced aneuploidy. Environ Mol Mutagen 10:183–188.

Maro B, Johnson MH, Pickering SJ, Flach G (1984): Changes in actin distribution during fertilization of the mouse egg. J Embryol Exp Morphol 81:211–237.

Maro B, Howlett SK, Webb M (1985): Non-spindle microtubule organizing centers in metaphase II-arrested mouse oocytes. J Cell Biol 101:1665–1672.

McGaughey RW, Chang MC (1969): Inhibition of fertilization and production of heteroploidy in eggs of mice treated with colchicine. J Exp Zool 171:465–480.

Miyazaki S, Hashimoto N, Yoshimoto Y, Kishimoto T, Igusa Y, Hiramoto Y (1986): Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization in golden hamster eggs. Dev Biol 118:259–267.

Mooseker MS, Coleman TR, Conzelman KA (1986): Calcium and the regulation of cytoskeletal assembly, structure and contractibility. In Evered D, Whelen J (eds): "Calcium and the Cell." Ciba Foundation Symposium 122. London: John Wiley, pp 232–249.

Nijhoff JH, de Boer P (1981): Spontaneous meiotic non-disjunction in animals. A study evaluating the various experimental approaches. Genetica 56:99–121.

Ohnishi ST, Flick JL, Rubin E (1984): Ethanol increases calcium permeability of heavy sarcoplasmic reticulum of skeletal muscle. Arch Biochem Biophys 233:588–594.

O'Neill GT, Kaufman MH (1987): Cytogenetic analysis of first cleavage mouse eggs following *in vivo* exposure to ethanol shortly before and at the time of conception. Development 100:441–448.

O'Neill GT, Kaufman MH (1988a): Influence of postovulatory aging on the segregation of chromosomes during the completion of the second meiotic division: A parthenogenetic analysis. J Exp Zool, 248:125–131

O'Neill GT, Kaufman MH (1988b): Cytogenetic analysis of ethanol-induced parthenogenesis. J Exp Zool, in press.

Sato K, Biandau KJ (1979): Second meiotic division and polar body formation in mouse eggs fertilized in vitro. Gamete Res 2:283–293.

Schliwa M (1981): Proteins associated with cytoplasmic actin. Cell 25:587–590.

Sellette C, Kuriyama R (1988): Distribution of pericentriolar material in multipolar spindles induced by colcemid treatment in Chinese hamster ovary cells. J Cell Sci 89:57–65.

Sugawara S, Mikamo K (1980): An experimental approach to the analysis of mechanisms of meiotic nondisjunction and anaphase lagging in primary oocytes. Cytogenet Cell Genet 28:251–264.

Szöllösi D (1971): Morphological changes in mouse eggs due to aging in the fallopian tubes. Am J Anat 130:209–226.

Tease C, Fisher G (1986): Oocytes from young and old female mice respond differently to colchicine. Mutat Res 173:31–34.

Thibault C, Szöllösi D, Gérard M (1987): Mammalian oocyte maturation. Reprod Nutr Dev 27:865–896.

Van Blerkom J, Runner MN (1984): Mitochondrial reorganization during resumption of meiosis in the mouse oocyte. Am J Anat 171:335–355.

Vig BK (1983): Sequence of centromere separation: Occurrence, possible significance and control. Cancer Genet Cytogenet 8:249–274.

Wasserman PM, Fujiwara K (1979): Immunofluorescent antitubulin staining of spindles during meiotic maturation of mouse oocytes in vitro. J Cell Sci 29:171–188.

Webb M, Howlett SK, Maro B (1986): Parthenogenesis and cytoskeletal organization in ageing mouse eggs. J Embryol Exp Morphol 95:131–145.

Whittingham DG (1971): Culture of mouse ova. J Reprod Fertil [Suppl] 14:7–21.