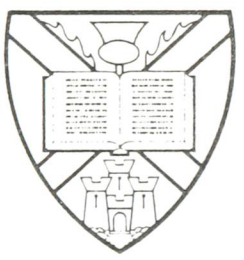


NON-DISJUNCTION IN MAMMALIAN GERM CELLS

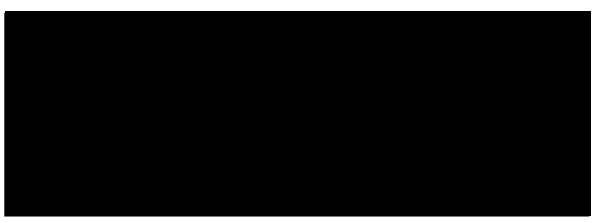
JOHN DAVID BROOK

DOCTOR OF PHILOSOPHY

UNIVERSITY OF EDINBURGH

1983

I hereby declare that this thesis has been composed by myself, and that the work is entirely my own, except for part of Chapter 3, where indicated, which was performed by Mrs. Sue Laing and has already been published by her as part of a M. Phil. Thesis at the University of Edinburgh.



23/8/83

To the memory of my mother, Ann Brook, without whose profound influence during my childhood, such work would never have been performed.

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 7.9)

Name of Candidate John David BROOK

Address [redacted]

Degree Doctor of Philosophy *Date* 23rd August, 1983

Title of Thesis NON-DISJUNCTION IN MAMMALIAN GERM CELLS

In the present thesis consideration has been given to some of the problems concerning human aneuploidy. Its frequency and origins are discussed and the hypotheses to account for it are presented.

Three different experimental approaches have been adopted to examine the aetiology of human aneuploidy. Two of these examine factors responsible for the maternal-age effect, i.e. the phenomenon of increasing aneuploidy with increasing age in the human female. In the first of these the XO mouse has been tested for suitability as a model for the human female; in the second, unilateral ovariectomy has been used as an experimental means of separating the effects of physiological and chronological ageing. This model proved more successful than the first and the implications for maternal-age-related aneuploidy are discussed.

In the third experimental Chapter the potential contribution to aneuploidy induction of germ cell exposure to chemicals is assessed. Three chemicals, one of which was known to be a strong aneuploidy inducer in lower organisms, were tested. The role of chemicals in mammalian aneuploidy induction, and the feasibility of routine testing for aneuploidy by chemical substances is discussed.

In the final chapter, consideration is given to the possible means of reducing the incidence of aneuploid conceptions in man, and to the development of future aneuploidy research.

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I am particularly grateful to Dr. Ann Chandley, my supervisor, for her help, support and for many useful discussions during the experimental work, and also for her reading and useful criticism of the manuscript.

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CONTENTS

Abbreviations

PMS(G)	Pregnant Mares Serum (Gonadotrophin)
HCG	Human Chorionic Gonadotrophin
uni-OVX or OVX	unilateral ovariectomy
E	oestrus
M	Met-oestrus
D	Di-oestrus
P	Pro-oestrus
LH	Leuteinizing Hormone
FSH	Follicle Stimulating Hormone
N.S.A.	Numerical Sex-chromosome Anomaly
EMS	Ethylmethanesulphonate
MMS	Methylmethanesulphonate
pFPA	p-Fluorophenylalanine
6MCP	6-Mercaptopurine
4CMB	4-Chloromethylbiphenyl
NaOH	Sodium Hydroxide
MI	Metaphase I
MII	Metaphase II
Pre-Lept	Pre-Leptotene
Zy	Zygotene
PMI	Pre-meiotic Interphase

PREFACE

TOWARDS AN UNDERSTANDING OF THE AETIOLOGY OF HUMAN ANEUPLOIDY

The term aneuploidy appears to have been introduced into the literature by Täckholm (1922) and describes a condition in which cells, or individuals, have one or more chromosomes, absent from, or in addition to, a euploid complement. The most common mechanism to give rise to aneuploidy is thought to be non-disjunction. Bridges (1913) first used the term non-disjunction to account for the process during *Drosophila* oogenesis in which the two X chromosomes failed to disjoin from each other resulting in the production of 'exceptional' flies with XXY and XO genotypes.

In a strict sense, non-disjunction can be defined as:

> "The failure of chromosomes contained in pairing configurations to separate regularly at anaphase I of meiosis or the failure of sister chromatids to be distributed to opposite cell poles at anaphase II of meiosis or at mitotic anaphase".

In all cases, aneuploid products are the result. Often, however, non-disjunction is a term which is used loosely to describe any defect in the cell process which gives rise to aneuploidy. For example, aneuploidy may also arise by "non-conjunction" associated with pairing failure at meiotic prophase (Belling, 1925) or by "defective centromere division" a phenomenon also known by such names as "pre-division" (Polani and Jagiello, 1976), "pre-segregation" (Hansmann and El-Nahass, 1979), and "precocious centromere division" (Threlkeld and Stolz, 1970). As in the case of 'true' non-disjunction, all these latter mechanisms produce aneuploidy by an error of segregation so that complementary (n+1) and (n-1) products result.

Some aneuploidy may arise through lagging of a chromosome on the metaphase spindle, such that the chromosome is lost from one of the products of the division. Chromosome loss leads solely to the production of (n-1) gametes.

Gametes arising from such errors of division are termed nullisomic (n-1) and disomic (n+1): the offspring resulting from these are monosomic (2n-1) and trisomic (2n+1) respectively.

The defects giving rise to aneuploidy are, for the purposes of the present thesis, of less interest than the factors which operate to bring them about. It is the aetiology of aneuploidy to which the present thesis will address itself.

Of all investigated species, man appears to show a peculiarly high level of aneuploidy among its conceptuses. Indeed it appears to show a spontaneous frequency of momosomy and trisomy of an order of magnitude greater than most other species (Ford, 1975). The contribution of aneuploidy to human foetal loss by spontaneous abortion, to perinatal death, and to the abnormalities among liveborns has been well documented (Carr, 1971; Jacobs *et al*, 1974; Warburton *et al*, 1980). Furthermore it is widely believed that numerical chromosomal abnormalities are responsible for foetal wastage even before pregnancy is recognised (Boue and Boue, 1973; Ford, 1975; and Kajii *et al*, 1978).

In man, there is a very marked increase in aneuploidy associated with maternal ageing, not only for such liveborn conditions as trisomy 21 (Down's Syndrome) but also for many of the other small chromosome trisomies which result in spontaneous abortion (Hassold *et al*, 1980). The cause of this age-related

rise in aneuploidy remains one of the major unsolved problems in human cytogenetics.

According to Penrose and Smith (1966) and Hook (1981) the maternal age dependent increase in aneuploidy accounts for approximately 40%-60% of all cases, consequently the remaining proportion must be associated with factors independent of maternal age. Suggestions to account for this maternal-age-independent aneuploidy include genetic predisposition, use of the contraceptive pill (Read, 1982), and exposure to X-irradiation and environmental chemicals. This latter category has been the subject of increasing interest in recent years. Industries involved in the production and marketing of drugs and other chemical substances, and government bodies have become increasingly concerned with the protection of both individual workers and entire populations from potential exposure.

The work described in the present thesis relates principally to two questions. Firstly; what factors are responsible for or associated with maternal age dependent aneuploidy? Secondly, could chemical substances induce aneuploidy in higher organisms?

In the first part of the thesis the problems of aneuploidy, its frequency, origins, the age-effect and hypotheses to account for it will be outlined. In the second part three different experimental approaches will be reported in which the mouse is used as a model to examine the aforementioned questions on aneuploidy induction.

The first of these three experimental chapters will address itself to the segregation of chromosomes in the XO mouse, an animal which has been suggested by some (Lyon and Hawker, 1973) as a good

model for the ageing human female in terms of aneuploidy production. The second experimental chapter again concerns the role of ageing in aneuploidy production. The use of unilateral ovariectomy on the CBA mouse, to shorten the reproductive lifespan, will be described. By this means physiological ageing of the ovary can be advanced in relation to the chronological age of the mouse. The aim has been to separate the influences of these two types of ageing in order to determine which is more important in influencing age related aneuploidy production. The third and final experiment involves the testing of four chemical compounds on mouse germ cells. All the chemicals were selected either on the basis of their known ability for inducing non-disjunction in lower organisms, or for some indication that they might have a potential for non-disjunction induction in the mouse. Furthermore, the potential of mouse germ cells in the routine testing of chemical substances for aneuploidy induction will be discussed.

PART 1 -- HUMAN ANEUPLOIDY - THE STORY SO FAR

CHAPTER 1

A GENERAL INTRODUCTION

1.1 THE FREQUENCY OF HUMAN ANEUPLOIDY

1.1.1 Evidence from Liveborns

From the data of ten independent chromosome surveys on the newborn human population, compiled by Bond and Chandley (1983), the overall level of aneuploidy at birth is put at 0.31% (see Table 1.1). The three principal autosomal trisomies found in the liveborn studies are those for chromosomes 13, 18 and 21. Other autosomal trisomies which occasionally also survive to term in man are No. 8 (Riccardi, 1977), No. 9 (Feingold and Atkins, 1973), and No. 22 (Bass *et al*, 1973), but these are rare and have never been found in any newborn survey.

Trisomy 13, or Patau's syndrome (Patau *et al*, 1960), has an incidence of approximately 1 in 20,000 births, with the majority of affected infants dying within the first few months of life. Infants with trisomy 18, or Edwards' syndrome (Edwards *et al*, 1960), with an incidence of about 1 in 10,000, also rarely survive for more than a few months. Both trisomic conditions have been found to be maternal age related (Magensis *et al*, 1968; Taylor, 1968).

The most common viable autosomal aneuploid condition, however, is trisomy 21 which constitutes nearly 90% of all the liveborn aneuploid individuals, occurring in about 1 in 600 newborns. This condition known as Down's syndrome, was named after John Langdon Down, a London physician credited with the first extensive clinical description of the syndrome (Down, 1866).

Table 1.1 Frequency of aneuploidy in the human newborn population. (Mosaics excluded) Results of ten independent chromosome surveys (Taken from Bond and Chandley, 1983)

Survey	Number of individuals karyotyped ♀	♂	Total	Sex aneuploids XYY	XXY	XXX	XO	Autosomal aneuploids +D	+E	+G	Total aneuploids	%
1 London (Canada) 1969	1015	1066	2081	4	1	0	0	0	0	2	7	0.34
2 New Haven (USA) 1970	2181	2184	4365	3	4	3	1	1	1	3	16	0.37
3 Edinburgh (UK) 1974	3831	7849	11,680	10	9	5	0	0	2	17	43	0.37
4 Moscow (USSR) 1974	1197	1303	2500	0	1	0	0	0	0	4	5	0.20
5 Winnipeg (Canada) 1975	6763	7176	13,939	4	6	5	0	1	2	14	32	0.23
6 Arhus (Denmark) 1975	5387	5761	11,148	5	8	8	3	1	1	16	42	0.38
7 Jerusalem (Israel) 1975	241	259	500	0	0	0	0	0	0	0	0	0.00
8 Calgary (Canada) 1976	437	493	930	2	1	0	0	0	0	0	3	0.32
9 Boston (USA) 1977	- *	13,751	13,751	11	9	-*	-*	0	0	19	39	0.28
10 Edinburgh (UK) 1980	1921	2072	3993	4	6	3	0	0	1	3	17	0.37
Totals	22,973	41,914	64,887	43	45	24	4	3	7	78	204	0.31

*Females not studied in this survey

References

1. Sergovich, F. *et al* (1969) New Eng. J. Med. 280: 851-855.
2. Lubs, H.A. and Ruddle, F.H. (1970) Science 169: 495-497.
3. Jacobs, P.A. *et al* (1974) Ann. Hum. Genet. 37: 359-376.
4. Bochkov, N.P. *et al* (1974) Humangenetik 22: 139-152.
5. Hamerton, J. *et al* (1975) Clin. Genet. 8: 223-243.
6. Nielsen, J. and Sillesen, I. (1975) Humangenetik 30: 1-12.
7. Cohen, M.M. *et al* (1975) Israel J. Med. Sci. 11: 969-977.
8. Lin, C.C. *et al* (1976) Hum. Genet. 31: 315-328.
9. Walzer, S. and Gerald, P.S. (1977) in "Population Cytogenetics. Studies in Humans", Ed. E. B. Hook and I. H. Porter, pp.45-61.
10. Buckton, K.E. *et al* (1980) Ann. Hum. Genet. 43: 227-239.

The association of Downs syndrome with an extra G-group chromosome, i.e. No. 21, was first demonstrated by Lejeune *et al* (1959) and subsequently confirmed by Jacobs *et al* (1959). Trisomy 21, like trisomy 13 and trisomy 18, arises principally by an error of segregation at meiosis in one or other parent, the aneuploid offspring thus being a simple "primary" trisomic carrying an extra whole chromosome 21. For Downs syndrome, about 95% of all cases arise in this way. The remaining few arise from parents carrying a $D/G^{(21)}$ or $G/G^{(21)}$ Robertsonian translocation, and are thus "tertiary" or "translocation" trisomics. Such cases are also described as "translocation Down's" individuals (Polani *et al*, 1960). There is also a minority of cases which arise by fertilization of a trisomy 21 germ cell produced by a trisomy 21 mosaic individual (Clarke *et al*, 1961). The discussion in this thesis will be confined to Down's syndrome individuals arising as primary trisomics.

A greater contribution to human liveborn aneuploidy is made by the sex-chromosome aneuploids, many of which are viable, although the phenotypic effects of these are much less severe than those of the autosomes. About 1 in every 500 males and 1 in 800 females show non-mosaic sex-chromosome aneuploidy (Table 1.1). This represents 1.7 per 1000 births which are aneuploid for a sex-chromosome compared with 1.4 per 1000 with an autosomal aneuploidy. Of the sex chromosome aneuploids which come to term in man, the four most common are XO and XXX in the female and XXY and XYY in the male.

The XO condition, first diagnosed cytologically by Ford *et al* (1959) for females afflicted with Turner's syndrome

(Turner, 1938) is the least common of the four, arising in the newborn surveys with a frequency of about 1 in every 6000 females (Table 1.1). The XO condition, however, is found frequently in spontaneous abortions (see next section), unlike the other sex-chromosome aneuploidies which are rarely found.

In the same year that the XO condition was linked to Turner's syndrome, Jacobs *et al* (1959)demonstrated the association and Klinefelter's syndrome (Klinefelter *et al*, 1942). This syndrome is characterised by aspermatogenesis and increased follicle stimulating hormone. The XXY karyotype is found with a frequency of about 1 in every 1000 liveborn males.

The XYY condition, associated with tall stature, was first described cytogenetically by Sandberg *et al* (1961). It is found at a frequency of about 1 in 1000 amongst newborn males, and occurs more frequently amongst inmates of reformatories and other penal institutions (Jacobs *et al*, 1965).

The most frequent sex-chromosome aneuploidy amongst liveborn females is trisomy-X, found in about 1 in every 1000 females (Table 1.1). Human XXX females show variable phenotypic features with no clear-cut syndrome. Most are sexually normal and fertile, but in many there is a moderate lowering of intelligence (Ratcliffe *et al*, 1979).

1.1.2 Evidence from Perinatal Deaths and Spontaneous Abortions

In 1974, Machin and Crolla published the first systematic study of the incidence and type of chromosomal abnormalities found amongst stillbirths and early neo-natal deaths. Subsequent studies

by Bauld *et al* (1974), Kuleshov (1976), Alberman and Creasy (1977) and Sutherland *et al* (1978) have established that the level of chromosome abnormality found amongst perinatal deaths is about 5%, i.e. about ten times higher than the level in the liveborn population. The most frequent autosomal trisomies found amongst the perinatal deaths are those for chromosomes 13, 18 and 21. Over 90% of trisomy 13's and 18's die perinatally compared with only 10% of trisomy 21 cases (Machin and Crolla, 1974; Alberman and Creasy, 1977).

Estimates of the spontaneous abortion frequency from all recognised pregnancies vary from 15% (Warburton and Fraser, 1964) to 24% (French and Bierman, 1962). Furthermore, spontaneous abortion surveys have shown that about 50% of all cases are due to chromosomal abnormality (Jacobs and Hassold, 1980). Figure 1.1, however, shows that there is both qualitative, as well as quantitative, variation in foetal loss at different stages of gestation, with the earliest losses including a large proportion which are chromosomally abnormal and the later abortions far less (Alberman, 1981).

Table 1.2, adapted from Bond and Chandley (1983), shows the frequencies of different types of chromosome abnormality found in seven spontaneous abortion surveys in which banding techniques were used. From this it can be seen that over 93% of the abnormalities are numerical, with 70% being aneuploid. The single most common chromosome anomaly is monosomy-X which represents about 20% of the total. Assuming that the minimum estimate, as suggested by Warburton and Fraser (1964), of 15% of all recognised pregnancies result in spontaneous abortion,

Fig.1-1

Percentage of chromosomal abnormalities in abortuses lost after different periods of gestation in three studies (Creasey, Crolla and Alberman, 1976 ; Kajii, personal communication ; Léridon and Boué, 1971) (Taken from Alberman, 1981)

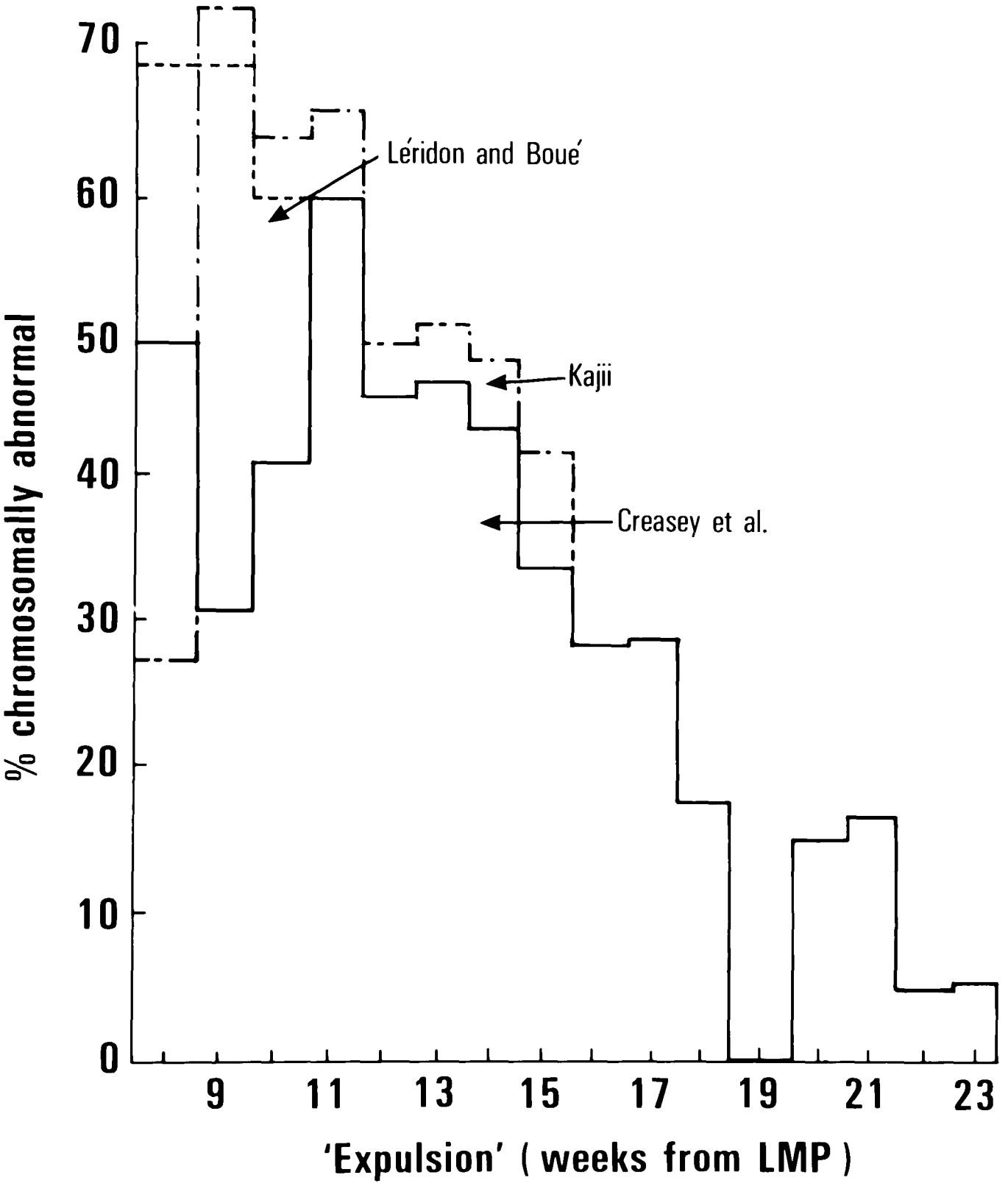


Table 1.2 Frequency of different types of chromosome abnormalities found in six spontaneous abortion surveys employing the use of banding techniques (Taken from Bond and Chandley, 1983)

Survey	45,X	3n	4n	Autosomal trisomy	Mosaicism	Structural abnormalities	Others	Total
Lauritsen *et al* (1972)	12	3	4	14	0	1	0	34
Therkelsen *et al* (1973)	39	14	10	66	4	6	0	139
Kajii *et al* (1973)	12	10	5	51	0	3	1	82
Boue *et al* (1975)	140	183	57	495	10	35	1	921
Creasy *et al* (1976)	68	38	12	143	12	10	4	287
Hassold *et al* (1980a)	112	70	33	212	12	20	4	463
Totals	383	318	121	981	38	75	10	1926
% of all abnormalities	19.9	16.5	6.3	50.9	2.0	3.9	0.5	

half of which are chromosomally abnormal, and 20% of these are XO, then it would appear that 1.5% of all recognised pregnancies are XO. Ford (1981) suggests 1.26 in 1000 (0.13%) as a reasonable frequency for XO zygotes arising through non-disjunction. It would appear, therefore, that the majority of XO zygotes arise by means other than non-disjunction (see later under 'origins' also). In contrast to the frequency of monosomy-X among the abortions, autosomal monosomics are rarely found. This is thought to be because there is very early selection against them *in utero*. In the mouse it is known that virtually all autosomal monosomics die at or around the time of implantation (Gropp *et al*, 1975).

Apart from the peculiar case of monosomy-X however, the contribution of the sex-chromosome aneuploids to spontaneous abortions appears to be negligible. From collective data obtained in several independent chromosome surveys on spontaneous abortions, it would appear that trisomics for the small chromosome (13-22) comprise 77% of the total, with trisomy 16 alone accounting for nearly one-third of the cases (Bond and Chandley, 1983). Trisomies for chromosomes 3, 5, 17 and 19 are rare and trisomy 1 has never been found in a spontaneous abortion (Jacobs and Hassold, 1980). Trisomy 21, despite being the most frequent liveborn trisomy, is also one of the most common trisomics among spontaneous abortions being found in approximately 2% of all cases. Creasy and Crolla (1974) suggest that about 70% of all cases of trisomy 21 are lethal before 28 weeks gestation. It must be remembered, however, that the rate of recovery of a particular trisomic condition amongst the spontaneous abortion sample will depend, not only on the extent to which that chromosome pair undergoes non-disjunction,

but also the extent to which the trisomy is selected against in early gestation.

1.2 THE ORIGINS OF HUMAN ANEUPLOIDY

1.2.1 Data from Liveborn Trisomics

Licznerski and Lindsten (1972) were the first to utilize fluorescent chromosome polymorphisms to trace the origin of the extra chromosome in a trisomy 21 infant to its mother. Such polymorphisms include alterations in size and/or staining properties of heterochromatic regions and chromosomal satellites. They are very stable features of a given chromosomal lineage (Schnedl, 1973) and appear to be without phenotypic effect in the individuals carrying them. As the great majority of such heteromorphic regions are situated at or near centromeres, they are virtually unaffected by crossing-over and are, therefore, ideal markers for tracing the origin of chromosomal anomalies (Caspersson *et al*, 1970). The method has now been used extensively, not only to trace the origins of many autosomal aneuploids among liveborns and spontaneous abortions, but also to trace the origins of polyploidy and some *de novo* structural re-arrangements. See Jacobs and Hassold (1980) for review. These authors have summarized the data on 368 cases of liveborn trisomy 21 for which the parental origin could be established in 158 (43%). Mikkelsen *et al* (1980) have also made extensive studies on liveborn trisomy 21 individuals, using a combination of several different staining techniques. From 110 families the non-disjunctional event was traced successfully by them in 76% of cases. The results from both studies, which include both fully and partially reported cases, are summarised in Table 1.3. Of the 237 cases in which

Table 1.3 The origin of liveborn trisomy 21 as detected by the use of polymorphic markers

	Study		
Origin	Jacobs and Hassold (1980)	Mikkelsen *et al* (1980)	Total
MAT I	92	49	141 (64.4)
MAT II	20	12	32 (14.6)
MAT ?	10	5	15
PAT I	16	10	26 (11.9)
PAT II	18	2	20 (9.1)
PAT ?	2	1	3
	158	79	

Figures in parenthesis represent the percentages of cases in which the origin of the error could be assigned to a specific parental division.

the parental origin could be determined, 188 resulted from maternal meiotic errors whilst only 49 arose from paternal errors (79.3% and 20.7% respectively). Of the 219 cases in which the error could be attributed to a particular division, 141 (64.4%) resulted from a maternal first division error, 32 (14.6%) from a maternal second division error, with 26 (11.9%) and 20 (9.1%) resulting from errors at paternal first and second divisions respectively.

The data in these studies, however, may be biased in two ways. Jacobs and Morton (1977) for example, have pointed out that trisomy due to postzygotic non-disjunction, with loss or non-detection of the monosomic line, would be indistinguishable from second division non-disjunction occurring randomly for maternal or paternal chromosomes. Although this would serve to inflate the relative contribution of second division errors to the aneuploidy level, this figure still remains below 25%. Jacobs and Hassold (1980) have also suggested that the reporting of only selected cases may lead to serious biases which favour paternal non-disjunction, although they are unable to explain why this should be so. In spite of these possible biases, the data in Table 1.3 show quite clearly that the majority of errors (64.4%) resulting in cases of liveborn trisomy 21, occur at the maternal first meiotic division. Roughly equal contributions are made by errors at the maternal second and paternal first and second divisions. First meiotic division errors predominate at all maternal ages for trisomy 21 (Mikkelsen *et al*, 1980) and, where data are available, for all other autosomal trisomic conditions found among liveborns and spontaneous abortions (Jacobs and Hassold, 1980).

The origins of the common sex chromosome aneuploids are shown in Table 1.4. The establishment of Xg blood group phenotypes in patients with sex chromosome abnormalities by Sanger *et al* (1971) and Race and Sanger (1975), has permitted the estimation of the relative contribution of the various maternal and paternal divisions to sex chromosome non-disjunction. Using this method, Cote (1973) has estimated that for XXY males, the relative contributions made by non-disjunction at the maternal first and second, and paternal first division are 38%, 21% and 41% respectively (Table 1.4). The X-chromosome in XO females is maternal in 77% of cases and paternal in 23%, according to Sanger *et al* (1977). Studies of Xg phenotypes in families with XXX children, however, give no information regarding the source of the extra chromosome (Ford, 1981).

1.2.2. Data from Trisomic Spontaneous Abortions

When considering the contribution of 1st and 2nd maternal and paternal divisions to non-disjunction it is somewhat surprising to realise that, in view of the identification of the chromosome involved in specific trisomics in over 1000 spontaneous abortions, the most comprehensive review to date (Jacobs and Hassold, 1980) determines the precise mechanism of origin for only 39 cases, and the parental origin only in a further 10 cases. The results of this survey are given in Table 1.5, which shows that irrespective of the autosome involved in the trisomy, it is almost always maternal in origin (92% of cases). Of the 39 cases in which the precise mechanism of origin was pinpointed, 33 (85%) were due to an error in the first maternal meiotic division. The contribution from errors at second maternal and first and second paternal divisions each made up 5% of the total.

Table 1.4 The origin of the common liveborn sex-chromosome aneuploids

Genotype	Liveborn Incidence (per 1000)	Sub-Group	Source of Error	% Occurrence of Sub-Group Relative to the Whole Type
XXY	1.07	X^mX^pY	Paternal 1st Division	41%
		X^mX^mY	Maternal 1st Division	38%
		X^mX^mY	Maternal 2nd Division	21%
XYY	1.03		Paternal 2nd Division	
XXX	1.04	$X^mX^mX^p$	Maternal 1st Division	
		$X^mX^mX^p$	Maternal 2nd Division	
		$X^mX^pX^p$	Paternal 2nd Division	
XO	0.17	X^mO	*Paternal 1st or 2nd Division	77%
		X^pO	*Maternal 1st or 2nd Division	23%

*See previous section (1.1.2) on aneuploidy in spontaneous abortions, as many XO's would appear to arise by mechanisms other than non-disjunction, although most are lost through spontaneous abortion.

Table 1.5 Origin of trisomy - spontaneous abortions
(124 cases examined - origin determined 40%)

Origin				Chromosome							
♂I	♂II	♀I	♀II	13	14	15	16	21	22	Total	
+	-	-	-				2			2	
-	+	-	-	1			1			2	8%
+	+	-	-							0	
-	-	+	-	3	1	2	12	4	11	33	
-	-	-	+				1	1		2	92%
-	-	+	+	1		2	1	3	3	10	
				5	1	4	17	8	14	49	

From: Jacobs, P.A. and Hassold, T.J. (1980). In "Human embryonic and fetal death" Ed. I. H. Porter and E. B. Hook. Acad. Press Inc. New York, San Francisco and London.

1.3 THE AGE EFFECT AND HUMAN ANEUPLOIDY

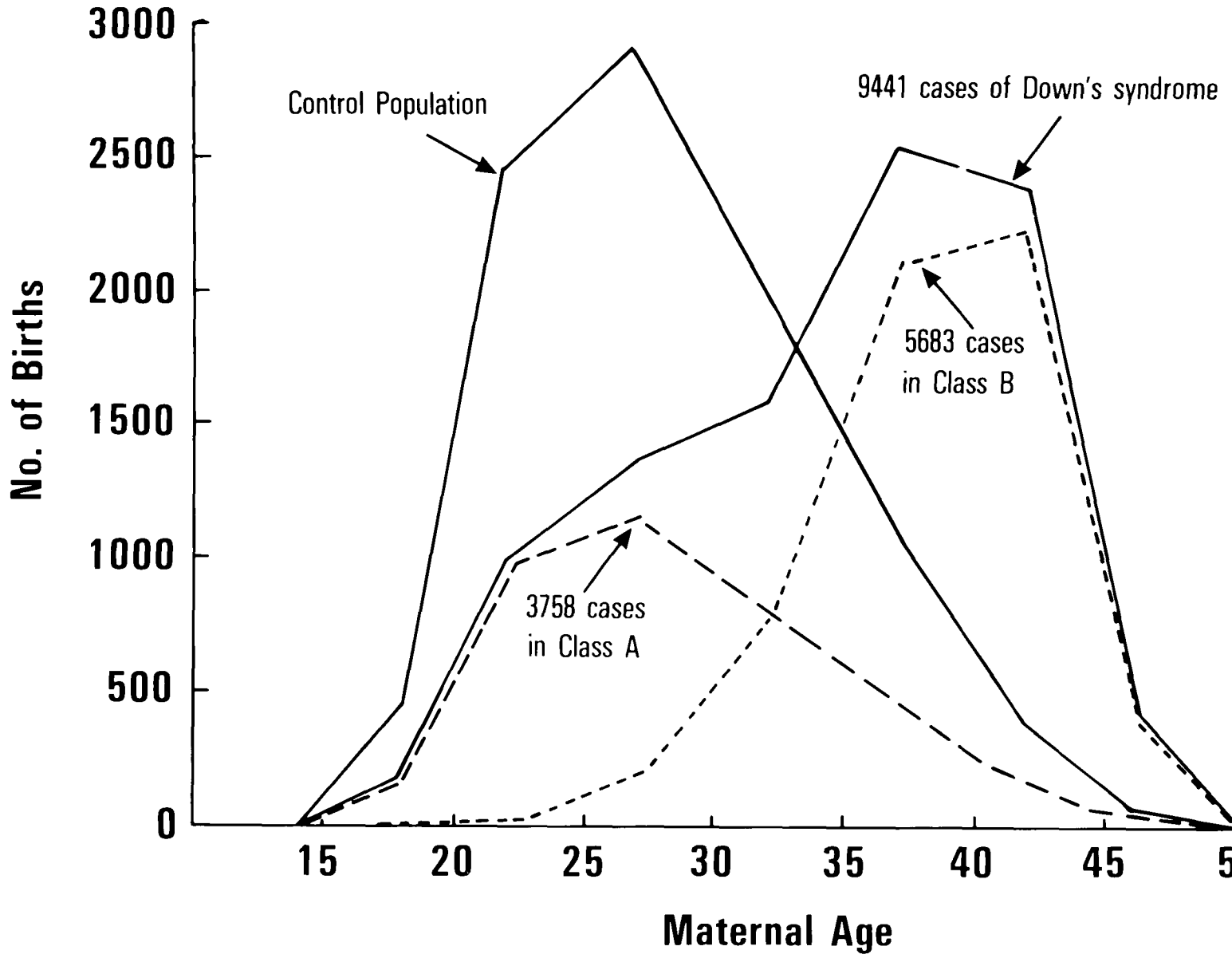
1.3.1 Evidence from the Studies of Liveborns

Despite the observation of Fraser and Mitchell (1876), over one hundred years ago, that a Downs baby was often the last child in a large family, it was not until the 1930's that the relationship between maternal age and Down's syndrome was clearly demonstrated (Jenkins, 1933 and Penrose, 1933, 1934). A mother aged 20 thus has a 1 in 2300 chance of producing a Down's baby compared with a 1 in 880 risk when 30-35 and a 1 in 54 risk over the age of 45 (Penrose and Smith, 1966). This proportion of cases which increase in frequency with maternal age is referred to as "maternal-age related" or "maternal-age dependent". Penrose (1961) has suggested from a proportional analysis that there are two types of Down's syndrome. Apart from the maternal age dependent group there is also a maternal-age independent category and this could well be the same for all other autosomal trisomics resulting from non-disjunctional events. Figure 1.2 shows the distribution of the two categories of Down's syndrome as suggested by Penrose (1961). Class 'A' events are maternal-age independent, show a peak frequency in women aged 28.5 years, corresponding to the peak for all births in the population, and represent 40% of all cases. These include all 'hereditary' cases (translocation trisomics). Class 'B' events, the maternal-age dependent group, represent 60% of all cases and show a peak around 43 years (Smith and Berg, 1976).

The "maternal-age effect" for trisomy 21 is now very well-documented and has been detected across national and racial

Fig.1-2

Maternal age distribution of 9,441 cases of Down's syndrome with control population (Taken from Smith & Berg 1976)



categories with remarkable uniformity in age-dependent incidence rates. According to Hook (1981) the proportions of maternal age independent cases from Swedish, New York and Massachussetts data sources are 62.7%, 57.5% and 49.3% respectively. The differences between them are due to differences in the proportion of older mothers in each of the three populations, nevertheless all are higher than the 40% of class 'A' events suggested by Smith and Berg (1976), perhaps reflecting a changing trend in the ages of women producing children. The proportion of Down's babies born to older mothers will be reduced as the proportion of livebirths to older women declines. Recently it has been suggested that non-disjunction may occur with equal frequencies at all maternal ages with decreased embryonic selection in older women being responsible for the apparent increase in Down's syndrome (Aymé and Lippman-Hand, 1982). This hypothesis, however, has been previously considered by Smith and Berg (1976), and more recently by Carothers (1983) and criticised on a number of grounds. It receives, therefore, little or no support at this time. If the maternal age effect represents an increased risk of non-disjunction in older mothers, rather than an increased chance of carrying a trisomic foetus to term, the 'class B' maternal age dependent group must, by implication, arise through maternal non-disjunction. Moreoever, this may well be due entirely to non-disjunction at the first meiotic division. The 50%, or so, of cases shown to be maternal-age independent could well arise through non-disjunction at first or second, maternal or paternal division occurring at approximately the same frequency. This would produce figures for trisomy 21 arising

through errors at maternal first division of 62.5% (50+12.5) with errors at maternal second and paternal first and second division each constituting 12.5%. These figures do not differ markedly from the levels observed for liveborn trisomy 21 cases in which the origin of the extra chromosome has been traced (see Table 1.3).

The existence of a paternal age effect for Down's syndrome has also been postulated but the subject is still somewhat contentious. Penrose (1933) and more recently Hook and Cross (1982) consider there to be no paternal age effect. Stene *et al* (1977) and Matsunaga *et al* (1978) on the other hand, have reported a significant increase in the incidence of Down's syndrome amongst offspring of very old (> 55 years) fathers. Erickson (1978, 1979) in 3 different samples, also found no evidence for a paternal age effect but the data have been criticised by Stene and Stene (1978) on the grounds that an ascertainment bias may have existed. As Mantel and Stark (1967) pointed out, the reason for the difficulty in demonstrating a paternal age effect is the close correlation between maternal and paternal ages. Although the available data do not rule out age effects in very old fathers, they do rule out age effects comparable in magnitude to those seen in the female (Sved and Sandler, 1981). The relative contribution made by paternal ageing is put into sharper perspective when the origin of the extra No. 21 chromosome in Down's syndrome, largely maternal, is considered (see Table 1.3).

Carothers *et al* (1978) have looked recently at the relationship between parental age and the aetiology of some sex chromosome aneuploids. In their view previous studies which had been made into this question had been complicated by sampling bias of one

kind or another (e.g. Ferguson-Smith *et al*, 1964, and Court-Brown *et al*, 1969). Carothers *et al* (1978) found that the incidence of XXY's and XXX's increased at high parental ages and for XXY's which only arise through paternal non-disjunction, there was a small but significant relationship between incidence and parental age. The close correlation between maternal and paternal age makes it difficult to establish the contributions of each independently. The XXY results could not, however, be explained without a maternal age effect, and could be explained with a maternal age effect alone. The XXX and XYY results could not be explained without either a maternal or a paternal age effect (Carothers *et al*, 1978). With regard to the XXY group of individuals, the maternal age effect may well be stronger than it would appear. As stated previously, about 40% of liveborn XXY males arise through paternal non-disjunction and only 60% are due to maternal errors. Assuming similar survival of XXY's of different parental origin at all gestational ages, it might be predicted that an even stronger maternal age effect would be found were it not for the relatively high proportion of paternally derived cases which tend to mask the effect. Unfortunately, data dealing with parental age and the specific meiotic origin of XXY cases are rare in the literature. Ferguson-Smith *et al* (1964) have reported 5 XXY cases in which the origin of the error and the maternal ages were given. Two of the cases, shown to be maternal in origin, were conceived by women of 39 and 41 years, compared with three XXY's shown to be paternal in origin which were conceived by women aged 17, 27 and 35. The paternal ages were not given, and it was not established at which meiotic division the two XXY cases, shown to be maternally

derived, originated. More data of this kind are required to establish a connection between age and source of error. When considering the effect of parental age on the aetiology of the XXX condition, Carothers *et al* (1978) did not distinguish between maternal and paternal origin of the extra X-chromosome (estimated at 62% and 38% respectively by Ford (1981)). Without further information on parental ages and the origin of the extra X-chromosome, it is not possible here either to establish the contribution made to X-chromosome non-disjunction by increased maternal age.

1.3.2 Evidence from Spontaneous Abortions

From a study of the origin of trisomics in human spontaneous abortions, Hassold and Matsuyama (1979) suggest that there are at least two mechanisms affecting non-disjunction at maternal meiosis I - one acting primarily on older women and certain chromosomes, the other being maternal-age-independent. More recently, Hassold *et al* (1980) have reported on the maternal age effect for autosomal trisomy from a series of over 1500 spontaneous abortions, of which 360 were trisomic. They found that trisomics, as a group, were associated with a substantial increase in maternal age, although considerable differences existed in the magnitude of the effect for different chromosomes. Increasing maternal age had a pronounced effect on trisomics involving the small chromosomes, although for trisomy 16 the mean maternal age was lower than for most other small chromosomes. In fact, Hassold *et al* (1980) found it to be significantly different from the mean maternal ages of chromosomes 13-22 combined. It still displayed, however, a significant, if reduced, relationship with maternal age. Trisomy for chromosomes in groups

A, B and C was associated with a moderate increase in maternal age, although the relationship was less clear than for the small chromosomes. The maternal age effect for autosomal trisomies found by Hassold *et al* (1980) amongst spontaneous abortions, may prove to be more pronounced when only trisomies of maternal first division origin are considered. To date, such an analysis has not been made owing to the limited amounts of data available.

1.4 ANEUPLOIDY AT CONCEPTION - The Projected Figure

In the previous sections it has been stated that aneuploidy levels among newborns, perinatal deaths and early spontaneous abortions are about 0.3%, 5.0% and 70% respectively. These figures have been derived directly by cytogenetic study on the appropriate individuals or material. Figures for the level of aneuploidy at conception, however, can only be estimated indirectly at present. Current best estimates are derived, by extrapolation, from the levels of aneuploidy recorded in early abortions. Such figures will represent the levels of aneuploidy at the time of the clinical recognition of pregnancy. What is not known, however, is the level of zygotic loss, due to aneuploidy, between fertilization and the earliest detection of pregnancy. In the mouse there are good data showing that most monosomics and some trisomics die at or around the time of implantation or even before (Gropp *et al* , 1975) and the same is likely to be the case also in man. Cytogenetic studies have never been performed on such early products of human conception, although recent developments in *in vitro* fertilization may provide the means by which these can be performed if, that is, the ethical problems can be overcome.

The only available direct information on the early errors of human reproduction is that of Hertig *et al* (1956) who examined 34 fertilized ova, all under 17 days gestation, from women undergoing hysterectomy operations. Of these 34 conceptuses, about one-third were stated to be morphologically abnormal. The authors were unable to evaluate how many of these pregnancies would have ended in abortion, although they predicted that at least some would have done so. These studies were carried out before the development of cytogenetic techniques, but, as suggested by Ford (1981), were they to be repeated now they could produce results of the greatest interest.

One method which gives a useful estimate of the frequency of aneuploidy in recognised pregnancies is that of Kajii *et al* (1978), who looked at very early *induced* abortions. They found that 3.2% of such abortuses with gestational ages ranging from 33 to 109 days post-ovulation, had a chromosome abnormality, although they indicated that 5% was the best estimate for a number of such studies combined. Discrepancies can arise, however, if only complete specimens are examined. Such studies produce much lower estimates, as in the case of 1.1% chromosome abnormality for the combined group of 'complete' induced abortuses quoted by Kajii *et al* (1978). Yamamoto and Watanabe (1978) have examined a group of very young abortuses with ages of 21-34 days post-ovulation, and found chromosome abnormalities in 10 out of 108 specimens (9.3%). Several other authors have estimated by extrapolation from the spontaneous abortion figures, that at the time of the clinical recognition of pregnancy 7-8% of all foetuses are chromosomally

abnormal (Jacobs, 1972; Alberman and Creasy, 1977; and Ford, 1981). Of these approximately half are expected to be trisomic.

Estimating the levels of chromosomal abnormality in general, and of aneuploidy in particular, at the time of clinical recognition of pregnancy, is a good deal easier than trying to estimate them at the time of conception. In order to estimate the aneuploidy level at conception from the abortion data, it is necessary to know what the levels of pre-implantational and early post-implantational losses are likely to be. There is considerable variation in estimation of these. The spontaneous abortion rate, based on personal interviews with women, is put at 15% by Roth (1963) and Warburton and Fraser (1964). Other estimates, based on life tables, which allow for early unrecognised losses, are higher with values of 14-29% (Erhardt, 1963; French and Bierman, 1962). Based on the results of Hertig *et al* (1956), Carr (1971) considers that a figure of 30% should be added to the estimate for the clinical abortion rate giving a figure of around 45% for the overall incidence of human conceptual losses. Based on actual birth figures and estimates of unprotected coitus within 48 hours of ovulation (assuming 50% fertilization), Roberts and Lowe (1975) have speculated that foetal loss could be as high as 78%.

One recent study into early conceptual loss carried out by Miller *et al* (1980) has attempted to use the rise in urinary levels of HCG to recognise very early post-implantation pregnancies before they can be recognised clinically. In a group of 197 normal women who were trying to conceive, a total of 623 menstrual

cycles were monitored and 152 women found to be pregnant. From these there were 102 clinically recognised pregnancies, with 50 women only showing biochemical evidence of pregnancy. This represents 33% of all pregnancies which never resulted in clinical recognition, the zygotes presumably being lost before implantation. Of the 102 clinically recognised pregnancies, 87 progressed beyond 20 weeks, 1 was terminated for therapeutic reasons, and 14 resulted in spontaneous abortion. The data are summarised in Table 1.6. This gives an apparent clinical abortion rate of 13.7%, remarkably close to the 15% suggested previously by Warburton and Fraser (1964). Furthermore, the total conceptual loss is put at 43%, virtually the same figure as that estimated by Carr (1971). The proportion of pregnancies progressing beyond 20 weeks, therefore, is only 57% of all those detected biochemically. Even this may be an over-estimate of total conceptual survival since any zygote dying *before* implantation would not be detected by this assay.

In view of the difficulties in estimating the total level of pregnancy loss, outlined above, it would seem that estimates of aneuploidy levels at conception, based on these figures, would be even more uncertain. Nevertheless, Boué and Boué (1973) have suggested that the level of aneuploidy at conception in man may be as high as 45%, and 50% for all types of chromosome abnormality. This is based on the assumption that for every 1000 clinically recognised pregnancies, 150 will abort and, of these, about 100 will be chromasomally abnormal. Amongst these chromosomally abnormal there are 15 with monosomy X and 15 with trisomy 16 on average. Assuming other chromosomes undergo non-disjunction at the same rate

Table 1.6 The outcome of biochemically detected pregnancies (Data of Miller *et al* (1980))

Category	Number of Women	% of all pregnancies detected biochemically	% of all clinically recognised pregnancies
Women detected biochemically as being pregnant	152		
Pregnancies clinically recognised	102	67.1	
Losses prior to clinical recognition	50	33.0	
Spontaneous abortions	14	9.2	13.7
Therapeutic abortions	1		
Pregnancies progressing beyond 20 weeks	87	57.2	85.3

they estimate that there should be 15 x 23 = 345 monosomics and similarly 345 trisomics, or a total of 690 anomalies resulting from non-disjunction alone. When the other chromosomal anomalies are added to the monosomics and trisomics it gives a figure for losses very close to the 850 births which go to term.

Some of the assumptions made in the above calculation are, however, somewhat uncertain and a more modest figure of 20% has been suggested by Ford (1975) for the frequency of all chromosome abnormalities at conception. This figure is based on the assumption that the rate of spontaneous abortion from clinically recognised pregnancies is about 15% (Warburton and Fraser, 1964) with the chromosome abnormality frequency among them being about 60% (Boué and Boué, 1973). This suggests that 12% of them are chromosomally abnormal since some trisomics and effectively all monosomics, which should be at least as frequent as trisomics, are likely to be eliminated without detection. Ford (1975) has concluded therefore that the proportion of human zygotes that are chromosomally abnormal at conception could be as high as 20%.

One alternative method of estimating aneuploidy levels at conception is to analyse the genomic complements in gametes. In man this has been attempted by several authors using two different approaches, in both cases on spermatozoa. Human oocytes in the required stage for analysis cannot be readily obtained.

Early work in this area stemmed from the chromosome banding techniques developed by Caspersson *et al* (1968). Zech (1969) observed that when metaphase spreads from human blood lymphocytes

were treated with quinacrine mustard, the distal portion of the Y chromosome fluoresced brightly. Barlow and Vosa (1970) then proposed that the fluorescent body, observed when human spermatozoa were similarly stained, also represented the Y chromosome. Pawlowitzki and Pearson (1972) attempted to estimate the frequency of meiotic errors involving the Y chromosome in a population of fertile men by estimating the numbers of spermatozoa showing two fluorescent Y-bodies. Subsequently staining methods were developed to identify other chromosome pairs such as No. 1 (Geraedts and Pearson, 1973) and No. 9 (Bobrow *et al*, 1972). Pearson *et al* (1975) estimated that the values for non-disjunction of the Y chromosome and chromosomes No. 1 and No. 9 were 2%, 3.5% and 5% respectively, although standard errors were high in each case.

The reliability of the fluorescent-body technique, however, has been criticised by Beatty (1977 and 1978) who pointed out that the estimated non-disjunction rates of the Y chromosome at the second meiotic division ranged from 4-22%, the magnitude of which he described as "intuitively unacceptable". He suggested that the rates were in fact much lower and that not all Y or 'F' bodies, as he called them, represented Y chromosomes. This was supported by Sumner and Robinson (1976) who found, on the basis of DNA measurements, that many 2F sperm were not in fact YY-bearing. Similar criticism may also apply to estimates of the rate of non-disjunction for other chromosome pairs calculated by these means. Moreover, these methods only allow the non-disjunction levels of individual pairs of chromosomes to be estimated. For an overall estimate it is necessary to assume similar rates of non-disjunction for all

chromosomes in the set. This may not in fact be justified. Different frequencies of trisomy for different chromosome pairs are seen among the human spontaneous abortions and this may reflect different levels of non-disjunction in the gametes giving rise to them. On the other hand, such differences may arise because certain trisomics are subject to early selective loss *in utero*. Warburton *et al* (1980) have argued that the former possibility seems the more likely. Ford (1981) also believes that a similar rate of non-disjunction for all chromosome pairs does not seem plausible, although he suggests that it cannot at present be excluded. One factor which might influence different chromosomes to undergo non-disjunction at different rates is their position in the ordered arrangement of the genome in the cell. Bennett (1982) has shown that in plants the frequency of a particular trisomy is related to the spatial order of the chromosomes. The same could well apply in man.

As the reliability of the Y-body, and other specific chromosome staining techniques has been justifiably criticised, the value of the results has been diminished, and more accurate and reliable techniques have been sought in order to establish the paternal contribution to human aneuploidy. Yanagimachi *et al* (1976) first described the technique of using hamster eggs as "reactivating-vehicles" for human spermatozoa, in an experiment to evaluate capacitation. The first systematic study to analyse directly the chromosome constitution of human spermatozoa was then performed by Rudak *et al* (1978). In this first report, 60 sperm were analysed and 3 were found to be abnormal, 2 of which were hypoploid and 1 hyperploid. More recently, however, Martin *et al* (1982 and pers. comm.) have analysed 948 spermatozoa

from 31 normal men and found 82 (8.6%) to be chromosomally abnormal. Of these 49 (5.2%) were aneuploid and a further 3.4% carried structural abnormalities, or a chromosome break gap or deletion. For the 49 described as aneuploid, 26 were hypoploid, 9 of which also carried an additional abnormality, and 23 were hyperploid, of which 6 also carried an additional abnormality.

Sufficient numbers to assess individual variation were not available, but it seemed that there was a range of 0-14% abnormalities among individual males. Of interest among the aneuploid complements was one 24, Y, +1 (no trisomy 1 abortus has yet been found), and only one complement 22, -C or Y that could have given rise to a 45, X embyro. All chromosome groups were represented among the aneuploid complements.

For an aneuploidy level of 5% in spermatozoa, and given at least an equal contribution from the female, an overall level of aneuploidy at conception in man would thus be estimated as 10%. However, the observations of Jacobs and Hassold (1980) and Mikkelsen *et al* (1980) suggest that the extra chromosome in patients with Down's syndrome appears to be of maternal origin in 75 to 80% of cases for both young and advanced age groups, with the vast majority occurring at the first meiotic division (see Table 1.3). Similarly for trisomics amongst spontaneous abortions (see Table 1.5) the additional chromosome is almost always of maternal origin (about 90% of cases) (Jacobs and Hassold, 1980). Assuming no differential loss of trisomics with respect to the parental origin of the extra chromosome prior to the clinical recognition of pregnancy, an approximately 4 times greater number of aneuploid eggs, than

aneuploid sperm might then be expected. The level of aneuploidy amongst oocytes, therefore, could be as high as 20% giving an overall level of aneuploidy at conception of about 25%. This is speculation however, and remains to be confirmed by direct cytogenetic observation.

1.5 HYPOTHESES TO ACCOUNT FOR ANEUPLOIDY IN MAN

Numerous hypotheses to account for the origin of aneuploidy arising by non-disjunction, and in particular that related to maternal age, have been proposed. Many, however, remain untested.

Probably the first idea was put forward by Bridges (1913) at the time he introduced the term non-disjunction. He suggested that the XXY and XO exceptions, found during his *Drosophila* experiments, arose as a result of "microtubular malorientation" or "chromosomal entanglement". Since that time, others have postulated a role for the incomplete or damaged spindle in aneuploidy production. Penrose (1965), for example, suggested that some meiotic errors might arise because of the decay of spindle fibres over the long period which elapses in women between prophase of meiosis in the foetal ovary, and ovulation of the egg in the adult, perhaps even decades later. Alberman *et al* (1972) questioned this on the grounds that spindle fibres were not formed during the long arrested diplotene stage of the egg, but suggested, as an alternative, that the components required to assemble a spindle may be subject to wear and tear over the years. Spindle failure has also been implicated by Mikamo (1968) to account for maternal age dependent aneuploidy, or, more specifically, aneuploidy occurring at the

first maternal meiotic division. In this case, however, rather than incomplete spindle assembly, it is suggested that non-disjunction might be caused by the degeneration of spindles in association with intrafollicular over-ripeness of the egg. Disintegration of spindle fibres especially at polar ends and hypertrophy of spindles was noted from sectioned *Xenopus* eggs which had been kept without undergoing ovulation for over one year (Mikamo, 1968). Mikamo and Hamiguchi (1975) have since made similar observations in the rat. Butcher and Fugo (1967) induced a delay of 48 hours in ovulation in the rat by administering phenobarbitol sodium and found a significant effect on fertilization rate, chromosomal anomalies (including aneuploidy) and embryonic death. Pre-ovulatory over-ripeness, arising as a consequence of spontaneously occurring irregular cycles in the aged female rat, has also been shown to have a detrimental effect on embryonic development, although chromosomal analyses were not performed in this case (Fugo and Butcher, 1971). Two studies in man which, although from limited data, further support intrafollicular over-ripeness as a cause of fetal wastage were both published over 15 years ago. Iffy (1963) presented data showing that of 19 cases of abortion in which the conception date was known, 14 of the women had conceived after day 17 of the menstrual cycle. Also Hertig (1967) reported that the human female who ovulates and menstruates regularly has a 92% chance of producing a normal offspring if she ovulates and conceives on or before day 14. If ovulation occurs on day 15 or later in the cycle however, the possibility of a normal conception drops to 43%. These two studies on humans, like many of those on the rat (e.g. Fugo and Butcher, 1966, 1971; Butcher *et al*, 1969) do not

provide cytological data to back up the idea that the increased levels of embryonic anomaly are due, at least in part, to increased levels of aneuploidy. This is inferred, however, in most cases, and is supported by the study of Butcher and Fugo (1967) in which an increased level of numerical chromosome abnormality was reported following delayed ovulation. Recently, however, Laing (1983) has looked at the effect of delayed ovulation in aged mice using two different means to produce the delay, and found no significant increase in the aneuploid complements at MII. Some pre-segregation errors were recorded but these did not reach significant levels. Substances which act directly on the spindle, e.g. Colchicine, have been demonstrated as marked aneuploidy inducers (Sugawara and Mikamo, 1980). Recently Kaufman (1983) has reported that the products of non-disjunctional errors arising at the second meiotic division can be seen in the female derived chromosomes of one-cell embryos of mice exposed to dilute solutions of ethanol. An incidence of aneuploidy, varying with dose, of up to 20% was found. He suggested that alcohol may act as a spindle disrupting agent and that it and other spindle-acting agents may be the cause of certain types of chromosomal defects (such as trisomy and monosomy) commonly observed in human spontaneous abortions. Kaufman (1983) argued that alcohol was equally likely to induce maternal first meiotic division errors, those predominantly found amongst spontaneous abortions and human liveborns (Jacobs and Hassold, 1980). The results, however, do not account for the increase in aneuploid offspring found with increasing maternal age unless one speculates that alcohol consumption increases in older women.

That hormones may be implicated in the induction of non-disjunction due to intrafollicular over-ripeness has been suggested by Fugo and Butcher (1971). They postulate that with ageing of the female there develop alterations in the secretion rate of the various gonadotrophic factors which control ovulation. This could result in irregularities in the length of reproductive cycles which could, according to Mikamo (1968), in turn lead to non-disjunction. There are numerous other reports suggesting hormonal involvement in non-disjunction induction. Rundle *et al* (1961) have reported increased androgenic hormone levels in mothers of Down's babies, and attempts have since been made to relate fetal chromosome abnormalities to maternal urinary oestradiol excretion (Jørgensen and Trolle, 1972; and Blumenthal and Variend, 1972). The observation of Carr (1967) that chromosome anomalies were found in 6 of 8 abortuses collected from women who become pregnant after taking oral contraceptives, led to numerous studies on the effects of hormones on chromosome abnormality induction, the results of which have proved equivocal. Bracken *et al*(1978) found, from a study involving 1,370 offspring with congenital malformations and almost 3,000 controls, that maternal oral contraceptive use in the year before conception, or even during pregnancy, was unrelated to malformations as a whole. They did report, however, a twofold excess of Down's syndrome amongst the offspring of women who had been taking oral conctraceptives at, or shortly after, the time of conception, although the numbers upon which this conclusion was based were very small. Evidence in support of the hormonal induction of non-disjunction in man comes from Harlap *et al* (1979) who have shown that in 814 babies resulting from breakthrough pregnancies, i.e. pregnancies resulting in women still using the contraceptive

pill, 3 had autosomal trisomics (3.69/1000) compared with 42 amongst 32737 controls (1.28/1000). They also show that in data collected from a number of studies on breakthrough prenancies the incidence of Down's Syndrome is four times that normally found at birth. Read (1982) has also argued in favour of an association between the taking of the contraceptive pill and an increase in Down's Syndrome among young mothers in recent years.

There have been a number of animal studies presenting evidence both for and against the hormonal induction of aneuploidy. McGauhey (1977) for example demonstrated increased levels of diploidy and hyperhaploidy at metaphase II in oocytes cultured in either progesterone or oestradiol 17 , although no effect was observed for oocytes cultured in both steroids. Jagiello and Lin (1972), Fechheimer and Beatty (1974), Maudlin and Fraser (1977), and Tease (1982b) have all failed to induce aneuploidy by hormone treatments in a variety of animals. Hansmann (pers. comm.) however, using the Djungarian hamster has shown a substantial increase in hyperploids (0-10%) amongst eggs ovulated using higher levels of PMS and HCG than those normally used for routine superovulation. (The effect of hormonal imbalance on aneuploidy induction is considered more fully in Chapter 3.)

Crowley *et al* (1979) have proposed a "Chiasma-hormonal hypothesis" relating to maternal age which suggests interaction between the hormonally-governed rate of meiosis (chromosomal alignment on the spindle) and the timing of chiasma terminalization. They suggest that as hormone levels and lengths of cycles change with advancing maternal age, so delays will occur in the attachment of bivalents to the spindle. Thus there will be an increasing chance

of losing chiasma (particularly from the smaller bivalents like the No.21 in man) by terminalization before proper alignment occurs. Consequently an increasing risk of non-disjunction will arise.

The latter part of the "Chiasma-hormonal hypothesis", implicating premature chiasma terminalization, incorporates ideas suggested earlier by Henderson and Edwards (1968) in their "Production-line hypothesis". This latter model, based on observations of decreased chiasma counts and increased numbers of univalents in oocytes of ageing female mice, envisages gradients (nutritional or developmental) in the foetal ovary, leading to differences in chiasma frequency along a production line of oocyte formation. Oocytes formed early in the foetal ovary, it is postulated, would be ovulated early in life; those formed later would be ovulated at advanced age. Furthermore, the univalents in aged oocytes with reduced chiasma frequency, would segregate randomly at first meiotic division producing aneuploid gametes. Subsequent work by Luthardt *et al* (1973), Polani and Jagiello (1976) and Speed (1977) has confirmed the decreasing chiasma frequency and/or increase in the number of univalents with age in the mouse. Whether the presence of such univalents would necessarily result in the subsequent production of aneuploid gametes has however been questioned. Polani and Jagiello (1976) and Speed (1977) failed to show any significant correlation between univalent presence at MI and aneuploidy at MII. More recently, Sugawara and Mikamo (1983) have looked at the correlation between changed chiasma configuration and meiotic non-disjunction in the aged Chinese hamster. As it is possible to classify the eleven meiotic chromosomes into four morphologically distinct groups,

these authors were able to determine whether the univalents seen at MI underwent normal segregation at anaphase I. Their results clearly showed that there was no correlation between the univalents seen at Metaphase I, which were all small in size, and the aneuploid complements at Metaphase II which involved chromosomes of all sizes. The production line hypothesis, therefore, remains the subject of discussion (Speed and Chandley, 1983). Similar uncertainties surround the Chiasma-hormonal hypothesis.

Another hypothesis to explain the age-related aneup loidy in women has implicated nucleolar persistence at meiotic prophase in the oocyte. Polani *et al* (1960) first suggested that nucleoli in aged oocytes might be more resistant to the normal nucleolar breakdown process at the end of meiotic prophase and this might be an important factor in the aetiology of trisomy involving acrocentric chromosomes such as chromosome 21 in Down's syndrome. Furthermore, following the findings of Collman and Stoller (1962), that a correlation might exist between Down's syndrome and infective hepatitis, Evans (1967) argued that any such increases in trisomy 21 might be due to an effect of the virus on the nucleoli of the ova, resulting in a reduced capacity for the dissolution of this organelle during meiosis and hence a failure of separation (non-disjunction) of half-bivalents sharing a common nucleolus. Even without viral infection, Evans (1967) has argued that this abnormal persistence of nucleoli during meiosis in older women may be a major aetiological factor in Down's syndrome. The accumulation of cytogenetic data on spontaneous abortions has shown, however, that this hypothesis cannot account for those frequent trisomies which involve chromosomes lacking nucleolus organiser regions, and yet are strongly age related, e.g.

trisomies 17 and 18. There are also other very common trisomies among the spontaneous abortions involving non-acrocentric pairs, such as trisomy 16, which are only weakly age related. Hassold and Matsuyama (1979) have pointed out that because of these inconsistencies, the general applicability of this hypothesis is limited.

To account for the maternal age effect for Down's syndrome in man, German (1968) has suggested that post-ovulatory over-ripeness of the egg resulting from delayed fertilization may arise with advancing maternal age as the frequency of coitus declines. It has long been known that the longer the time interval between ovulation and fertilization, the greater the chance of a developmental anomaly. Blandau and Young (1939) showed that in the guinea-pig, delayed insemination of up to 20 hours caused a reduction of 50% in subsequent litter size and a seven-fold increase in abnormal pregnancies. Furthermore they suggested that delayed fertilization might be a cause of early abortion in the human female. Delayed fertilization in the rabbit was shown by Austin (1967) to increase the numbers of hypoploid blastocysts over control levels, but number looked at were small in both groups. Vickers (1969) demonstrated a 50% increase in the incidence of chromosome anomalies amongst mouse embryos for which fertilization had been delayed 7 to 13 hours with a 9-fold increase in the incidence of triploidy. There was, however, little increase in aneuploidy, particularly hyperploidy, apart from the occasional case. Delayed fertilization is no longer considered to be the cause of the maternal age effect in man for a number of reasons. Firstly there is the failure of several of the previously mentioned experimental studies to

demonstrate an increase in aneuploidy with delayed fertilization. Secondly, other studies have shown that the age dependency for the frequency of coitus is not closely enough related to the incidence of Down's syndrome to support the German hypothesis (Penrose and Berg, 1968; Cannings and Cannings, 1968). This argument has recently re-surfaced (Mulcahy, 1978; Milstein-Moscati and Beçak, 1983; James, 1983). Another piece of evidence, and probably the most significant, against this hypothesis comes from those studies in which the origin of the extra chromosome in trisomy 21 conceptuses (both liveborn and amongst spontaneous abortuses) has been traced. These have shown the error to occur largely at the first maternal meiotic division, i.e. prior to ovulation, so that any subsequent delay in fertilization could not influence events. Delayed fertilization may, however, still be responsible for some aneuploidy induction at the second division. Rodman (1971) has shown that, after prolonged sojourn in the oviduct, the disjunction of MII chromatid pairs sometimes occurs before fertilization. This could thus account for some aneuploidy arising at the second meiotic division.

There are two further hypotheses which have received a great deal less attention than those already mentioned. Both attempt to show a correlation between physiological factors and Down's syndrome, although neither hypothesis attempts to explain the 'maternal age effect'. One was put forward by Fialkow (1966) who suggested that the presence of thyroid auto-immune phenomena in women might be associated with a higher risk of Down's syndrome in the offspring. It was suggested that the maternal immunologic abnormality more directly produced the child's aneuploidy, although

no mechanism of origin is advanced (Fialkow, 1966). Fabricant and Schneider (1978) have looked at aneuploidy levels in oocytes from two inbred mouse strains - A/J and NZB/J - which both manifest autoimmunity, and have, however, found opposite effects. They concluded that the failure of thymectomy, to increase level of aneuploidy in the A/J strain, together with the lack of a substantial increase in aneuploidy in the NZB/J strain, suggests that auto-immunity indicated by the presence of anti-nuclear antibodies, does not play an important role in aneuploidy induction.

In recent years there have been a number of reports suggesting a correlation between α_1-antitrypsin type and non-disjunction. α_1-antitrypsin (α_1-protease inhibitor (PI)) is a major inhibitor of the activity of a variety of proteolytic enzymes, and is made up of a number of different variants. Compared to the common PI type 'M' (representing 80% of the population), some of the 'non-M' variants, e.g. 'S' and 'Z' show lower serum levels. The first report of a correlation between chromosome anomalies and PI type was made by Aarskog and Fagerhol (1970) who found a higher number of 'non-M' variants amongst subjects with sex chromosome mosaicism (4 of 7) compared with 10% in the general population. An increased 'non-M' frequency in patients with Down's syndrome has been found by Fineman *et al* (1976) and Jongbloet *et al* (1981). Contrary findings have been produced, however, by Arnaud *et al* (1976) and Guanti and Di Loreto (1980) who found a normal frequency of variant PI types amongst Down's syndrome patients.

Of more interest perhaps is the observation that there is an increased frequency of 'non-M' variants amongst the mothers of

Down's children (Aarskog and Fagerhol, 1970; Jongbloet *et al*, 1981). A significantly increased frequency of non-M variants was found by Jongbloet *et al* (1981), for mothers of Down's babies in whom the non-disjunctional event had occurred at the first meiotic division. It was suggested that proteolytic imbalance due to a lack of natural inhibitor in heterozygous mothers may affect the stability of the microtubule networks and result, therefore, in non-disjunction. However, no increase was found in the frequency of 'non-M' variants amongst the fathers of Down's children in whom the extra chromosome was paternally derived. These findings, although interesting, tend to be based on small sample sizes and should therefore be viewed with some caution. Further studies could perhaps provide greater insight into the relationship between α_1 antitrypsin type and non-disjunction.

Finally it should be mentioned that despite a drop in the birth rate of older women in Denmark between 1960 and 1971, the expected corresponding decrease in the incidence of trisomy 21 over the same period was not found (Mikkelsen *et al*, 1976). Similar observations had been reported by Uchida (1970) in Canada, and indications also come from studies in the U.K. (Read, 1982), and Sweden (Nordensen,1979). In all these reports environmental factors such as X-irradiation, chemical substances or contraceptive hormones (Read, 1982) have been implicated in aneuploidy being induced independently of maternal age. Exposure to X-irradiation has long been known to induce non-disjunction (Mavor, 1924; Uchida and Lee, 1974; Tease, 1982a) although no increase in sensitivity to radiation appears to occur with increasing maternal age, at least in the mouse (Tease, 1982a).

Certain chemicals will also induce non-disjunction (Sugawara and Mikamo, 1980) although chemical effects have been less well investigated than those of X-rays. It is, however, the subject for further consideration in Chapter 4. The possible role of hormones and hormonal imbalance in non-age related aneuploidy, as well as that related to physiological ageing of the ovary, will be discussed in Chapter 3.

PART II

HUMAN ANEUPLOIDY - AN EXPERIMENTAL APPROACH

CHAPTER 2

X-CHROMOSOME SEGREGATION, MATERNAL AGE AND ANEUPLOIDY IN THE XO MOUSE

2.1 INTRODUCTION

Unlike XO women, XO mice are fertile, albeit subject to reproductive impairment (Lyon and Hawker, 1973). They do, however, show a similarity to the human female in that fertility ends through a depletion of oocytes (Faddy *et al*, 1983), unlike most other mouse strains which still have many oocytes left in the ovaries at the cessation of fertility. Lyon and Hawker (1973) suggest that XO mice may pass through a period of irregular oestrous cycles towards the end of their reproductive life, during which time hormonal imbalance may occur leading to aneuploidy.

That the XO mouse should undergo a period of irregular cyclicity towards the end of reproductive life is not so remarkable in itself. Studies on other strains show that before cycling ceases, a period of irregular cyclicity occurs (Thung *et al*, 1956; Thung, 1961; Nelson *et al*, 1982). This irregular cyclicity, however, combined with the virtual depletion of oocytes from the ovary of the XO mouse, indicate that it could provide a useful model for the situation in human pre-menopausal females, where non-disjunction occurs with a high frequency leading to the birth of aneuploid children.

The segregation of the single X chromosome in the XO oocyte has been the subject of controversy ever since Cattanach

(1962) established the first breeding stock of XO mice. From his own breeding data, Cattanach (1962) observed that although litter size was near normal for this particular stock, a shortfall of some 30-37% in XO compared to XX offspring occurred from XO mothers. He was unable to determine the reason for the reduction in XO progeny, but as one possibility, suggested that preferential loss of the chromosome sets lacking an X chromosome to the polar bodies in the meiotic divisions of the ova might have occurred. The alternative was that death of the missing classes during embryonic development had taken place. To account for the higher than expected litter size in XO mothers, he did not, however, discount the possibility of early loss of inviable embryos, compensated by the development of all individuals of the viable classes, some of which would have been lost in larger normal litters as a result of overcrowding in the uterus.

In a subsequent study Morris (1968) examined reproductive performance and embryonic mortality in a large series of XO and XX females. One series of pregnant females of both genotypes was dissected after 15 days gestation and another series after 3½ days. From his finding, he concluded that there could be both an abnormally low segregation of nullo-X gametes in XO females and a reduction in viability of XO foetuses during the early stages of gestation. This lower viability of XO's *in utero* contrasted with their seemingly normal viability after birth. Strong circumstantial evidence was also found for the death of all OY zygotes before implantation.

Direct cytological information of the segregation of the X chromosome has since been obtained by several groups of authors

analysing chromosomal complements in the metaphase II oocytes ovulated by XO females. The results however, are conflicting (see Russell (1976) for review). According to Evans and Ford (unpublished data), segregation of the X to egg or polar body is random. The data of Kaufman (1972) and Luthardt (1976), however, suggest that it is non-random, with the X-bearing chromosome sets being preferentially included in the egg nucleus.

A further complicating factor is the claim made by Deckers *et al* (1981), from breeding data on XO mice, that the phenomenon of non-random segregation is maternal-age related. These authors found a greater recovery of XO progeny relative to XX as the age of the mother (or litter number) advanced.

In the present chapter an attempt is made to clarify some of these issues by combining breeding data on a large series of XO mice at a range of ages, with a cytological analysis of ovulated metaphase II oocytes in young and old animals.

2.2 METHODS AND MATERIALS

2.2.1 Animals Used

The colony of XO mice used was set up from mice kindly supplied by Dr. Mary Lyon, MRC Radiobiology Unit, Harwell, England. The sex-linked gene, Tabby, was used as marker. Normal-coated XO females mated to Tabby males produced three types of phenotypically distinct offspring; normal-coated males (+/Y), greasy-coated females (Ta/O) and striped females (Ta/+). The Ta/O and +/Y offspring were subsequently used as breeders to regenerate +/O and Ta/Y animals, with striped females (Ta/+) again being produced.

In this way all offspring could be identified from their coat colours. Brother-sister matings were avoided. The stock was checked occasionally, by blood karyotying, to ensure that all supposed phenotypic XO females were in fact of the XO genotype.

Offspring were classified at weaning and female breeders used until they reached 36 weeks of age, after which time they were killed and used for oocyte chromosome analysis.

2.2.2 Oocyte collection and chromosome preparation

The female mice used for chromosome analysis in metaphase II oocytes constituted six groups, divided according to age and phenotype. There were two XO groups, i.e. +/O and Ta/O with Ta/+ sibs serving as controls. Analysis was carried out over two different ages within each group, i.e. 8-20 weeks (young) and 30-40 weeks (old) (Lyon and Hawker, 1973 have shown that in XO mice, both age-related ovarian changes and reduced reproductive performance are detectable by 28 weeks).

Each female was superovulated using 2.5iu pregnant mares serum (PMS) and 2.0iu human chorionic gonadotrophin (HCG) given 48 hours later. The oocytes were sampled 15 hours after HCG injection at a time corresponding to metaphase II. Hansmann and El Nahass (1979) have previously shown that these hormone doses do not affect the incidence of non-disjunction during the first meiotic division in the mouse oocyte. Mice were killed by cervical dislocation, the oocytes being removed from the ampullae of the fallopian tubes and fixed by the method of Tarkowski (1966). The preparations were C-banded according to the method of Sumner (1972) and chromosome counts made.

2.3 RESULTS

2.3.1 Birth Data

Birth data were obtained from both Ta/0 and +/0 mothers (Table 2.1). Analysis showed that there was a significant difference between the two breeding groups ($X^2_3 = 9.73$; $p < 0.05$), this being due to a higher level of deaths among offspring from Ta/0 mothers than +/0 mothers between birth and weaning. Ta/0 females are generally less robust than +/0 females and appear to be less competent as mothers. The data at weaning, showing increased death of offspring from Ta/0 mothers compared with +/0 mothers, supports the findings of Cattanach (1962), Morris (1968) and Deckers *et al* (1981), although the difference between the two types of mother is lower in the present study than has been found by these other authors.

There was no significant difference in the distribution of XY; XX; XO offspring at weaning from the two types of XO mother. Neither was there any difference in the birth ratio of XX; XO offspring between the two maternal genotypes, the proportion being 1.88:1 for Ta/0 mothers and 1.89:1 for +/0 mothers. Both ratios were lower than those found by earlier investigators (Table 2.2) indicating that comparatively more XO progeny were born to XO mothers in our stocks.

2.3.2 Chromosome Counts

A total of 379 metaphase II (MII) counts were made from 82 XO females and 179 counts from 28 XX females. The results have been grouped in Tables 2.3 - 2.5 according to maternal age and genotype. Cells giving counts of less than 17 were few in number, tending to

Table 2.1 Birth data from XO mice

Mating Type	Pairs	Litters	Total Animals at Birth	Animals at Weaning XY	XO	XX	Dead
Ta/O ♀ X +/Y ♂	50	177	624	188 (30.1%)	113 (18.1%)	212 (34.0%)	111 (17.8%)
+/O ♀ X Ta/Y ♂	48	248	940	339 (36.1%)	166 (17.7%)	314 (33.4%)	121 (12.9%)

Figures in parentheses represent percentages of total births

Table 2.2 Ratios of XO to XX offspring at weaning

Author	+/O Mothers		Ta/O Mothers	
	No. of ♀♀	Ratio Ta/O:Ta/+	No. of ♀♀	Ratio +/O:Ta/+
Cattanach (1962)	661	1 :2.74	276	1 :3.30
Morris (1968)	966	1 :2.37	926	1 :2.67
Russell (1976)	118	1 :2.17	192	1 :3.23
Deckers *et al* (1981)	362	1 :2.45	119	1 :2.84
Brook (Present Study)	480	1 :1.89	325	1 :1.88
Leonard & Schroder (1968)	-	-	2,029	1 :1.97

be from poor quality preparations: They were discounted as unreliable.

A breakdown of the chromosome counts from the XX (Ta/+) females is given in Table 2.3. This shows a proportion (22.03%) having counts below the expected n = 20 number. It is assumed that the vast majority of these hypomodal counts are attributable to artefactual loss of chromosomes during slide preparation.

Chromosome counts from the two genotypically different groups of XO mice (Ta/O and +/O) are given in Tables 2.4a and 2.4b respectively. These showed no significant differences ($\chi^2_{12} = 9.94$; $p > 0.5$) and the counts for the two genotypes were thus pooled (Table 2.5). From Table 2.5 it would appear, at first glance that segregation of the X chromosome, to egg or polar body, in XO females, is occurring entirely at random; equal numbers of n = 19 and n = 20 being recorded. From a consideration of the data obtained in XX females, showing a 22% level of cell breakage and chromosome loss due to preparative technique, it is by no means justifiable, however, to reach such a straightforward conclusion. If artefactual loss of a single chromosome occurred, it would result in oocytes with 20 chromosomes being spuriously classified as having only 19, thus helping to inflate the n = 19 total. At the same time, some oocytes with 19 chromosomes would be spuriously classified as having only 18. The net result would be to deplete the number of counts in the 20-chromosome category whilst leaving the number in the 19-chromosome category approximately the same. A correction factor is thus clearly necessary in order to arrive at a true figure for the ratio of nullo-X to X-bearing ova. This has been devised in the following way, taking into account the possibility

Table 2.3 Chromosome counts from MII preparations from Ta/+ mice

	Chromosome number n =					
	17	18	19	20	21	Total
Young (8-20 weeks)	1	4	13	87	0	105
Old (30-40 weeks)	1	9	11	51	0	72
TOTAL	2	13	24	138	0	177

Table 2.4 Chromosome counts from MII preparations from XO mice

	Chromosome number n =					
a) Ta/O	17	18	19	20	21	Total
Young (8-20 weeks)	1	13	29	39	0	82
Old (30-40 weeks)	1	11	46	44	1	103
Total	2	24	75	83	1	185

	Chromosome number n =					
b) +/O	17	18	19	20	21	Total
Young (8-20 weeks)	3	11	42	39	0	95
Old (30-40 weeks)	3	11	44	39	2	99
Total	6	22	86	78	2	194

Table 2.5 Chromosome counts from MII preparations from Ta/O and +/O mice combined

	Chromosome number n =					
	17	18	19	20	21	Total
Young (8-20 weeks)	4	24	71	78	0	177
Old (30-40 weeks)	4	22	90	83	3	202
Total	8	46	161	161	3	379

that each chromosome count has arisen by a two-step process involving firstly the segregation of chromosomes at anaphase I and secondly, possible breakage and loss of a chromosome (or chromosomes) by technical artefact. If it is assumed (1) that all those cells with less than 20 chromosomes in the control group (Ta/+) have arisen through breakage, and (2) that the probability of oocyte breakage is the same in both XO and XX mice,then, it is possible to estimate the proportion of all spreads from XO mice with 19 or less chromosomes which have arisen through breakage as follows:

If p_o represents the proportion of unbroken cells in the control group (Table 3), p_1 the proportion losing 1 chromosome and $1-p_o-p_1$ the proportion losing more than 1 chromosome, then the following values can be assigned to each group: -

$$p_o = 0.7797$$

$$p_1 = 0.1356$$

$$1-p_o-p_1 = 0.0847$$

$$1-p_o = 0.2203$$

For the XO oocyte spreads, the number found in the 20-chromosome group (n = 20) is made up of the actual number ovulated with 20 chromosomes (prior to breakage) multiplied by the proportion of unbroken spreads. Similarly the number of counts in the 19-chromosome (n = 19) group comprises the number of non-broken 19-chromosome bearing spreads plus the number ovulated with 20 chromosomes which have subsequently lost 1 chromosome. The number with 18 (or less) chromosomes (n = 18) is made up of the number ovulated with 20 chromosomes which subsequently lose

more than one chromosome plus the number ovulated with 19 losing one or more chromosome subsequently.

This can be expressed algebraically as shown below: -

$$n20 = Mp_0$$

$$n19 = Mp_1 + (N-M)p_0$$

$$N18 = M(1-p_0-p_1) + (N-M)\ (1-p_0)$$

where N = total number of oocyte preparations scored, and

M = the number in the n = 20 group prior to breakage.

These equations can be used to estimate M, most conveniently by the modified minimum chi-square method (Kendall and Stuart, 1961).

For the XO females the segregation ratio is 205:171 for oocytes with counts of 20 and 19 chromosomes respectively (Table 2.6). This 1.2:1 ratio does not differ significantly from the 1:1 ratio expected if random segregation is occurring (χ_1^2 = 3.07: 0.1 > p > 0.05).

To simplify the calculation, the effect of non-disjunction was ignored, as there were only three disomic eggs with counts of n = 21. The effect of chromosome gain on the segregation ratio would be in the opposite direction to that of chromosome loss, thus slightly decreasing the 1.2:1 ratio, taking it even closer to a 1:1 ratio.

Table 2.6 also shows a comparison with data obtained by Kaufman (1972) and Luthardt (1976). These authors did not introduce a correction factor into their results to allow for artefactual breakage. Their data have, however, been subjected to the correction

Table 2.6 Segregation ratios found by various authors, before and after correction

Author	Original findings			Corrected findings		
	20	≦19	% X-bearing	20	≦19	% X-bearing
Evans and Ford (unpublished)	61	59	50.8	-	-	-
Kaufman (1972)	65	40	61.9	76	29	72.4
Luthardt (1976)	52	37	58.4	61	28	68.3
Brook (present study)	164	215	43.3	205	171	54.6

model allowing for their own levels of control breakage. When this is done the data show an even greater bias towards non-random segregation than when the uncorrected figures are considered. The discrepancy between their data and those obtained in the present study will be dealt with in the Discussion. It is not possible to adjust the data of Evans and Ford (unpublished) to allow for breakage as no control data were given by these authors.

For the stock of mice used in the present study, the ratio of X-bearing to nullo-X eggs at ovulation (1.2:1) differs from that found at weaning, the ratio to XX to XO offspring at that time being 1.88:1. Assuming there to be an equal chance of fertilization of X-bearing and nullo-X eggs, it would thus appear, from the altered ratios, that 36.2% of XO mice die between fertilization and weaning. Cattanach (1962) has shown that XO offspring have as good a chance of survival between birth and weaning as do XX offspring, and it can thus be assumed that the 36.2% death of XO's occurs during gestation.

2.3.3 XO Segregation and Maternal Age

In view of the claim made by Deckers *et al* (1981), that a greater number of XO offspring are born to mothers of advanced age, the cytological data were considered, not only in relation to genotype, but also to maternal age. The data presented in Tables 2.3 and 2.4 show no significant differences however, either for Ta/O or +/O mothers, in distribution of chromosome counts in the young group compared with the old. Tables 2.7 and 2.8 moreover, show the numbers of offspring of each genotype weaned from +/O and Ta/O mothers respectively, in terms of litter order. χ^2 tests for

Table 2.7 Genotype of offspring weaned from +/0 mothers in terms of litter order

Litter	No. of Mothers	Total	Ta/0	Ta/+	+/Y	$\frac{Ta/+ + +/Y}{Total} \times 100$
1	48	186	39	71	76	79.03 ± 2.99
2	45	139	26	50	63	81.29 ± 3.33
3	39	123	25	43	55	79.67 ± 3.61
4	35	110	28	39	43	74.55 ± 3.60
5	28	98	14	44	40	85.71 ± 3.51
6	23	82	21	33	28	74.39 ± 4.84
7	14	42	5	20	17	88.10 ± 5.01

Table 2.8 Genotype of offspring weaned from Ta/0 mothers in terms of litter order

Litter	No. of Mothers	Total	+/0	Ta/+	Ta/Y	$\frac{Ta/+ + +/Y}{Total} \times 100$
1	52	186	32	62	92	82.80 ± 2.75
2	41	110	25	43	42	77.27 ± 4.01
3	34	95	21	39	35	77.89 ± 4.25
4	21	72	17	32	23	76.39 ± 4.71
5	15	45	7	20	18	84.44 ± 5.47
6	9	33	9	13	11	72.73 ± 7.73
7	4	13	2	5	6	84.62 ± 9.90

heterogeneity, between the two sets of breeders showed no change in the relative proportions of offspring with litter order - so the two sets of data can be combined. Regression analysis on the combined data shows there to be no significant change in the proportion of progeny born to older mothers ($t = 0.356$: $p < 0.5$). This finding, together with the cytological evidence, gives no indication in our stock of a changing pattern of X-segregation with age of the mother. This contrasts with claims made by Deckers *et al* (1981) for an increasing recovery of XO progeny with increasing maternal age (see Discussion).

2.3.4 Aneuploidy

As can be seen from Table 2.5 three disomic eggs (n = 21) were found in the old age group of XO females compared with none in young XO or in control XX females (Table 2.3). These disomic eggs are assumed to have arisen by non-disjunction in the X-bearing oocytes, and constitute 3/86 (3.5%) of the total eggs assumed to be X-bearing. Their frequency was not significantly greater, however, than in the other two groups of female (young XO and control XX). If the assumption is made that a similar level of non-disjunction occurs among nullo-X eggs (the hyperploid (n = 20) products however being hidden among the normal X-bearing (n = 20) totals), a projected figure of 7 out of 202 hyperploid counts for old XO mothers would be obtained. This enlarged figure is again not significantly different from the zero level of aneuploidy of young XO and control XX females. It is also expected that for each non-disjunctional event producing a disomic egg, there would be a comparable X-bearing nullisomic (n = 19) egg produced. These would be hidden in the naturally occurring nullo-X bearing

total. Similarly, non-identifiable double nullisomics (n = 18) may be produced by non-disjunction in nullo-X oocytes but these could not distinguished from oocytes which had lost chromosomes through breakage. If the overall level of aneuploidy were thus derived by doubling again, there would then be 14/202 or a 7% frequency for the aged females and this would be statistically significant ($p < 0.05$). The assumption is made in the above calculation that for every non-disjunctional event producing a disomic egg, a corresponding event would produce a nullisomic. This, of course, is the conventional view of aneuploidy production by non-disjunction. Recent data of Maudlin and Fraser (1978) indicate, however, that trisomy might arise in ageing female mice without equivalent monosomy. How this could come about is not stated, but if it were to be true, our calculations would not, of course, be valid.

2.4 DISCUSSION

In view of contradictions in the literature concerning the XO mouse, the present study was set up in an attempt to answer three basic questions. Firstly, does the segregation of the X-chromosome, during first meiotic division of the oocyte, occur entirely at random? Secondly, if the X-chromosome is preferentially incorporated into either egg or polar body, does this directed segregation phenomenon change with maternal age? Thirdly, does the XO mouse constitute a good model for the pre-menopausal human female in terms of maternally age-related aneuploidy production?

The ratio of XX to XO offspring at weaning (1.88:1) in the present study is considerably lower than the ratios observed by

others (Cattanach, 1962; Morris, 1968; Russell, 1976). Similarly, the ratio of ovulated X-bearing to nullo-X eggs (1.18:1) is lower than has been found in previous cytological studies. In fact, unlike the studies of Kaufman (1972) and Luthardt (1976), the corrected figures in the present study are consistent with a 1:1 segregation ratio, in agreement with Evans and Ford (pers. comm.). The large difference between the results of this study and those of Kaufman (1972) and Luthardt (1976) cannot be easily reconciled. When both previous sets of data are corrected for breakage however (see Table 2.6) the proportion of X-bearing gametes becomes so high that, to be reconciled with the birth data from our own and other studies, it would be necessary to postulate preferential survival of XO's during gestation. This clearly is not the case. The present study indicates a 36.2% loss of XO progeny during gestation, and others have shown that there is excess death for XO litters during early gestation, as compared with XX's (Morris, 1968 and Russell, 1976). The early loss of OY embryos accounts for part of this but loss of a considerable proportion of XO's prior to day 12 post-conception also seems to occur (Russell, 1976; Luthardt, 1976). As pointed out by Russell (1976), the further from randomness one postulates the segregation of the X chromosome to be, the lower need be the prenatal loss of XO embryos. To reconcile his findings, Morris (1968) concluded that there was preferential segregation of the X-bearing set of chromosomes into the gamete and death of some XO's during the early stages of gestation. However, Evans and Ford (pers. comm.) on re-analysing Morris' data, subsequently suggested that they could be interpreted as showing a 1:1 segregation ratio, and even an increased production of nullo-X, as compared to X-bearing gametes.

It would appear from these contradictory results that the cytological studies are unsatisfactory because of the problem of breakage and chromosome loss. Obviously it would be ideal if it were possible to identify the X-chromosome in the oocyte and then eggs could be simply scored as X-bearing or nullo-X. Nevertheless, it seems unlikely that the different results obtained by various authors can be explained on the basis of differing amounts of breakage encountered in each different study. One possibility is that there is a drive mechanism, which is responsible for the excess production of X-bearing gametes but which varies in strength from one stock to another. Genetic background may be important. Thus, in the present study there may be little, if any, preferential loss of the chromosome set lacking an X to the polar body, whereas in others - such as those used by Kaufman (1972) and Luthardt (1976) the drive mechanism may be stronger. It would, however, seem unlikely that the amount of death of XO progeny during gestation should differ significantly in other stocks from the 36.2% found in the present study.

The second point which arises out of this study concerns the question of whether preferential segregation of the X to the egg changes with maternal age. Since the data in the present study give no indication of any such change, they are at variance with those of Deckers *et al* (1981). Both the birth data and the MII counts found in the present study show no reduction in the transmission of X-bearing gametes with age in XO mothers. Regression analysis on X-segregation data in successive litters of Deckers *et al* (1981) however, showed a significant negative slope, indicating change with maternal age. Similar treatment of these data gave no such significant result, with the slope in fact being

slightly positive. As a χ^2 test for heterogeneity proved negative, regression analysis of the combined data was performed producing a non significant - even though slightly negative, slope. This would suggest that the two sets of data are homogeneous but the anomalous result of Deckers *et al* (1981) is due to their small sample size.

Finally, to the question of aneuploidy in ageing XO mice and their suitability as a model for the pre-menopausal human female as suggested by Lyon and Hawker (1973). Although an increase in disomic oocytes was observed with increasing age in the XO mice used in the present study, this alone was not found to be statistically significant. Only 3 eggs which had chromosome counts of 21, could be confidently classified as disomic, but presumably X-bearing nullisomics were also produced in similar quantity. Non-disjunction in nullo-X eggs would not be noted as nullo-X eggs with 20 chromosomes would be scored as normal X-bearing cells. Specific X-staining combined with total chromosome counts would provide a means of identifying all disomic eggs although it would still be impossible to distinguish between 'true ' nullisomics, that is those arising through non-disjunction, and those arising through breakage during fixation. The usefulness of the XO mouse as a suitable model for human aneuploidy and the maternal age effect is therefore questionable, in view of the problems arising from the estimation of the true aneuploidy frequency.

CHAPTER 3

THE EFFECT OF UNILATERAL OVARIECTOMY AND AGE ON ANEUPLOIDY INDUCTION IN CBA MICE

3.1 INTRODUCTION

Hypotheses to account for chromosomal non-disjunction and age related aneuploidy have been outlined in Chapter 1. Changes in hormone levels and the irregularity of the ovarian cycles preceding the menopause have frequently been implicated, although the underlying mechanisms are highly speculative and lack a firm experimental basis (Fugo and Butcher, 1971; Lyon and Hawker, 1973; Crowley *et al*, 1979).

During the 6-8 years preceding the menopause in the human female there is typically a marked increase in the variability of intermenstrual intervals (Treloar *et al*, 1967). The duration of this transitional phase, during which unusually long and short cycles are often interspersed, varies considerably amongst women. Levels of circulating gonadotrophins are greatly increased. Adamopoulos *et al* (1971) found a seven-fold increase in LH and a three-fold increase in FSH levels in women approaching menopause. Papanicolaou *et al* (1969) consider that in late reproductive life the reciprocal relationship believed to exist in younger women between pituitary gonadotrophic function and ovarian oestrogen secretion no longer operates. Contradictory evidence exists on the level of oestradiol prior to menopause.

Most authors consider that oestrogen excretion is reduced during the climacteric (Furuhjelm, 1966; Pincus *et al*, 1954), although England *et al* (1974) report significantly higher concentra-

tions of oestradiol 17β from women in the 4th decade of life compared with either younger or older women. Sherman *et al* (1976) however, considered that hormonal changes associated with follicular maturation and corpus luteum function occurred in the presence of high menopausal levels of FSH and LH, but with a diminished secretion of oestradiol and progesterone. Treloar *et al* (1967) observed shorter cycle length in women 40-41 years than in women aged 18-30 which was attributable to a shorter follicular phase. The lower levels of oestradiol found by Sherman *et al* (1976) in both follicular and luteal phases are contrary to the findings of England *et al* (1974).

Hormonal imbalance at menopause has been documented in a number of other species as well as in the human female. Hodgen *et al* (1977) reported that menopause in the rhesus monkey presented sustained elevations of serum gonadotrophin and low circulating levels of oestradiol and progesterone. In the mouse Nelson *et al* (1982) found that an increase in cycle length with age began at about 7 months with a decreased frequency of 4 day cycles, although irregular cycles were occasionally observed in 3-4 month old animals. Most animals entered an acyclic state at 12-16 months in which vaginal cornification persisted. The transition to longer cycles was associated with a delayed rise of pre-ovulatory oestrogen, although the pre-ovulatory levels attained did not differ from young animals at pro-oestrus (Nelson *et al*, 1982). Present evidence suggests that the onset of acyclicity in these animals is due to a combination of factors; both a decline in number of ovarian oocytes and failure of the neuroendocrine system to produce an ovulatory stimulus (Gosden *et al*, 1983; Felicio *et al*, in press). Ovulation can be delayed in animals treated with pentobarbitol, and during this

extended phase Butcher (1975) has shown that plasma levels of oestradiol were elevated but dropped back to base level at the usual time in relation to ovulation. An association between irregular oestrous cycles, hormonal imbalance and increased aneuploidy with increasing maternal age has been suggested by several authors (Fugo and Butcher, 1971; Lyon and Hawker, 1973; Crowley *et al*, 1979). Recently it has been suggested that hormonal imbalance in younger women might also give rise to aneuploidy. Indeed use of the contraceptive pill has been implicated in a number of studies (Koulischer and Gillerot, 1980; Read, 1982).

Changes similar to those associated with the menopause can be induced at an earlier age by unilateral ovariectomy. Thung (1961) originally reported that unilateral ovariectomy (uni-ovx) in the mouse aggravated or exaggerated normal ovarian ageing such that normal oestrous cycles are replaced by irregular oestrogenic activity, effectively reproducing menopause at an earlier age. Since that time unilateral ovariectomy has been the subject of numerous studies in a variety of species. Mandl and Zuckerman (1951), for example, looked at the numbers of normal and atretic oocytes in unilaterally spayed rats and confirmed results reported earlier by Arai (1920) showing that the ovary doubles in weight and produces as many mature Graafian follicles as found in the two ovaries of littermate controls. The number of primordial oocytes remains at the level normal for one ovary (Mandl and Zuckerman, 1951). More recently, Hirshfield (1982), looking at follicular recruitment in long-term hemicastrate rats, found that at metestrus there were half as many small and medium antral follices in long-term hemicastrates as in controls. The total number of large antral

follicles, however, was the same in both hemicastrate and intact rats.

Following unilateral ovariectomy the overall number of oocytes ovulated does not diminish. Moreover, in most cases, compensatory ovulation occurs by the first oestrus following operation so that the total number of ova shed is the same as in control animals (Asdell, 1924). McLaren (1966) using the mouse, found a two-fold increase in ovulation rate from the remaining ovary, three days after the removal of its partner. She suggested that this was due to the single ovary consuming twice as much of the available FSH than it otherwise would, rather than to the absolute level of hormone in the blood. Bast and Greenwald (1977) examined the effect of acute and chronic elevations in serum levels of FSH after unilateral ovariectomy in the cycling hamster and found significant increases 24 hours post-operatively. However, these elevations were not the sole cause of compensatory ovulation as this was also found in certain cases where serum levels were not acutely altered.

Although unilaterally ovariectomized animals shed as many ova at ovulation as intact controls, the total number of offspring produced during reproductive life is, nevertheless, found to be halved (Jones and Krohn, 1960). Biggers *et al* (1962) suggested that this reduced breeding potential might be due to uterine ageing as a result of repeated overloading. This was substantiated experimentally by Gosden (1979) using the mouse.

The means by which compensatory ovulation occurs has pre-occupied a number of authors in recent years. Peppler and Greenwald (1970) suggest that it is brought about by a doubling of the number

of large follicles which ultimately mature during the course of the oestrous cycle. This arises out of an increased proliferation of small follicles, rather than from a decreased level of follicular atresia. According to Peppler (1971) an absence of compensatory ovulation in the hemicastrated rat after 6 or 12 months is supported by a general decrease in follicular development at these ages compared with younger ones. Hirshfield (1982) however, reported that the ovaries of long-term hemi-castrate rats contain far fewer atretic follicles than ovaries of intact rats, and Welschen *et al* (1978), showed that in the rat, atresia could be prevented or even reversed by unilateral ovariectomy, and is perhaps comparable with the effects of PMSG in rescuing follicles from atresia in rats (Braw and Tsafriri, 1980). Thus the effects of the operation depend upon the stage of the cycle at which it is performed.

Neither of the conflicting hypotheses (increased follicular growth vs. decrease of atresia)has been resolved in studies of other species. Greenwald (1974) reported that following unilateral ovariectomy in the golden hamster, the number of follicles becoming atretic is reduced, whereas in the guinea-pig it is the number of small follicles proliferating to larger ones which is increased. From a study of vaginal smears and growing follicles in mice, Thung (1961) suggested that unilateral ovariectomy increased the rate of ageing in the remaining ovary by increasing the rate of oocyte depletion. However, this is contrary to the earlier report of Jones and Krohn (1960) that the failure of semi-spayed mice to produce litters for as long as normal mice, was not related directly to a precocious disappearance of oocytes from the ovary. Gosden (1979) also considered that unilateral ovariectomy in mice did

not accelerate senescence of the remaining ovary. Baker *et al* (1980) looked at the number of oocytes and rate of atresia in unilaterally ovariectomized mice in order to clarify the means by which increased output of eggs from the single ovary is controlled in the long term. They consider that this may be brought about either by an increase in the rate at which follicles leave the pool, or by a reduction in the number of oocytes lost by atresia. Their findings, however, were equivocal. They observed that during the first four weeks following unilateral ovariectomy there was an increased progression of follicles from the pool to form growing follicles. After this time, however, the rate of depletion from the pool was no different to that in controls. If increased recruitment cannot account for the output of additional oocytes then reduced atresia at some stage of follicular growth must be implicated. However, they were also unable to find support for this, as there was no difference in the proportion of oocytes undergoing atresia between control and treated mice for any follicle class. They did, however, suggest that they may have been unable to detect a change in the rate of atresia because of their classification of atretic follicles. Alternatively Baker *et al* (1980) suggest that 'rescue' from atresia may only be detectable at a specific stage in the oestrous cycle. This, however, cannot be assessed from their results, and further work is necessary to clarify this point.

In the present chapter, the relationship between hormonal imbalance, irregular cyclicity and the increase in maternal age-related aneuploidy is examined. By means of unilateral ovariectomy, the reproductive lifespan in CBA females has been shortened in order

to create a situation in which an aged ovary (in physiological terms), is present in a young (chronologically speaking) animal. Thereby the influences of physiological and chronological ageing of the female on aneuploidy production can be separately assessed. In addition, the postulate, made by Henderson and Edward (1968) in their "production line" hypothesis, that the frequency of ovulation of chromosomally abnormal oocytes may be higher when the overall number of eggs available is reduced by unilateral ovariectomy, will be examined.

3.2 METHODS

3.2.1 Operation

Female mice aged from 6-8 weeks were anaesthetised using 'Avertin' which was prepared from its constituent ingredients shortly before the operation. This consisted of 0.315 g Tribomoethyl alcohol and 0.25 ml 3 methyl-1-butanol per 10 mls saline. 0.25 ml was injected per mouse (weight approx. 20 g). A small dorsal area was shaved, swabbed with alcohol and a short incision made in the flank. The ovary was located and pulled out by the ovarian fat pad. In those cases of sham operation the ovary was pushed back and the wound closed with 'Michel' clips. For those mice underoing unilateral ovariectomy the ovary was tied-off using Ethicon 'Meresilk' suture and cut off with scissors. The fallopian tube was replaced and the wound closed with 'Michel' clips as for the sham operation. Two weeks after the operation the clips were removed.

3.2.2 Chromosome Preparation

In order to assess the effect of maternal age and unilateral ovariectomy on the incidence of aneuploidy, chromosome counts were

made on 3½-day old embryos from mothers sub-grouped according to age and operation. These sub-groupings were as follows:

Young	(9-13 weeks)	:	Sham and Uni-ovx
Mid	(22-26 weeks)	:	Sham and Uni-ovx
Old	(35-40 weeks)	:	Sham and Uni-ovx
Very old	(45-50 weeks)	:	Sham

Swiss male mice, of proven fertility, aged 12-52 weeks were placed in cages containing females from the above groups. The females were examined for the presence of a vaginal plug in the mornings. Around noon on the third day following the appearance of a vaginal plug each female was injected with 1 µg Colcemid (Ciba) per gram body weight, and killed three hours later. Uni-ovx females were examined to ensure that the operation had been complete. Those animals showing the partial presence of a supposedly excised ovary were discounted. The ovaries and uterine horns were removed, placed in a plastic universal tube containing physiological saline (0.15 m NaCl) and transferred to the laboratory. The uterine horn was dissected away from the ovary and the vagina, and a small incision was made at the ovarian end. A blunt needle was inserted into the uterine horn from the vaginal end and six or seven drops of saline were passed through the horn from a syringe. The contents of the horn were flushed out into a small plastic dish. The embryos were located under the dissecting microscope, counted and placed in hypotonic solution (0.8% sodium citrate) for ten minutes. They were then placed on a clean slide and fixed with a few drops (five or six) of a mixture of methanol and glacial acetic acid (3:1, v:v)

according to the method of Tarkowski (1966). Optimum fixation and spreading was obtained using six drops of fixative, allowing the blastocyst almost to dry before the application of the next drop. Furthermore, when the blastocyst was placed on the slide any hypotonic solution also deposited, was carefully removed in order to prevent the blastocyst from shooting off the slide as the fixative was added. The position of the fixed preparation was indicated by a scratch with a diamond marker below the slide.

The ovaries were examined under the dissecting microscope (x10) and the number of corpora lutea counted.

The blastocyst preparations were stained with 5% Giemsa for 5 minutes, coded and scored 'blind' under the microscope (x1000, oil immersion). Attempts were made to band the chromosomes when an aneuploid embryo was detected, but these failed.

A group of up to 10 stud males were used and their success in both mating and fertilizing eggs was monitored. Females not producing plugs with different males within 30 days were killed.

Sufficient animals were used to produce about 50 analysable spreads per sub-group.

3.2.3 Smearing

A separate group of 41 mice was set up for smearing in order to establish the effect of uni-ovx on the regularity of the oestrous cycle. 20 were uni-ovx and 21 were shams. Smearing was started four weeks after the operation and performed daily, six days a week. A smooth glass pipette containing a drop of distilled water was

inserted into the vagina, and the water was gently expelled and drawn back into the pipette a couple of times. The fluid was spread across a glass slide and allowed to dry. For each mouse the smears for one week were made on the same slide. The slides were coded and analysed by Mrs. S. Laing of the Department of Physiology, University of Edinburgh.

When over 50% of the mice in each group no longer showed recognisable oestrous cycles, smearing was discontinued. The mice were killed and autopsied. Those mice in the uni-ovx group, for which the operation had been incompletely performed, i.e. the ovary had not been fully removed, were discounted, as were mice in either group showing ovarian tumours.

At all times males were kept in the same room as the females to ensure exposure to the influence of pheromones.

3.2.4 Ovarian Histology and Hormone Levels

Equivalent groups of females to those used in the aneuploidy study were set up for ovarian histology and serum hormone assay, with approximately 10 animals in each group. They were smeared in the mornings, at daily intervals until a regular oestrous cycle pattern was established, and then killed at noon on the day of pro-oestrus. The mice were first anaesthetised with ether and blood was removed by cardiac puncture. Then the ovaries were dissected out and placed in aqueous Bouin's fixative for 24 hours after which they were transferred to 80% alcohol. They were then transferred to the Department of Physiology, University of Edinburgh,

for further processing and examination by Dr. R. Gosden. The ovaries were paraffin embedded and serially sectioned at 7 μm intervals. Slides were stained with haematoxylin and eosin and coded to avoid observer bias. Every tenth section from each ovary was examined in detail at x400 and the number of primordial follicles were counted. Only those follicles in which at least 50% of the oocyte nucleus was visible were recorded so as to avoid overcounting. The numbers were recorded in both ovaries of the sham-operated groups and combined for comparison with those in the uni-ovx mice. (Counts were multiplied x10 and log transformed (see results).)

The blood was allowed to stand at 4°C for 1 hour and then spun down in a microfuge for 2 minutes. The serum was removed and stored frozen at -20°C. The levels of oestradiol-17β are presently being assayed by Dr. C. E. Finch (University of Southern California, Los Angeles), but the results are, as yet incomplete.

3.3 RESULTS

3.3.1 Chromosomal Anomalies in 3½ day Embryos of CBA Mice

The number of corpora lutea, embryos and unfertilized eggs obtained from females of each experimental group is shown in Table 3.1. At all ages the unilaterally ovariectomized females produced fewer corpora lutea than did their sham-operated counterparts. The lower numbers of corpora lutea and embryos recovered from older females implied a considerable loss of fecundity; this effect of ageing began earlier in unilaterally ovariectomized than intact animals. There was a parallel effect of age and treatment on the proportions of embryos and unfertilized eggs

Table 3.1 The recovery of 3½ day embryos from mated females

Experimental Group	Maternal Age (weeks)	No. of Animals	*Corpora Lutea* (A)	Mean (A)	Unfertilized Eggs (B)	No. of Embryos Recovered (C)	$100^{*}\frac{[A-(B+C)]}{A}$	% Embryos Analysed
OVX	9-13	29	261	9.00±0.25	3	243	5.7	29.0
SHAM	9-13	26	277	10.65±0.24	13	251	4.7	27.2
OVX	22-26	33	295	8.94±0.29	9	265	7.1	29.2
SHAM	22-26	21	214	10.19±0.43	10	187	7.9	37.1
OVX	35-40	99	240	2.42±0.16	3	146	37.9	28.1
SHAM	35-40	65	284	4.37±0.26	10	238	12.7	24.9
SHAM	44-50	66	190	2.88±0.27	1	137	27.4	33.8

*Percentage of ova which are lost as a result of failure of ovulation or of early development

recovered as a percentage of corpora lutea, which suggests that there were additional losses during development between the times of ovulation and blastulation, possibly involving one or more of the following: failure of follicles to rupture, failure of fertilization or failure of blastulation. This proportion increased from 5.7% to 37.9% in uni-ovx animals compared with an increase from 4.7% to 27.4% in shams. About 30% of all blastocyst preparations were analysable, the range being from 24.9% in 35-40 week-old shams to 37.1% in the sham 22-26 week old group. A chi-squared test for heterogeneity of the proportion analysable from each group was not significant, indicating a similar level of efficiency of preparation in each group, thus discounting the possibility of bias due to preparation losses.

Table 3.2 shows chromosome counts from the preparations of 3½-day old embryos. Only those cells in which the chromosomes were clear and well-spread were scored. Cells were not scored if a precise count could not be made. As many cells as possible were scored for each embryo, although in the majority of cases only one was analyzable. This was because only one or two of the cells per embryo would be in metaphase at the same time, and often there was insufficient spreading of chromosomes to allow analysis of both. In approximately 20% of embryos it was however possible to count more than one cell, and in some cases up to 5 spreads were analysable. Those embryos showing two or more cells with 39 chromosomes were recorded in order to distinguish them from those spreads of 39 chromosomes which may have arisen through breakage and artefactual loss. The two-cell 39 scores are marked with an asterisk in Table 3.2. Chromosome spreads from embryo preparations are shown in Figures 3.1 and 3.2.

Table 3.2 Chromosomal Anomalies in 3½-day old embryos of CBA mice

Experimental Group	Maternal Age (weeks)	Total Embryos Analysed	4n	3n	2n/3n	41	40	39	38	37	% Aneuploid
OVX	9-13	67	-	-	-	-	59	8	-	-	11.9
SHAM	9-13	66	-	1	-	-	61	4	-	-	6.1
OVX	22-26	75	-	1	-	3	60	10[*1]	-	-	17.3
SHAM	22-26	65	-	-	-	-	62	3[*2]	-	-	4.6
OVX	35-40	41	2	-	1	2	25	7[*2]	1	1	22.0
SHAM	35-40	58	-	1	-	1	46	5	2	2	12.1
SHAM	44-50	46	-	1	-	1	36	8[*2]	-	-	19.6

*Two cell 39's

Fig. 3-1

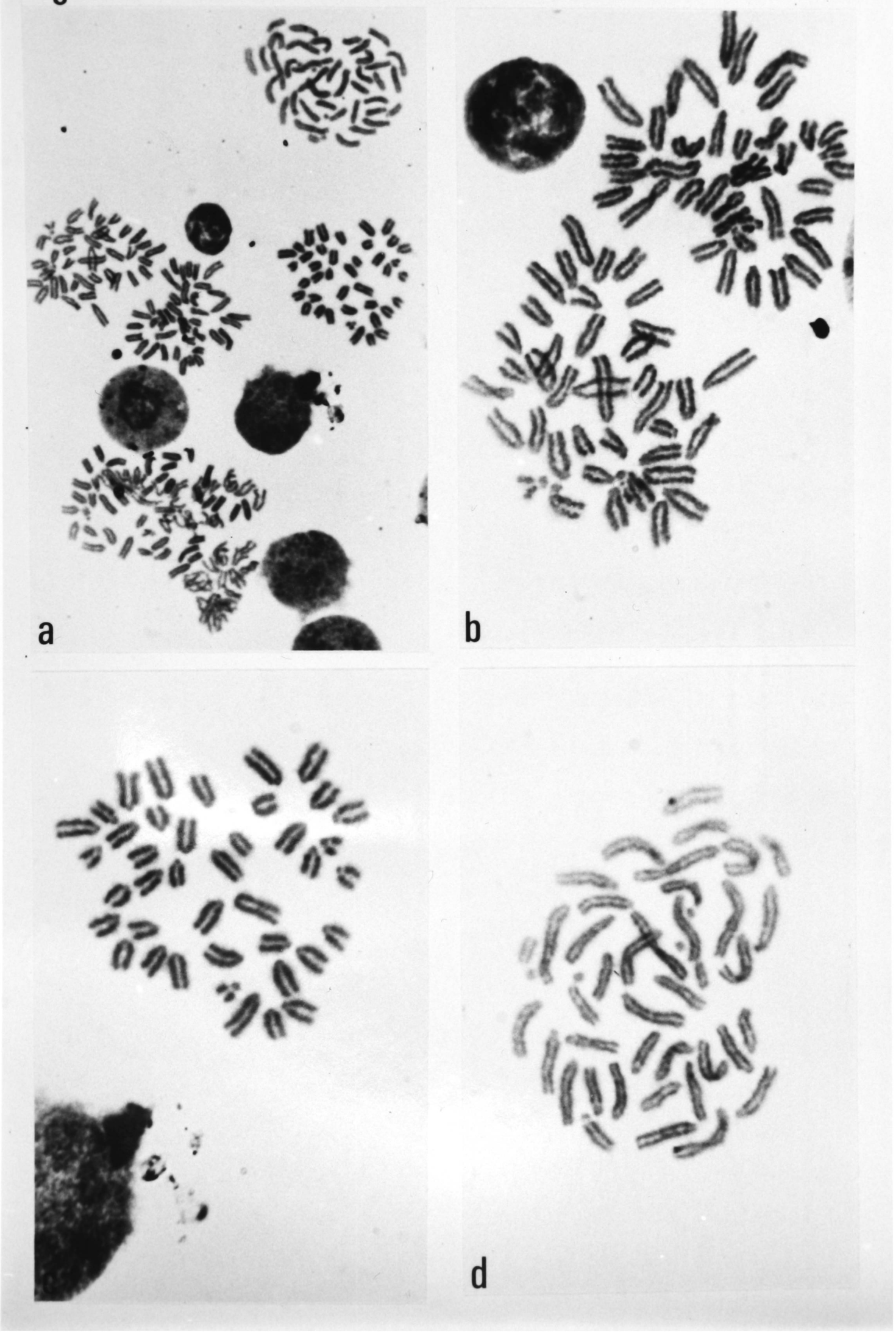
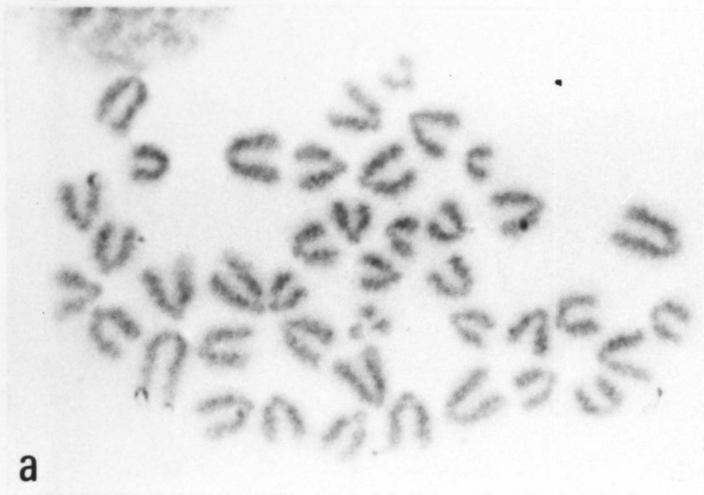
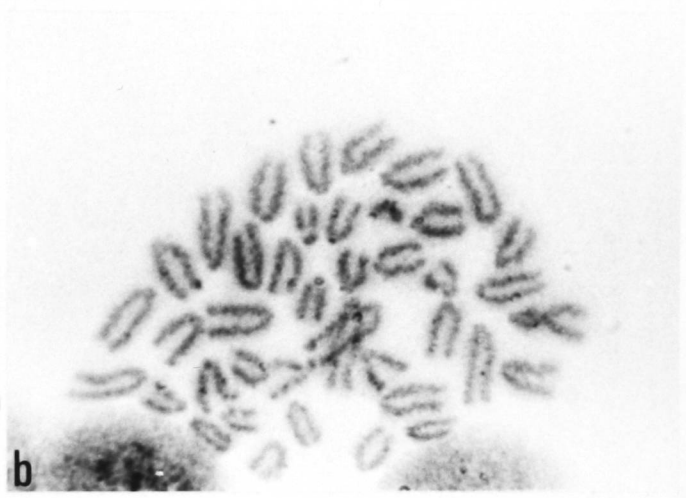


Fig.3-2


a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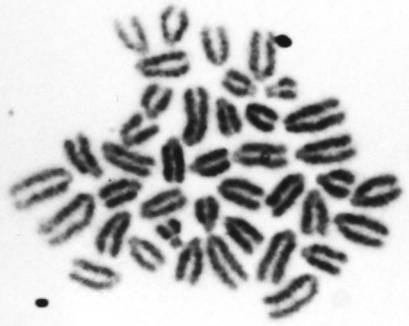
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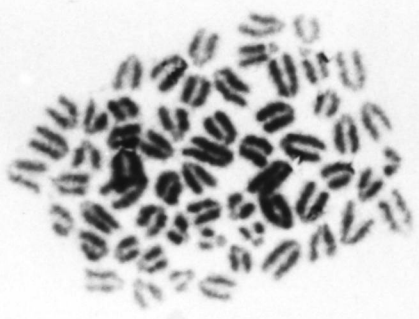
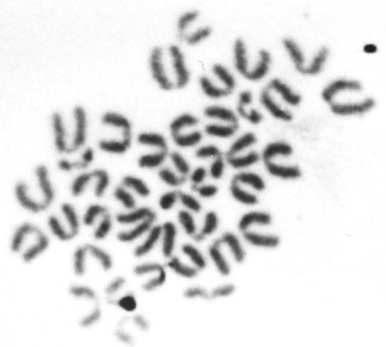

d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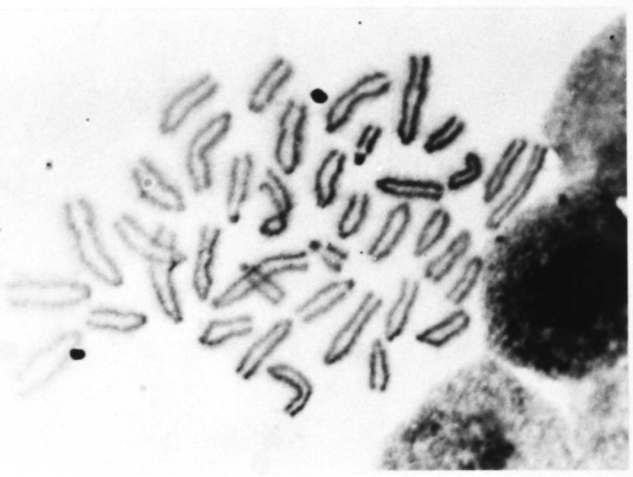

f

The great majority of embryos in mothers aged 9-13 weeks old were diploid (2n = 40) and trisomic embryos were not found at this age. The incidence of monosomic embryos was 11.9% in the unilaterally ovariectomized sub-group at this age range and 6.1% in the age-matched controls. In the next oldest group (22-26 weeks), the proportions of monosomic embryos were virtually unchanged, but three trisomic embryos (4.0%) were found in the uni-ovx group. Thus the overall incidence of aneuploid embryos with either 39 or 41 chromosomes had increased to 17.3% in the latter sub-group whereas it was only 4.6% in the sham-operated controls. The highest incidence of aneuploidy was found in uni-ovx animals at 35-40 weeks of age (22.0%). The values in the corresponding group of controls had risen to 12.1% at this age and increased further at 44-50 weeks of age to 19.6%. Trisomic embryos were recovered from both uni-ovx and intact animals at these advanced ages. They were always less plentiful than monosomic embryos though not open to the criticism of the latter that a chromosome could have been lost during slide preparation.

Although there were insufficient data to deduce the mathematical relationship between maternal age and the incidence of aneuploidy, when linear regression analysis was used as a first approximation, the regressions for both uni-ovx and control groups were found to be significantly greater than zero ($p \sim 0.01$). Chi-squared analysis of the distribution of aneuploid embryos at particular ages showed no significant differences between treatment groups at 9-13 weeks or 35-40 weeks. There was, however, a higher proportion of monosomics and trisomics in uni-ovx mice aged 22-26 weeks than in their controls ($\chi^2 = 5.74$, d.f. = 1; $0.02 > p > 0.01$). The raw data suggested that

the effects of age and treatment applied to trisomic and monosomic embryos alike, although the relatively lower frequency of the former type, combined with the difficulties of obtaining sufficient material, resulted in the statistics falling just short of conventional levels of significance ($p < 0.05$) when trisomy alone was analysed. The overall incidence of polyploidy was much lower than that of trisomy and monosomy combined (1.4% cf. 12.7%) and was distributed differently and approximately randomly with respect to age and treatment. There were no uni-ovx results for the very old (44-50 weeks) group because very few of the animals were still ovulating at this age.

3.3.2 The Time Interval between the Introduction of the Male and the Appearance of a Vaginal Plug in the Female

The data were examined to assess whether there was any relationship between the karyotype of the embryo produced by any particular mother and the time interval from the introduction of a male into her cage and mating (the 'plugging' time). Table 3.3A shows the effect of age and operation on the plugging time for mothers producing only normal ($2n = 40$) embryos. For the uni-ovx animals the longest plugging time was found for the 22-26 week old age group, however, as for sham operated controls, none of the plugging times differed significantly.

The data in Table 3.3B are classified without regard to maternal age or treatment sub-group and show that the mean plugging time was longer for mothers producing aneuploid ($2n = 41$ and/or $2n = 39$) embryos than it was for those mothers producing only normal euploid ($2n = 40$) embryos. Mothers producing two aneuploid offspring had a mean plugging time of 4.50 days compared with 2.76

Table 3.3(a) The Effect of Age and Operation on the Time Taken to Plug* for Mothers Producing only Normal Euploid (n=40) Embryos

Age	Operation	Number of Mothers	Mean Time Taken to plug in days ± S.E.
9-13 weeks	OVX	18	2.40 ± 0.37
9-13 weeks	SHAM	17	2.29 ± 0.56
22-26 weeks	OVX	17	3.94 ± 0.63
22-26 weeks	SHAM	18	2.56 ± 0.23
35-40 weeks	OVX	15	2.73 ± 0.23
35-40 weeks	SHAM	23	2.87 ± 0.34
44-50 weeks	SHAM	14	2.50 ± 0.49

Table 3.3(b) The Time Taken to Plug* for Mothers Classified According to Embryonic Genotype

Genotype of Embryo Produced	Number of Mothers	Mean Time Taken to plug in days ± S.E.
Only those with 40 chromosomes (see Table 3.3(a))	122	2.76 ± 0.15
Two aneuploid offspring†	6	4.50 ± 1.34
One embryo with 41 chromosomes	6	3.33 ± 1.02
One embryo with 39 chromosomes where 2 cells were examined	6	3.17 ± 0.70
One embryo with 39 chromosomes where 1 cell only examined	28	3.04 ± 0.49
One embryo with 38 chromosomes	6	2.50 ± 0.34
One embryo with either 80 or 60 chromosomes	5	2.40 ± 0.51

*That is the time recorded from introduction of the male to mating

†Embryos produced were either 2 x 39 or 41 and 39

days for mothers producing only normal euploid offspring. This difference, however, was not significant with a Student 't' test ($t = 1.296$; $p > 0.10$), as the groups were small in size with large standard errors. The trend to a longer time interval between introduction of the male and mating, in those females producing aneuploid conceptuses is nevertheless of interest.

3.3.3 Oestrous Cycle Regularity

3.3.3.1 The Frequency of all Oestrous Cycles

From the morphology and distribution of cell-types from any smear, it is possible to determine the stage of the oestrous cycle for that particular female. Figure 3.3 shows the various cell types found on each day of the oestrous cycle. A complete cycle is classified as a sequence from epithelial/cornified cells through leukocytes back to cornified epithelial cells, e.g. Oestrus (E); - Met-oestrus (M); - Di-oestrus (D); - Pro-oestrus (P); - Oestrus (E) would be classified as one complete cycle regardless of the number of days spent in either 'M', 'D' or 'P'. The frequency of all complete oestrous cycles per 20 day interval for both ovx and sham operated animals is shown in Figure 3.4. The two groups followed the same general trend of fewer cycles with increasing age, although spurious - not significant increases were observed from time to time in both cases.

In the intact animals there was no difference in the frequency of oestrous cycles per 20 day interval from 90-249 days. During the interval from 250-269 days however, there were significantly fewer oestrous cycles than during the 90-109 day interval ($t = 2.32$,

Fig.3-3

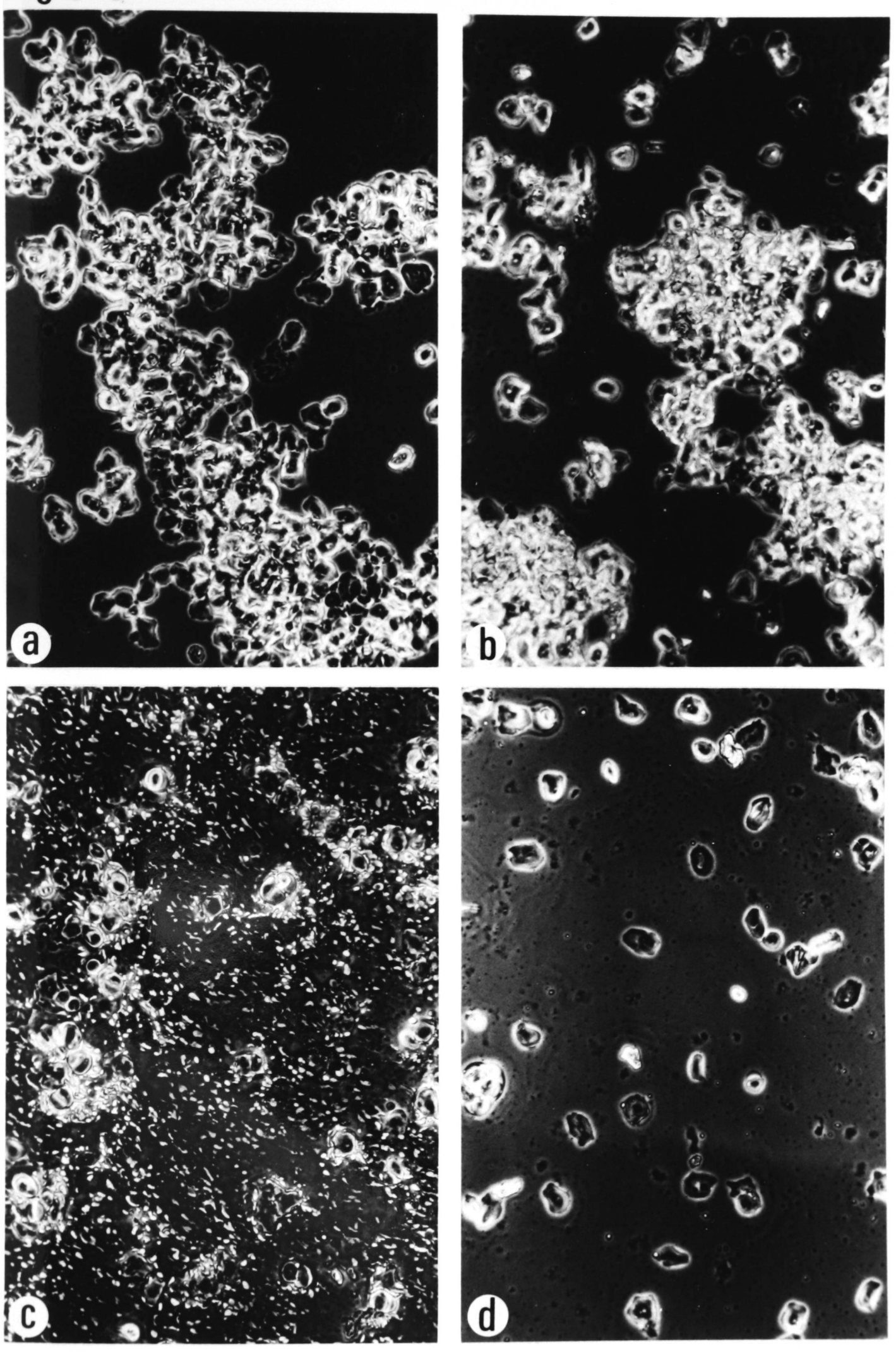
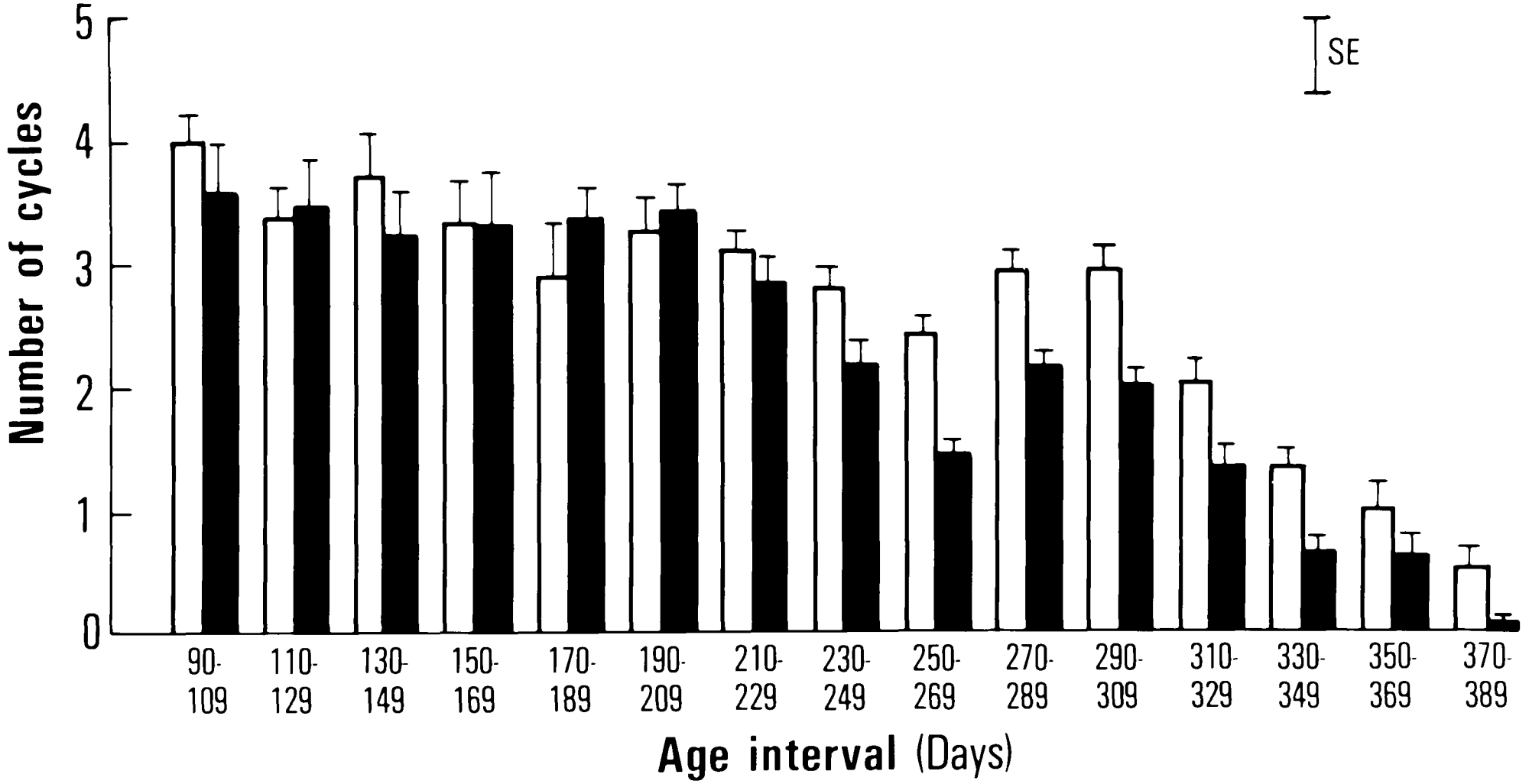


Fig.3-4

The frequency of all oestrous cycles from intact and unilaterally ovariectomized mice.



Legend: Open bars = intact animals
Closed bars = unilaterally ovariectomized animals.

0.05 > p > 0.01, using 1 tailed 't' test). During the two 20 day intervals from 270 to 309 days there was no significant difference in the number of oestrous cycles when compared to any preceding 20 day interval. All 20 day intervals after day 310 however, showed markedly fewer cycles than earlier intervals (Days 90-109 cf 310-329; t = 2.4, 0.05 > p > 0.01).

A similar trend was noted for the ovx group with significantly fewer cycles occurring during the 230-249 day period compared with days 110-129 (t = 3.6; 0.01 > p > 0.001). Each 20 day interval subsequent to day 230 showed significantly fewer cycles than days 110-129.

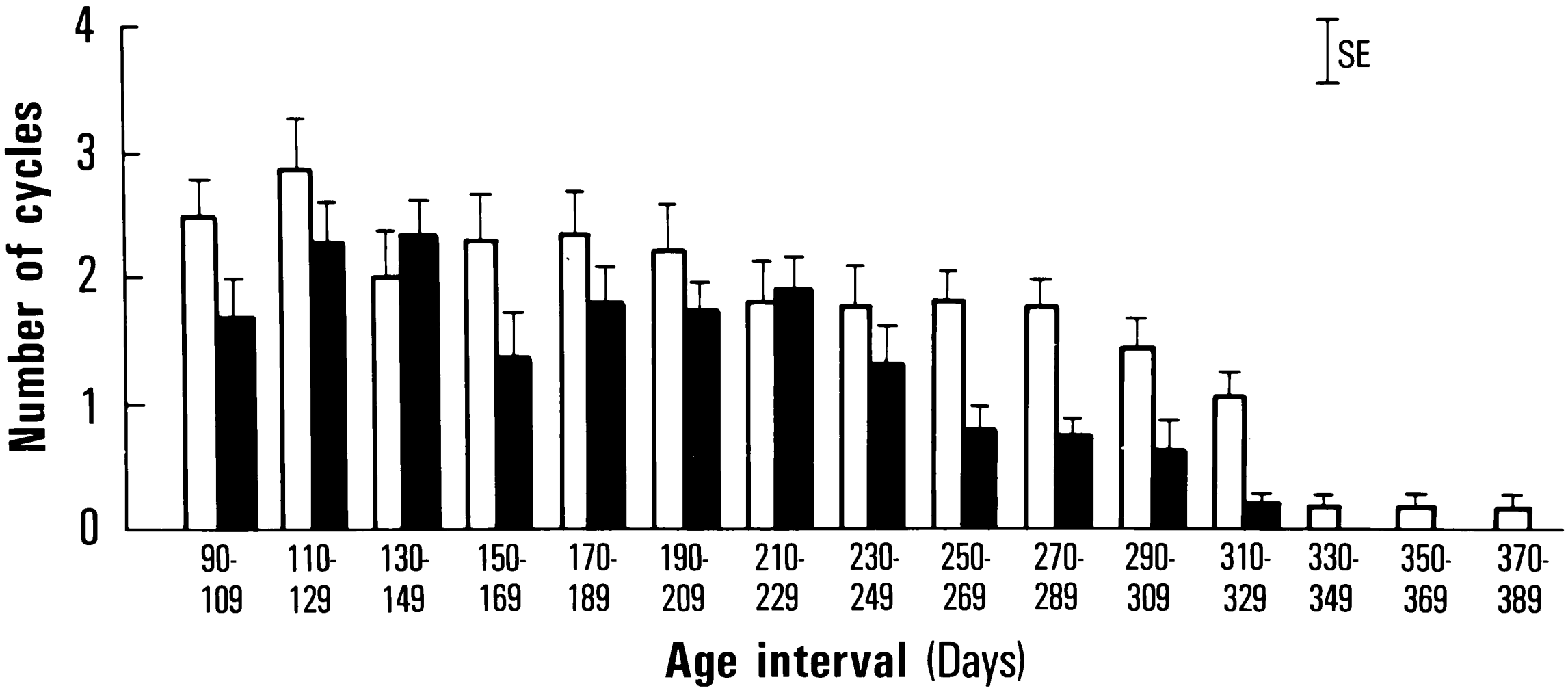
For most intervals the mean number of cycles per animal in the intact group exceeded the mean number in the ovariectomized group. This was always true after day 210. Furthermore, after day 230 with the exception of the 350-369 day interval, there were significantly more cycles per 20 day interval in the intact animals than in their ovariectomized counterparts (e.g. Days 230-249; t = 3.12: 0.01 > p > 0.001) using two-tailed unpaired 't' test.

3.3.3.2 Frequency of 'Normal' 4 or 5 Day Cycles

Cycles were classified as normal if they showed either a 4-day pattern of Pro-oestrus(P), Oestrus (E), Met-oestrus (M) and Di-oestrus (D) or either of the following 5-day cycles P,E,E,M,D or P,E,M,D,D. These 4 or 5-day cycles were the most common types found and tend to be intermittent, rather than occurring in long strings. Figure 3.5 shows the frequency of 'normal' 4 or 5-day cycles for both ovariectomized and intact animals. In both groups

Fig.3-5

The frequency of 'normal' 4 - or 5 - day oestrous cycles from intact and unilaterally ovariectomized mice.



Legend: Open bars = intact animals
Closed bars = unilaterally ovariectomized animals.

the trend was similar to that shown in Figure 3.4 for all types of oestrous cycles.

For the intact animals there was no significant difference in the number of 'normal' cycles per 20-day interval up to day 289. From day 250 onwards, however, there was a gradual decline in the number of 'normal' cycles with the 290-309 day interval being significantly different to the 110-129 day interval (with a 1 tailed 't' test; $t = 4.48$; $p < 0.001$).

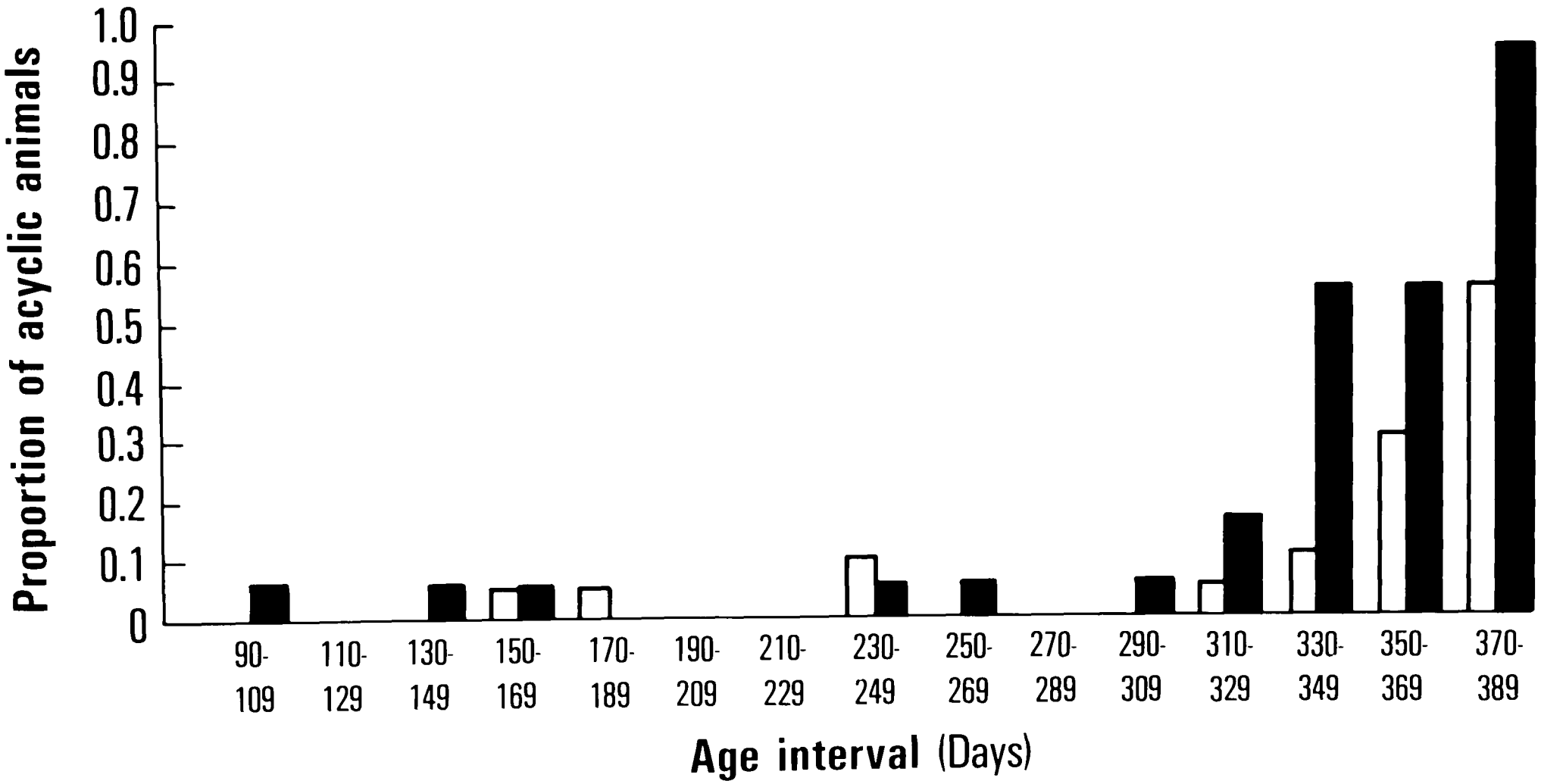
The frequency of normal cycles per 20-day interval in ovariectomized animals was slightly more erratic than for the intact animals, with the 150-169 day interval showing significantly fewer 'normal' cycles than the previous 20-day interval ($t = 2.79$; $0.01 > p > 0.001$). However, during each of the next 3 20-day intervals the number of 'normal' cycles was higher than for the 150-169 day interval. During the 239-249 day interval there were significantly fewer normal cycles than from days 210-229 ($t = 2.22$; $0.05 > p > 0.01$). These continued to decrease for each of the following 20-day intervals, and by day 330 none of the OVX mice showed 'normal' 4 or 5-day cycles.

There were significantly more normal cycles in intact animals than their OVX counterparts for each interval from day 250-329 (e.g. for 250-269 days using a two-tailed unpaired 't' test; $t = 3.59$; $0.01 > p > 0.001$).

3.3.3.3 Proportion of Acyclical Animals

Animals were classified as acyclical if they failed to show a complete oestrous cycle during any 20-day interval. Figure 3.6

Fig. 3-6
Proportion of acyclic animals in groups of intact and unilaterally ovariectomized mice at different ages.



Legend: Open bars = intact animals
Closed bars = unilaterally ovariectomized animals

shows that up to 310 days of age, few intact or uni-OVX animals were acyclical throughout any given 20-day interval, although individual animals did occasionally have extended periods of persistent vaginal cornification. After day 310 however, the proportion of acyclical animals found in each group increased although there were consistently more acyclical mice in the uni-OVX group at any given age. At 330 days of age, approximately half of the uni-OVX group were acyclical, but a comparable proportion of the intact group was not found to be acyclical until 40 days later.

3.3.4 The Distribution of Primordial Follicles within the Ovaries of Sham and Uni-OVX Animals at Various Ages

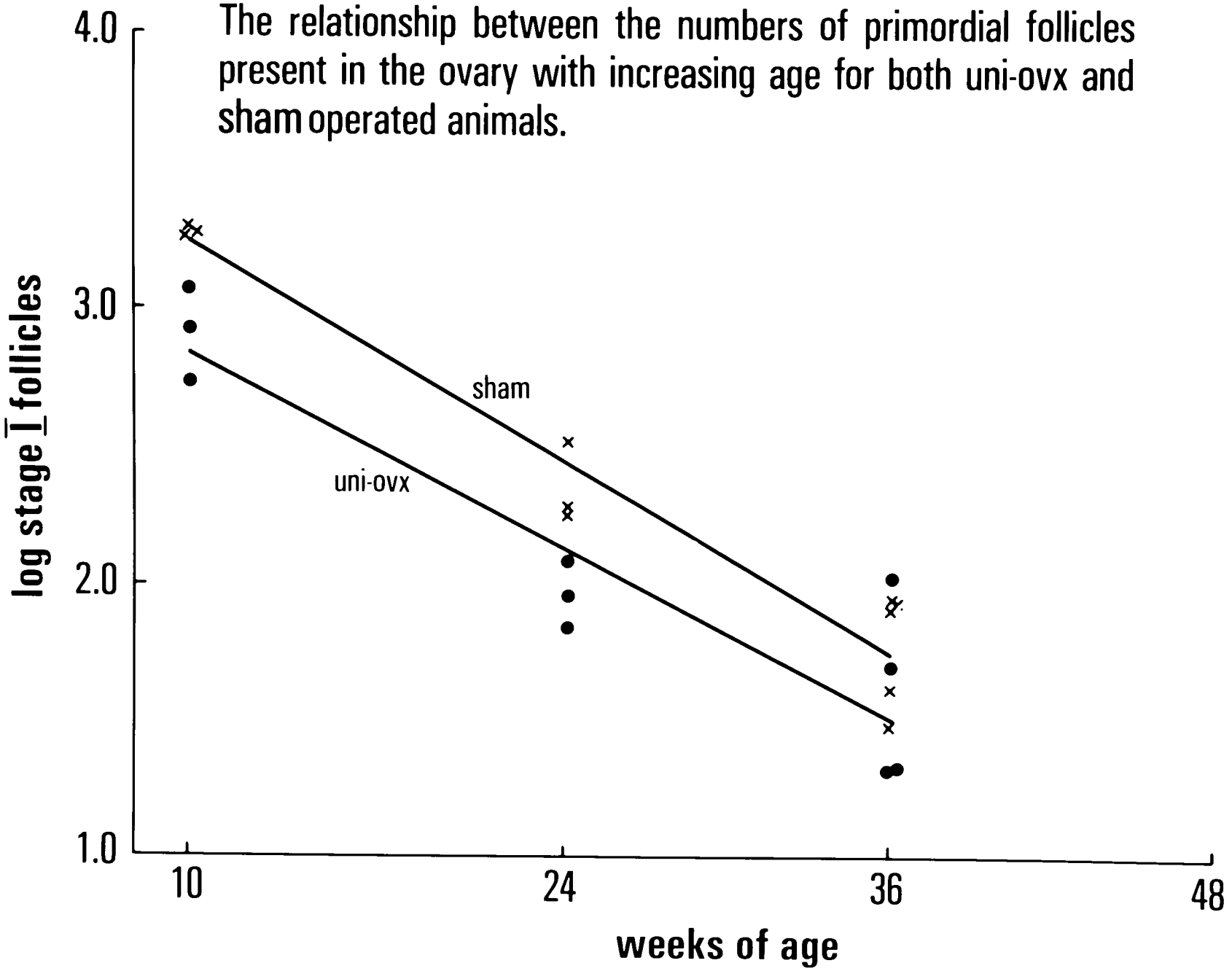
The data obtained for the primordial follicle counts, based on 21 animals from 3 ages is not complete. However, it does represent progress so far. Figure 3.7 shows the relationship between age and log total number of oocytes for uni-OVX and sham operated mice. The data were transformed semi-logarithmically since there is widespread evidence that the decline in number of oocytes is exponential with respect to age. The fit to a straight line was satisfactory for both experimental groups (uni-OVX, $r = 0.97$; sham, $r = 0.84$). The slopes are not significantly different for the two groups of mice, indicating that although the uni-OVX mice had fewer primordial follicles than intact animals, the rate of oocyte loss was the same in both groups.

3.4 DISCUSSION

In the previous chapter an attempt was made to use the XO mouse as a model for the menopausal human female. The characteristics of the XO mouse which made it particularly useful

Fig. 3-7

The relationship between the numbers of primordial follicles present in the ovary with increasing age for both uni-ovx and sham operated animals.



in such studies - i.e. shortened reproductive life (Lyon and Hawker, 1973) which is perhaps a consequence of reduced numbers of oocytes at birth, and early depletion of the stock of oocytes in middle age with virtual exhaustion at the cessation of fertility (Burgoyne and Baker, 1981) are also common to the CBA mouse strain (Faddy *et al*, 1983). Unlike the XO mouse, however, the CBA strain under normal circumstances produces only haploid oocytes (n = 20), so that aneuploid and euploid gametes or embryos can easily be distinguished.

The CBA mouse has been used in the present study, to test whether as claimed for other strains (Thung, 1961), that uni-OVX accelerates the normal progression of age changes of the ovarian cycle, and also to determine whether there is an associated change in the age-specific incidence of embryonic aneuploidy as a result of the removal of an ovary in early life. It was hoped that the results might also provide a means by which the validity of the production-line hypothesis (Henderson and Edwards, 1968) and hormonal hypothesis (see Chapter 1) could be examined.

An increase in aneuploidy with maternal age was found for both uni-OVX and sham operated animals in the present study. Similar increases in aneuploidy with increasing maternal age for the CBA mouse strain have been reported by other authors. Gosden (1973) for example, reported a significant increase in the number of trisomic embryos from 8-12 month old CBA/H-T6 mothers compared with mothers aged 2-7 months. Martin *et al* (1976) using *in vitro* maturation of oocytes found 5.2% disomy amongst oocytes from 5-8 month old animals compared with zero disomy in both 2-5 and 8-11 month old

animals. Chebotar (1978), however, has claimed a fourfold increase in hyperploidy amongst superovulated oocytes from older (8-11 month old) CBA's when compared with younger groups. Fabricant and Schneider (1978), looking at 10-14 day old embryos from CBA mothers, have demonstrated an increase in aneuploidy from 7.2% to 15.3% with increasing maternal age from 4.7 to 8.0 months. A similar increase in aneuploidy with increasing maternal age was also reported by Max (1977).

In all work designed to assess the level of non-disjunction by chromosome counting lies the problem of distinguishing between true aneuploidy and that which is due to artefacts of preparation. How best to express the aneuploidy figure is always a problem. In the present study the aneuploid counts for all groups, regardless of age and operation, are inflated by and the distributions skewed towards the hypoploids. These can arise not only by non-disjunction or chromosome lagging, but also as artefacts through chromosome loss during preparation. Such artefactual aneuploids should occur in all groups to a more or less equal extent (assuming a symmetrical distribution of probabilities for segregation of chromatids) and therefore diminish any age effect for aneuploidy which may exist. (Furthermore, some trisomic embryos might also be lost in the classification if one chromosome was lost by artefact. They would be then included in the diploid (2n = 40) group). In order to overcome the problem of artefactual hypoploids in this study, those spreads with two or more cells showing 39 chromosomes were recorded as 'true -39 s' as the probability of loss, through breakage, of 1 chromosome from each of 2 cells in an embryo is very small.

The usefulness of this however, has been reduced as the number of analysable cells per preparation was not recorded for other chromosome scores. Hence it is not possible to determine what proportion of the 1-cell 39's are also 'true-hypoploids'. Probably the best assessment of non-disjunction can be obtained from the 'hyperploidy' figure, indeed a doubling of the hyperploid total has been used by several authors as the best means whereby the frequency of non-disjunctional events can be estimated (e.g. Ford, 1975). In the present study the hyperploidy count in the sham animals increased from zero in both the 9-13 week and 22-26 week old animals to 1.7% and 2.2% in the 35-40 and 44-50 week old animals giving aneuploidy frequencies (by doubling) of 3.4% and 4.4% respectively. In the uni-OVX animals this aneuploidy figure increased from zero to 8.0% and to 9.8% for the 9-13, 22-26 and 35-40 week old groups respectively. These increases however, are not statistically significant, reflecting the problems of obtaining sufficient data in studies of this kind. For the statistical comparisons therefore, all 41, 39, 38 and 37 chromosome embryos have had to be included in the aneuploidy total regardless of the fact that a proportion of the latter category undoubtedly arose by artefactual loss.

The results show that with increasing maternal age these total aneuploidy levels increase three-fold amongst sham controls and become almost doubled in the uni-OVX group. This increase is apparently progressive (continuous) rather than stepwise. At all ages the level of total aneuploidy was greater amongst uni-OVX than sham animals, and it increased consistently with increasing maternal age in the former group. In the sham-operated animals,

however, there was no increase in aneuploidy between 9 and 26 weeks of age although an increase did occur in subsequent age-groups. These results show agreement with the findings of Gosden (1973) and Fabricant and Schneider (1978), rather than those of Martin *et al* (1976) who found a peak aneuploidy level in MII oocytes from middle-aged CBA females (i.e. 5-8 month old). Sopelak and Butcher (1982) have looked at the effect of decreased ovarian tissue, on embryonic development in rats and found an increase in abnormal and retarded embryos following uni-OVX, which supports the present findings. No cytogenetic data was collected by Sopelak and Butcher (1982).

With increasing maternal age there was also an increase in the proportion of ova lost due to a failure of ovulation or early development. Unfortunately, present results cannot precisely assess the contributions of each. It would seem likely, however, that most of this loss of ova is due to the failure of ovulation as Jones and Krohn (1961) have documented "Corpora Lutea accessoria (or atretica)" in old ovaries in which ova are retained. Furthermore, dead embryos should still have been detectable 3½ days after copulation, as unfertilized eggs were still obvious after this time. In the case of the human female too, the frequency of anovular cycles is known to increase with maternal age (Sharman, 1962; Döring, 1969).

From the present results it appears that the frequency of oestrous cycles, whether classified as 'normal' 4 or 5-day cycles, or as all types of cycle, decreases in both uni-OVX and intact animals with age. This is not a continuous decrease, however, as there is a period from 90 to about 300 days when the cycle

frequency in both groups remained at consistently high levels. This is similar to the pattern observed by Nelson *et al* (1982) for ageing intact C57BL/CJ mice, although in that strain the period preceding irregular cyclicity was longer. Cycle frequency declined earlier in the uni-OVX animals, with a significant difference in cycle length obvious after 230 days. For sham controls the frequency of normal cycles started to decline from day 250, with all types of cycle, regardless of the method of classification, being significantly longer than preceding intervals from about day 300. Thung (1961) demonstrated that the frequency of normal cycles declined more rapidly in ageing uni-OVX animals than in their sham-operated counterparts. In the present study the decline in regular cyclicity did not occur until at least 150 days after operation. This delayed effect of uni-OVX may be explained by the flexibility of the ovarian system in young animals which continue to cycle regularly with the remaining ovary shedding an increased number of ova at oestrus. As the ovarian follicle population becomes reduced during ageing the compensatory response wanes and cycles become irregular and cease before those of intact animals (Thung, 1961). This might explain why the fertility of long-term uni-OVX mice is lost earlier in life than intact control animals (Biggers *et al*, 1962; Gosden, 1979).

The finding that there was little difference in the time taken to plug for mothers of normal offspring with increasing age, regardless of operation sub-group, was of some interest. With the exception of the 22-26 week old uni-OVX group all plugging times were remarkably consistent. This time interval could be expected to show some similarity to the changing pattern of cycle frequency

with advancing age as the mean time taken to plug for any group should reflect the regularity of oestrus. The data, however, are presented differently in each case. The fact that the mothers were classified on the basis of the offspring they produced may account for the lack of increase in plugging time with age, particularly as the cycle frequency judged from the smear data, did change with advancing age. Another factor may be that generally those animals with the more regular cycles are the ones which plug at any age. From the raw data there appeared to be a trend towards longer plugging times for mothers producing aneuploid offspring. None of these, however, were significantly longer than for mothers producing euploid offspring, although this conclusion is based on small samples and more information needs to be collected before a firm conclusion can be reached. It may be that populations with a greater risk of producing aneuploid offspring are actually comprised of an increased number of specifically 'at-risk' individuals, i.e. those with a relatively high frequency of irregular cycles. Those individuals cycling regularly may continue to produce normal offspring regardless of age. In this respect Mikamo and Hamiguchi (1975) have looked at young rats mated during a naturally-occurring six-day cycle. They found that such prolonged cycles often resulted in a lowering of implantation rates and in an increase in the numbers of degenerated embryos. This deleterious effect of a spontaneous delay of ovulation in the rat is not necessarily associated with advanced maternal age. The fact that older females tend to have longer oestrous cycles, however, suggests that irregularities associated with prolonged cycles are, as a consequence, more likely to occur in older females

(Mikamo and Hamiguchi, 1975). These authors have also induced delayed ovulation with pentobarbitol using a method similar to that described by Butcher and Fugo (1967). When the results of these two studies were pooled, they showed 11 aneuploids from 627 in the delayed ovulation group compared with 0 from 640 controls ($p < 0.005$). Mikamo and Hamiguchi (1975) concluded that the increased incidence of aneuploidy in the 6-day cycle group was very likely due to the delay of ovulation causing over-ripeness of the ovum. Butcher (1975) considers that major factors of abnormal embryonic development are probably associated with intra-follicular hormone levels and the sequence of events during a few days immediately prior to ovulation. Furthermore, Butcher (1975) suggests that, as found by Mikamo and Hamiguchi (1975) for the rat, intra-follicular ageing of the oocyte as a result of prolonged menstrual cycles in the human female could also result in birth defects at *any time* during reproductive life when a delay in ovulation occurs.

Studies have been made to determine the plasma concentration of LH, FSH, prolactin, progesterone and oestradiol-17β throughout the 4-day oestrous cycle of the rat (Butcher *et al*, 1974). Butcher (1975) also reported on the changes which occur in the oocyte and in circulating levels of hormones as a result of pentobarbitol delayed ovulation. Pro-oestrus peaks of LH, FSH and progesterone were supressed on both days of treatment but occurred at the expected time on the afternoon prior to ovulation. Plasma levels of oestradiol were elevated on all 3 days but dropped back to base level at the usual time in relation to ovulation. None of the hormone concentrations differed significantly from those found in controls during this

period. Since oestradiol was elevated throughout the period of experimentally delayed ovulation, Butcher (1975) suggests that it is this hormone which should be studied for its possible role in alterations in the oocyte during intrafollicular ageing. Furthermore, it should be determined whether a prolonged secretion of oestrogen occurs during the lengthened oestrous cycles found in naturally aged animals. More recently Butcher and Pope (1979) have used an antiserum against œstradiol for oestrogen absorption, and replacement with diethylstilbestrol to study the role of oestrogen during prolonged oestrous cycles of the rat on subsequent embryonic death or development. Their results suggest that the early rise, or prolonged elevation of pre-ovulatory levels of oestrogen in relation to the time of ovulation is responsible for alterations in the oocyte and intrauterine environment which result in subsequent abnormal development and embryonic death following delayed ovulation. It would appear that extended elevation of oestradiol might be more important than hormone concentration *per se*, and certainly for the human female there appears to be a natural overall lowering of oestrogen levels during the peri-menopausal period (Barlow *et al*, 1981). The hormone assays from serum taken on the day of pro-oestrous in the present study will indicate such lowering in CBA females, if it occurs, but will only reflect changes on this one specific day with increasing age.

The relationship between the rate of meiosis and the timing of ovulation, both of which are hormonally controlled, would appear to be critical. Resumption of meiosis and ovulation is normally synchronised (Donahue, 1972). In the intact fully developed

follicle the ovary remains in the dictyate stage until ovulation is imminent. If the oocyte is removed from the follicle meiosis is resumed. It would appear that the resumption of meiosis without ovulation leads to atresia, and certainly oocytes in many follicles undergoing atresia show evidence that meiosis is resumed (Foote, 1975). A breakdown in the relationship between the rate of meiosis and ovulation, might result in the production of aneuploid oocytes. Indeed Crowley *et al* (1979) have considered a breakdown in this relationship due to changing hormone levels to be responsible for the increase in Down's syndrome with increasing maternal age in man. In their "Chiasma-hormonal" hypothesis they propose that hormone levels, which both trigger the resumption of meiosis, control its timing in the ovum, and control cycle length, change with advancing maternal age. They suggest that meiosis may slow down, making premature chiasma terminalisation in meiotic bivalents (and especially the small acrocentric pairs) more likely to occur so that aneuploidy may result from random segregation of univalents.

The latter part of this hypothesis incorporates ideas of Henderson and Edwards (1968) from the 'production-line' hypothesis which the present study may also test. There are two basic suppositions on which this hypothesis rests: Firstly, that those eggs laid down first in the fetal ovary are those ovulated first, while those laid down last are ovulated last. Secondly, that those laid down last are subject to inferior conditions (developmental or nutritional) leading to a reduction in numbers of chiasmata and thus an increase in the numbers of univalents found at metaphase I of meiosis. This they postulate could lead to increased aneuploidy

with increasing maternal age. Recent data from the mouse (Polani and Jagiello, 1976; Speed, 1977) and Chinese hamster (Sugawara and Mikamo, 1983) indicate a lack of correlation between univalent occurrence at MI and aneuploidy incidence in MII oocytes, thus calling into question the 'production line' hypothesis as well as the latter part of the 'chiasma-hormonal' hypothesis which invokes the random segregation of univalents. On the basis of their hypothesis, Henderson and Edwards (1968) suggest also that the frequency of ovulation of chromosomally abnormal oocytes may be higher when the overall number of eggs available is reduced by unilateral ovariectomy. By this they imply that the store of ovarian oocytes is utilized more rapidly after removing the contralateral ovary, and the defective oocytes are therefore ovulated earlier. Quite clearly the present data show an increase in aneuploidy levels with increasing maternal age for uni-OVX animals which precedes the similar rise in sham-operated controls. From the counts of primordial follicles, however, it would appear that the rate of loss of oocytes from the ovary is the same for both groups. Baker *et al* (1980) reported that after an initial 4-week period following operation the rate of depletion of follicles from the pool was no different in uni-OVX animals compared with intact controls. This is supported by the present findings and also those of Jones and Krohn (1961) who also obtained some limited evidence of this. Contrary to the assumption made in the 'production-line' hypothesis therefore, the earlier increase in aneuploidy following uni-OVX would not appear to be due to a more rapid utilization of oocytes.

As well as testing the production-line hypothesis, the present chapter has examined the relationship between hormonal

imbalance, irregular cyclicity and the increase in aneuploidy with increasing maternal age. The potentiation of the maternal age effect by uni-OVX implies that abnormal segregation of meiotic chromosomes in ageing oocytes is an epiphenomenon of physiological ageing of the ovary rather than dependent upon the chronological age of the oocyte or mother *per se*. The earlier increase in irregularities of the cycle and the increase in aneuploidy might signify a causal relationship since embryopathies are more common in rats which cycle irregularly (Fugo and Butcher, 1971). The results imply that uni-OVX may be an additional risk factor for Down's syndrome in older women. Indeed, any factor which induces an early menopause may present additional risks of increased aneuploidy (Emanuel *et al*, 1972). For example the correlation of auto-immune disease and increased aneuploidy (Fialkow, 1966) may be due to an indirect effect of this condition as Irvine *et al* (1968) and Vallotton and Forbes (1969) have reported premature menopause in some females with auto-immune disease. As suggested by Butcher (1975) prolonged menstrual cycles with delayed ovulation could result in birth defects at any time during reproductive life. Irregular cycles will obviously become more frequent in older females, due to hormonal imbalance, and hence aneuploidy levels will also be expected to be higher from such mothers. Even in young women, however, there could exist a risk of a Down's conception at any irregular cycle. Current interest in Down's syndrome research is focussing on the apparently increased incidence over recent years of Down's births to younger women (Mikkelsen *et al*, 1976; 1980; Lowry *et al*, 1976). The suggestion that the contraceptive pill may be implicated as a causal factor has been made by Read (1982).

It is also tempting to speculate that the probability of conceiving a Down's fetus will be determined primarily by the time of approaching menopause. This question was investigated several years ago (Oster, 1953; Sigler *et al*, 1967), although it has not yet received an unequivocal answer. A logical extension of this hypothesis is that any factor, environmental or ideopathic, which depletes the oocyte population, and results in premature menopause, could advance the maternal-age-effect for aneuploidy.

CHAPTER 4

THE INDUCTION OF ANEUPLOIDY BY CHEMICAL TREATMENT

4.1 INTRODUCTION

The first discovery that aneuploidy could be induced experimentally appears to have been made by Mavor (1921, 1922) using X-irradiation. Since that time numerous studies into aneuploidy induction have been made and a range of organisms has been tested both with radiation and chemical compounds. Chemical induction studies in mammalian germ line systems are, however, not numerous.

Some authors consider that exogenous agents may be responsible for the induction of nondisjunction in humans. Mikkelsen *et al* (1980) have suggested, for example, that the increasing incidence of Down's offspring born to younger mothers in the heavily urbanized districts around Copenhagen might be due to increased exposure of the population to environmental pollutants. There are also epidemiological data indicating that extra human aneuploidy, superimposed on that attributable to maternal ageing, could arise from exposure to radiation (therapeutic or diagnostic) (Uchida, 1979; Alberman *et al*, 1972). These data are, however, somewhat equivocal (Uchida, 1979) and certainly in the mouse there would appear to be no increase in sensitivity to radiation induced aneuploidy with maternal age (Tease, 1982a).

The heightened concern over human exposure to hazards of this kind has led, in recent years, to a surge of mutagenicity testing to detect substances of potential danger to man. Aneuploidy has been used as an end-point in some of the tests, although to a much lesser degree than structural abberation or point mutation. When aneuploidy induction has been used as an end-point, however, fungal or *Drosophila* test systems have been utilized most often, since these are generally quicker and less tedious than those employing mammalian germ cell systems. Extrapolations of such studies in lower eukaryotes, to man, would seem questionable; testing mammalian germ cells would certainly seem more appropriate. In view of this lack of information from mammalian systems the present studies were undertaken. Four different chemical compounds have been tested on male and female germ cells of the mouse, at various stages of meiosis, in order to ascertain their aneuploidy inducing potential.

4.1.1 The Choice of a Test System

There are a number of different systems described in the literature which can be used as non-disjunction indicators in mammals and all of which have been employed at one time or another to study both spontaneous and induced levels of aneuploidy. None, however, is without limitation. Choice of a particular system will be governed by whether direct estimates of non-disjunction in germ cells at the first or second meiotic divisions (or both) are required, or whether the levels of aneuploidy in zygotes, foetuses or liveborns is to be determined. In the mouse, cytological or

genetic techniques are available for the detection of aneuploidy at all these stages, and have been employed by a number of workers. Russell (1979), for example, has developed a system for the detection among liveborns, of numerical sex-chromosome anomalies (N.S.A's) utilizing X-linked genetic markers which render all viable types, except XYY, recognisable on external examination. One advantage of this method is that spontaneously occurring sex-chromosome aneuploids are extremely rare, so that there is little background 'noise' for the end-point. Methods are also available for the detection of certain autosomal aneuploids among liveborns by complementation testing (Lyon *et al*, 1976). Such systems use marker genes in the detection of the non-disjunctional event, two gametes with complementary aneusomies giving rise to chromosomally balanced viable offspring. Other authors have estimated levels among liveborns by karyotyping newborn mice (Goodlin, 1965).

Systematic karyotyping of mid-term foetuses has also been performed (Ford and Evans, 1973; Yamamoto *et al*, 1973: Speed and Chandley, 1981). This technique yields several metaphases per conceptus, which gives a reliable finding if all have the same number of chromosomes, and it permits identification of the particular chromosome involved in non-disjunction. Mosaicism, which can sometimes be observed, does however present a problem since the origin of the error, be it meiotic or mitotic may not be discernible. A further disadvantage is that there is a reduced chance of recovering autosomal trisomics and virtually no chance of finding monosomics. According to Gropp (1982) most autosomal trisomics in the mouse die from about day 10 of gestation onwards, while monosomics are eliminated before, or shortly after implantation.

Some of these problems can be overcome by scoring 1-cell embryos or early cleavage divisions in morulae or blastocyts. Maudlin and Fraser (1978) have used the former and Gosden (1973) the latter to look at the relationship of aneuploidy with maternal age in the mouse. Others (e.g. Röhrborn *et al*, 1971; Watanabe and Endo, 1982) have used early cleavage divisions to score structural and numerical abnormalities induced by various chemicals given to the parents. This technique tends to be more difficult than other methods which employ the use of older embryos since fewer analysable cells are present on the slides. The great advantage is, however, that few if any early losses should be missed, depending of course on the gestational stage examined.

Non-disjunction at the first meiotic division in either males or females can be assessed by dyad counting at metaphase II. Metaphase II oocyte analysis has been used for aneuploidy assessment by Hansmann (1974) and Sugawara and Mikamo (1980). It is the protocol used by Hansmann (1974) upon which the present study of induced non-disjunction in female mice is largely based. The scoring of aneuploidy levels in MII oocytes is particularly relevant in view of the high levels of non-disjunction found to occur at the first maternal meiotic division in humans compared with at all other times (Jacobs and Hassold, 1980). It does however, have the disadvantage that it cannot provide a measure of non-disjunction occurring at the second meiotic division.

In males too, MII analyses have been carried out both in untreated (Ohno *et al*, 1959; Beatty *et al*, 1975) and treated animals (Szemere and Chandley, 1975; Szemere and Marczinovits,

1977). Good testicular preparations from male mice normally contain relatively large numbers of metaphase II cells and so the system provides a quick and simple method for aneuploidy analysis. It has been adopted for use in the present study. Tates (1979) has developed an alternative system for measuring sex-chromosome non-disjunction using the germ cells of the male field vole *Microtus oeconomus*. In that species it is possible to identify the X and Y chromosomes in round spermatids because of the heterochromatic nature of the Y and a large block of centromeric heterochromatin on the X, both of which stain darkly and can be separately identified by C-banding. This technique, like the NSA technique of Russell (1979), only detects sex-chromosome non-disjunction with the further disadvantage that it may also detect anomalies additional to whole chromosome aneuploidy.

4.1.2 Previous Findings for Aneuploidy Induction in Mammalian Germ Cells using Chemical Compounds

The data concerning the chemical induction of aneuploidy in mammalian meiotic systems are not extensive, and where studies have been carried out, the effects have not been great. The one exception to this is the effect of spindle inhibitors.

Sugawara and Mikamo (1980) scored MII oocytes in the Chinese hamster to assess the aneuploidy inducing potential of colchicine, administered during spindle formation at concentrations which are not completely inhibitory. In a total of 2124 oocytes analysed, an increase in aneuploidy was reported from 2% (35/1742) at control levels to over 25% (99/382) in treated animals. Both anaphase lagging and non-disjunction were observed. Other chemicals have

been selected for aneuploidy testing because of their known effects on chromosomes or DNA, for example in terms of chromosome breakage. (In *Drosophila melanogaster* (Parker and Busby, 1973) and the mouse (Tease, 1982a) there are data which implicate chromosome breakage phenomena in aneuploidy induction.) In this respect the alkylating agents EMS (ethylmethanesulphonate) and MMS (methylmethanesulphonate) were chosen for testing by Szemere and Marczinovits (1977). They compared the results obtained with these two compounds with those previously obtained for X-irradiation (Szemere and Chandley, 1975). MMS (50 mg/Kg body wt.) gave 3.3% (13/295) cells with more than 20 dyads at MII following treatment at the pre-leptotene stage of meiosis, this being two-thirds the effect produced by 100 rad X-rays. EMS (240 mg/Kg) produced half the effect found with X-rays i.e. 7/414 (1.7%) of cells with more than 20 dyads.

Hansmann (1974) looked at the stage sensitivity of mouse oogenesis to amethopterin and cyclophosphamide and found that the induction of aneuploidy was related to treatment at highly sensitive stages where chromosome segregation takes place, such as in oogonia and during the immediately pre-ovulatory (diakinesis/MI) phase. This stage was also found by Tease (1982a) to be sensitive to X-ray induced non-disjunction in the mouse. The dictyate stage treated 1 week prior to superovulation, was insensitive.

Jagiello and Lin (1973) examined metaphase II oocyte spreads to assess the effect of mercury treatment of dictyate oocytes. The results were negative, in spite of previous demonstrations of an *in vitro* effect. Another heavy metal which has been tested for aneuploidy induction is cadmium. For the golden hamster

female, Watanabe *et al* (1979) reported 8.4% aneuploidy following treatment at diakinesis/MI, with cadmium chloride (4 mg/Kg). This was a significant increase over control levels. In the mouse an earlier study (Watanabe *et al*, 1977) had, however, produced no significant increases in MII aneuploidy when treatment was applied at this stage. Subsequent studies (Watanabe and Endo, 1982) however, showed an increase in aneuploid and triploid blastocysts in the mouse following treatment at diakinesis/MI in the female parent using a dose of 6 mg/Kg. The increase in triploidy however was the most prominent effect.

4.1.3 The Choice of Chemicals for Testing

(a) p-fluorophenylalanine (pFPA) and phenylalanine

pFPA was chosen for testing because of its known potency as an aneuploidy-inducer both at mitosis and meiosis in a wide range of fungal systems. Lloas (1961) for example showed that pFPA could induce mitotic haploidization in diploid *Aspergillus niger* while Griffiths (1979) using *Neurospora crassa* and Bond and McMillan (1979) using *Sordaria brevicollis*, showed that pFPA was very effective at inducing meiotic aneuploidy even though it had not been shown to induce gene mutation (Griffiths, 1979). Tates (1979) using the germ cells of *Microtus oeconomus* demonstrated that pFPA (300 mg/Kg over 24 hrs) was capable of inducing sex-chromosome non-disjunction in a mammalian system. As pFPA is an analogue of the amino-acid phenylalanine, both being structurally very similar, it was considered worthwhile to use phenylalanine as a control.

(b) 6-Mercaptopurine (6MCP)

6MCP, the non-alkylating anti-leukaemic purine analog, was also selected for use on the test system. Holden *et al* (1973) and Generoso *et al* (1975) have shown that 6MCP can induce chromosome breakage in late-differentiating spermatogonia and very early spermatocytes of the mouse. There is also some evidence to suggest that it may induce non-disjunction; Cacheiro and Generoso (1975) found three XYY males amongst 615 F_1 progeny of males treated with 6MCP at late spermatogonial or early spermatocyte stages.

$_p$FPA and phenylalanine were dissolved in physiological saline whereas 6MCP was dissolved in sodium hydroxide in physiological saline, therefore it was necessary to use this as a control.

(c) 4-Chloromethylbiphenyl (4CMB)

The selection of the other chemical for testing on the system was somewhat fortuitous. 4CMB was tested as part of a United Kingdom Environmental Mutagen Society national toxicology trial. The chemical was provided by I.C.I. and was tested by numerous groups on the widest possible range of mutagen testing systems. 4CMB is known to be a carcinogen although there was no evidence to suggest it had aneuploidy incuding properties. Because of problems dissolving 4CMB it was applied in suspension in a mild solution (0.05%) of the detergent Tween 80, consequently it was necessary to use this as a control also.

4.1.4 Choice of Spermatogenic and Oogenic Stage for Chemical Testing

It was decided to test the sensitivities of three different meiotic stages in males and two different stages in females. Choice of stage was based on the following considerations.

Pre-leptotene in the male was chosen because of previous demonstrations of its apparent sensitivity to non-disjunction induction following treatment with X-rays (Szemere and Chandley, 1975) EMS and MMS (Szemere and Manzinovits, 1977). Zygotene was also chosen in the male because it is the stage in meiotic prophase at which pairing of homologous chromosomes occurs. In *Drosophila* Savontaus (1975) showed that aneuploidies arising from the irradiation of oocytes were often non-crossovers, and suggested that this effect was due to a failure of pairing. This stage was not examined in the female because it occurs pre-natally thus presenting problems from possible teratogenic effects with maternal exposure.

Diakinesis/Metaphase I in both males and females was chosen because this is the stage immediately preceding the meiotic division where possible effects on the spindle might be produced. It was also chosen because in female mice, an increased sensitivity to aneuploidy induction by X-rays has been demonstrated following treatment at the diakinesis/metaphase I (pre-ovulatory stage) (Tease, 1982a) compared with treatment at the dictyate stage (Tease, 1981). Also it was a stage found sensitive to aneuploidy induction by Amethopterin and cyclophasphamide in the mouse (Hansmann, 1974) and by cadmium chloride in the hamster (Watanabe *et al*, 1979).

Dictyate was also treated in the present study in both young and old females in order to test for any further enhancement in aneuploidy levels over and above those attributable to ageing.

4.2 METHODS

4.2.1 Males

4.2.1.1 Chemical Treatments

The chemicals tested for aneuploidy induction, 6MCP, pFPA, phenylalanine, and 4CMB with sodium hydroxide (NaOH) and Tween 80 as controls, were all injected intraperitoneally.

6MCP was dissolved in a solution of 0.01 m NaOH and was tested at a dose of 150 mg/Kg body weight. It was necessary to heat the 6MCP solution prior to administration in order to achieve the necessary dose in a single 0.4 ml injection. Both pFPA and phenylalanine were dissolved in physiological saline and given as 0.3 ml injections at a dose of 100 mg/Kg body weight. the 4CMB was solubilized in a solution of 0.05% Tween 80 in distilled water. Prior to the injection of 4CMB sonication was necessary, to achieve an even distribution of undissolved chemical, for an approximate dose of 100 mg/Kg body weight. The vehicle for this last test, Tween 80, was used as a control, as was a 0.01 m solution of sodium hydroxide.

4.2.1.2 Chromosome Preparations

Metaphase II (MII) chromosome spreads from air-dried testis preparations vary between mouse strains in both quality and

quantity (R.M. Speed, personal communication). As MII was chosen as the end-point for the male study, it was desirable to use the strain of mouse giving maximum numbers of analysable MII counts per slide. Two random-bred strains of mice, Swiss and Q, which were available, were compared. A random-bred colony of Swiss-albino mice (Schofields. Delph, Odlham) was set up at the Animal Unit of the Western General Hospital, Edinburgh. A random-bred colony of Q-strain mice had previously been established from mice supplied by Professor D. S. Falconer (Institute of Animal Genetics, University of Edinburgh. For further information on Q-strain see Falconer, 1973).

Air-dried spreads of testicular cells were obtained using a slight modification of the technique of Evans *et al* (1964). Males of the two strains were killed at 8-12 weeks of age, by cervical dislocation. The testes were dissected from the tunica, and placed in 2.2% isotonic sodium citrate. The seminiferous tubules were teased apart in fresh 2.2% sodium citrate, and the cell suspension drawn off using a pasteur pipette. The cells were centrifuged at 400 r.p.m. for 8 minutes, the supernatant being discarded. The pellet of cells was then resuspended in 1.0% hypotonic sodium citrate solution for 8 minutes before being centrifuged again at 400 r.p.m. for 8 minutes. The supernatant was again discarded and fixative (3:1, methanol: glacial acetic acid) was slowly added down the side of the tube with constant agitation of the pellet. This was done by gently flicking a finger against the side of the test tube. After the addition of 20 drops of fixative, the cells were gently pipetted up and down, and more fixative was added up to a

volume of 5 mls. The cells were pelleted and resuspended in fresh fixative twice more. The final volume of fixative used varied between 2 and 3 mls depending upon the size of the pellet. The cell suspension was then left for a few minutes to allow clumps of cells to settle to the bottom of the tube.

Micro-slides were cleaned in alcohol and wiped with a clean dry cloth. Breathing on the slides produced a surface layer of condensation which aided cell spreading as the cell suspension was dropped from a height of about 10 cm. One drop was placed at each end of the slide and the slide shaken vigorously to give drying with optimum spreading.

The spreads were stained with carbol fuchsin (Carr and Walker, 1961) for 5-10 minutes, and after rinsing off with distilled water were differentiated briefly with 100:1; ethanol: glacial acetic acid and dried on a hot plate. The choice of this stain was made for several reasons. Firstly it was found by experience to give excellent criteria by which meiotic cell stage identification could be carried out in air-dried mouse testis preparations. Secondly it allows clear identification of the X and Y chromosomes at metapahse II. Thirdly it can be used as a prior stain for autoradiography as, unlike Giemsa, it does not wash out in developer and does not interfere with autoradiographic emulsion.

4.2.1.3 Identification and Timing of the Spermatogenic Sequence

Swiss males were chosen for the experiment as they were found to yield a higher number of analysable metaphase II spreads than the Q-strain (see later). The various spermatogenic stages

were classified on the basis of nuclear size and morphology. Microscopic examination and cell staging were carried out using a x100 objective and a x12.5 eyepiece lens. Nuclear diameter was measured by means of a calibrated eyepiece scale. Nuclear morphology is the more reliable of the two methods as nuclear size is dependent upon the hypotonic time used during cell preparation. Nuclear size alone, however, had to be used to identify cells in which the morphology of the nucleus was obscured by heavy tritium labelling when meiotic sequence analysis was performed (see below). A hypotonic treatment of 1% sodium citrate followed by an 8 minute spin was used throughout, although slight variation is inevitable. Size measurements can only be considered accurate ± 15%.

To establish the temporal sequence of spermatogenesis for the Swiss (Schofield) strain, the following schedule was used. Twenty-eight male mice aged 8-12 weeks, each weighing approximately 30 g, were injected with 100 µCi of tritiated thymidine (Amersham, s.a. 44 c/m mol) in 0.2 mls of distilled water. Two males were killed by cervical dislocation at 2 hrs and 24 hrs and subsequently at daily intervals for a further 12 days. Air-dried testis preparations were made from each male and stained with carbol fuchsin as described previously.

Autoradiographs were made using Kodak NTB2 liquid emulsion, diluted to half strength in distilled water. The slides were dipped in the emulsion for approx. 3 secs, dried in a current of air, and left to expose in light-tight boxes containing a small amount of silica-gel. The boxes were stored at 4°C for 14 days after which time they were developed using Kodak D19 developer at 20°C.

At each of the killing times the fate of the labelled cells was determined and the meiotic progression followed. Not only was the most advanced labelled cell type in the sequence recorded, but also, and more importantly, the progression of the "majority" cell types. In order to establish the precise time at which the majority of MII spreads showed label it was necessary to include some extra and more precise injection-to-killing intervals. Labelled MII cells were first seen on day 11: males were therefore killed at two-hourly intervals from day 11 to day 11 plus 16 hours post injection with tritiated thymidine.

4.2.1.4 The Effect of Chemical Treatment on Spermatogenic Timing

To ensure that cells sampled at MII had actually been treated at the desired stages earlier in the spermatogenic cycle, i.e. that delays in spermatogenesis had not been caused, the autoradiographic study was repeated on males which had been injected with the chemicals under test.

Twenty eight males were injected with tritiated thymidine as described earlier. Pairs of males were then treated with the appropriate doses of pFPA, 6MCP, phenylalanine and NaOH, chemical injection being given at appropriate times, based on timings in untreated males, to hit labelled cells in pre-leptotene, zygotene and metaphase I. For 4CMB and Tween 80, tests were confined to cells in metaphase I. All mice were killed at the time when treated cells should have been expected to reach MII. Air-dried preparations were made, and after development of the autoradiographs, mII spreads were examined for the presence of label. Comparisons

were made between control and treated animals. The pFPA treatment at MI was repeated with a further six males, pairs of males being killed at hourly intervals from 11 days 10 hours to 11 days 13 hours after the initial injection of tritiated thymidine.

4.2.1.5 Testing for Aneuploidy

Once the effect of chemical treatment on the timing of spermatogenesis had been established experiments to assess the aneuploidy incuding potential of the chemicals were performed.

Male mice were injected with either 6MCP, pFPA, phenylalanine, NaOH, 4CMB or Tween 80 at the desired doses (see Section 4.2.1.1) and subsequently killed at appropriate intervals in order to sample cells at MII which had received treatment at pre-leptotene, zygotene or MI. Air-dried preparations were made and the slides stained with carbol fuchsin. MII spreads were examined for numerical abnormalities by counting of dyads. Loss or gain of chromosomes was noted, if it occurred, as were structural aberrations, breaks and fragments.

4.2.2 Females

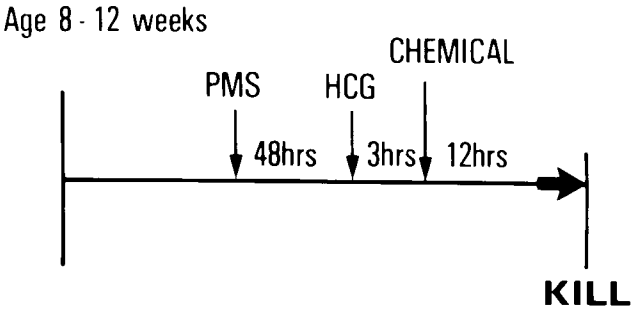
4.2.2.1 Chemical Treatments

Female mice of the Swiss strain were divided into four experimental groups, with treatments being made, in each case at either the dictyate or metaphase I (MI) stage of oogenesis in young (8-12 week old) and aged (36-40 week old) animals. The various treatment regimes employed are summarised in Figure 4.1.

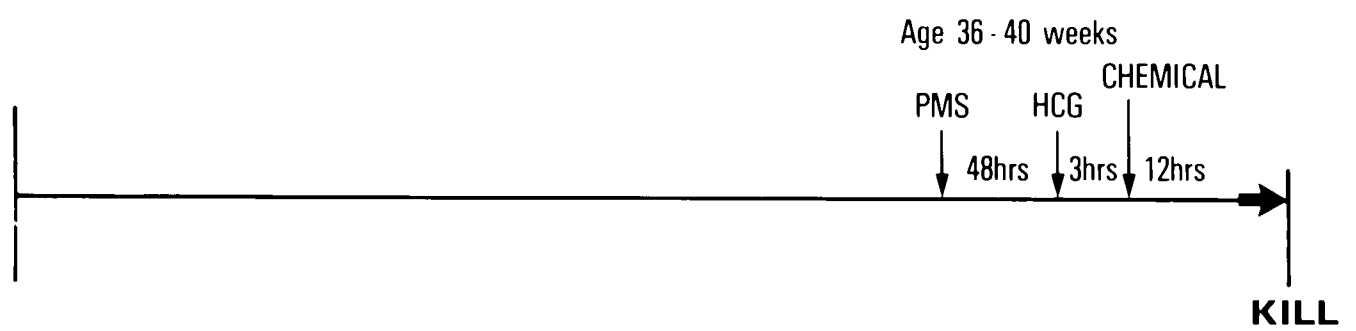
Fig.4-1

THE CHEMICAL TREATMENT OF FEMALE MICE

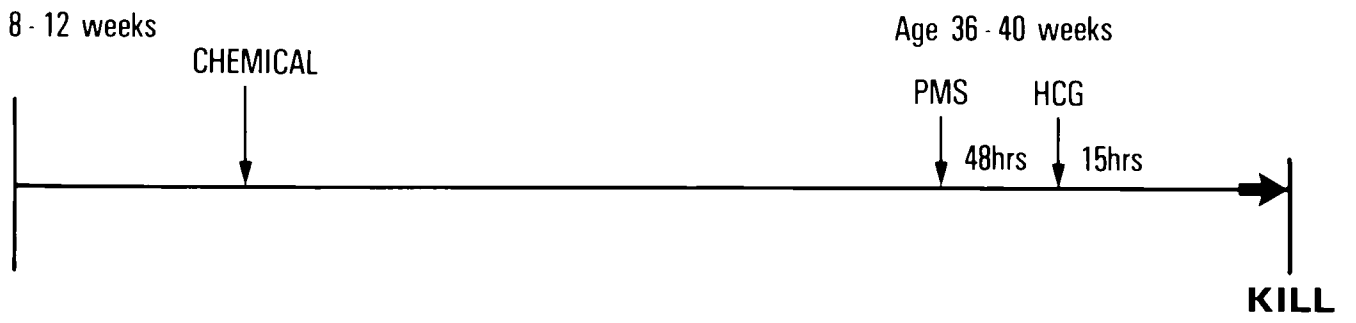
a) GROUP A Young animals treated at diakinesis/MI



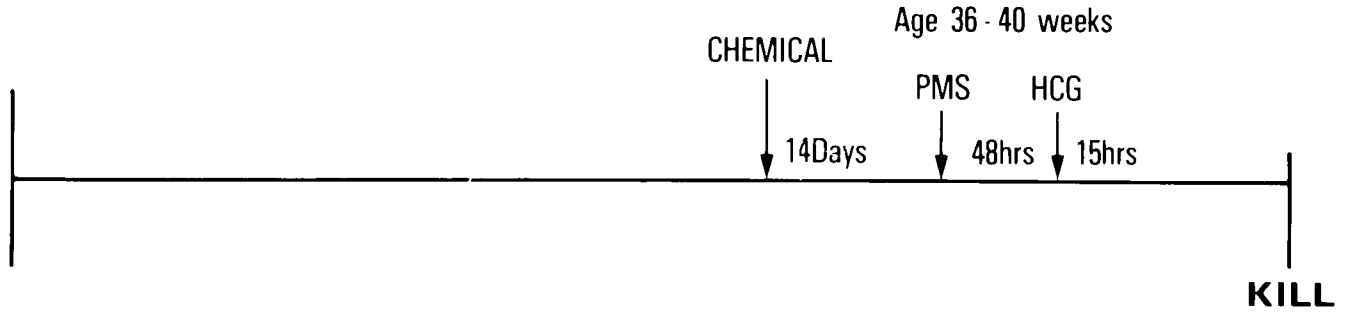
b) GROUP B Aged animals treated at diakinesis/MI



c) GROUP C Aged animals treated at the dictyate stage whilst young



d) GROUP D Aged animals treated at the dictyate stage when aged



In all cases the mice were superovulated using 3.0 iu pregnant mares serum (PMS) and 3.0 iu human chorionic gonadotrophin (HCG) 48 hours later. These doses of hormone have been shown to have no effect on the incidence of non-disjunction during the first meiotic division (Hansmann and El-Nahass, 1979). All oocytes were sampled at metaphase II (MII) - 15 hours after HCG injection. Females were killed by cervical dislocation, the oocytes removed from the ampullae of the fallopian tubes and fixed according to the technique of Tarkowski (1966). Chromosome spreads were C-banded using the method of Sumner (1972) and analysed at MII with regard to numerical abnormalities.

4.2.2.2 Treatment Groups

Group A; Young Animals Treated at MI

8-12 week old females were superovulated as described above, and 3 hours after HCG injection they were injected with 4CMB, at either 50 or 100 mg/Kg, suspended in 0.05% Tween 80. Eggs to be sampled at MII would thus be at a stage of oogenesis corresponding to diakinesis/MI (the preovulatory stage) at the time of treatment (Hansmann, 1974). Tween 80 alone was used as a control. 12 hours after 4CMB treatment the mice were killed, the number of eggs ovulated was counted, and chromosome spreads prepared.

Group B; Aged Females Treated at MI

Females aged 36-40 weeks were superovulated as before, and oocytes treated 3 hours later at diakinesis/MI with either 100 mg/Kg pFPA, 100 mg/Kg phenylalanine, or 150 mg/Kg 6MCP dissolved in

0.01 m NaOH which was also given as a control. In order to dissolve the 6MCP completely it was necessary to heat the NaOH solution slightly. All injections were given intra-peritoneally (i.p) in volumes of 0.3 ml or 0.4 ml. The mice were killed and chromosome spreads made 12 hours after injection of 4CMB.

Group C; Aged Females Treated at the Dictyate Stage whilst Young

Females aged 8-12 weeks were injected i.p. with pFPA, 6MCP, NaOH and phenylalanine at previously stated doses. They were then left to age. At 36-40 weeks superovulation with PMS and HCG was carried out, and MII chromosome spreads prepared. In this experiment oocytes sampled at MII, would have been at the dictyate stage when treated in the young females.

Group D: Aged Females Treated at the Dictyate Stage

Females aged 36-40 weeks were treated with pFPA, phenylalanine, 6MCP and NaOH. They were then left for 14 days before superovulation and chromosome preparation. In this case oocytes sampled at MII would have been in the dictyate stage in the aged animals when treated.

4.3 RESULTS

4.3.1 Males

4.3.1.1 Identification and Timing of Spermatogenic Stages

The number of dividing cells found on air-dried preparations from both Swiss and Q strain mice is shown in Table 4.1. As stated earlier, a higher number of dividing cells (spermatogonial metaphases

Table 4.1 Numbers of Dividing Cells per Slide in Air-Dried Testis Preparations from Mice of Different Strains

Mouse Strain	No. of Animals	Total No. of Slides Scored	Mean No. of Dividing Cells per Slide		
			Spermatogonial Metaphases	Meiotic MI	Meiotic MII
Swiss	4	16	6.3	27.8	18.9
'Q'	4	16	4.2	18.0	8.5

and 1st and 2nd meiotic metaphases) was found in mice of the Swiss strain than in Q males. The stage of most importance from the point of view of aneuploidy analysis, i.e. meiotic metaphase II, was seen more than twice as frequently on slides prepared from Swiss males. The total number of dividing cells per slide will be dependent on the technique used for preparation, and particularly on the volume of fixative in which the cells are finally suspended. These two factors were kept constant for both strains, the observed differences thus indicating an inherent difference between strains. In view of this finding the Swiss strain was selected for the aneuploidy testing experiment.

Figures 4.2, 4.3 and 4.4 illustrate the characteristics of the various spermatogenic stages. The identification of the pre-leptotene, zygotene and MI stages was particularly important for the aneuploidy induction test. The distribution of grains, and hence radioactive label, amongst the various cell stages at daily intervals is shown in Table 4.2. None of the mice killed 2 hours after the tritiated thymidine injection showed labelled leptotenes, whereas after 24 hours 33.5% of labelled cells were in this stage. For pre-leptotene treatment, therefore, the best time was considered to be greater than 2 hours but less than 24 hours following the injection of radioactive precursor. Six hours was chosen, somewhat arbitrarily as a suitable time to consider most labelled prophase cells still to be in pre-leptotene. On day 2 only 18.6% of cells were in zygotene, but by day 4 nearly half the labelled cells had passed through zygotene. On day 3, however, 48.9% of cells showing grains were still in the zygotene

Fig.4-2

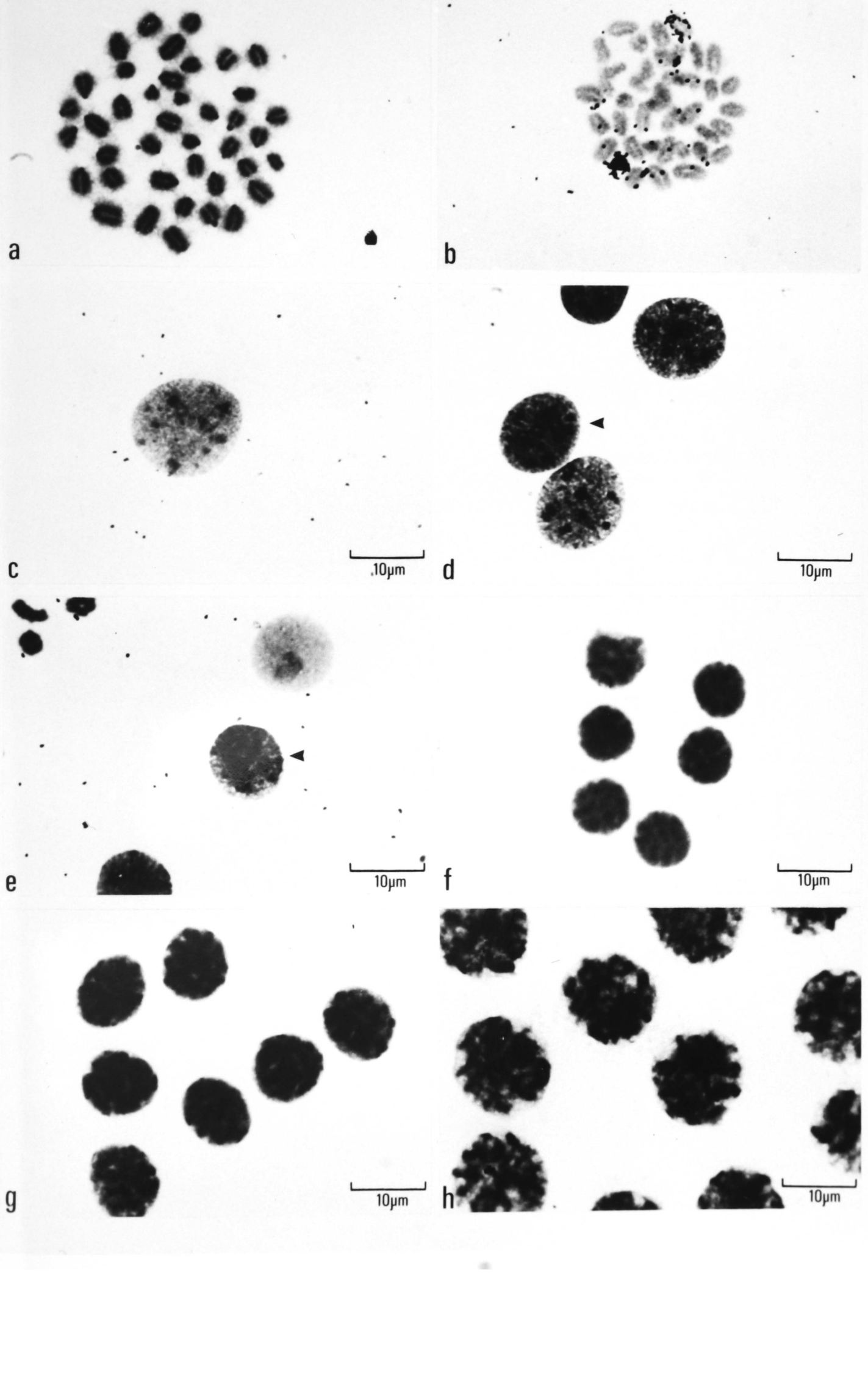
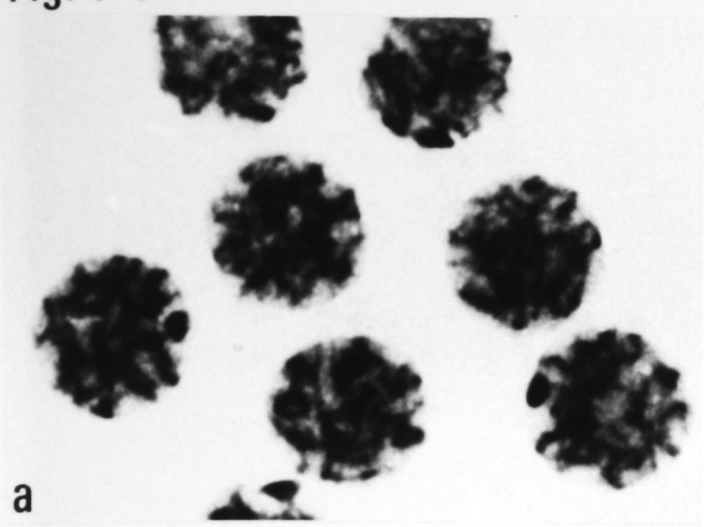
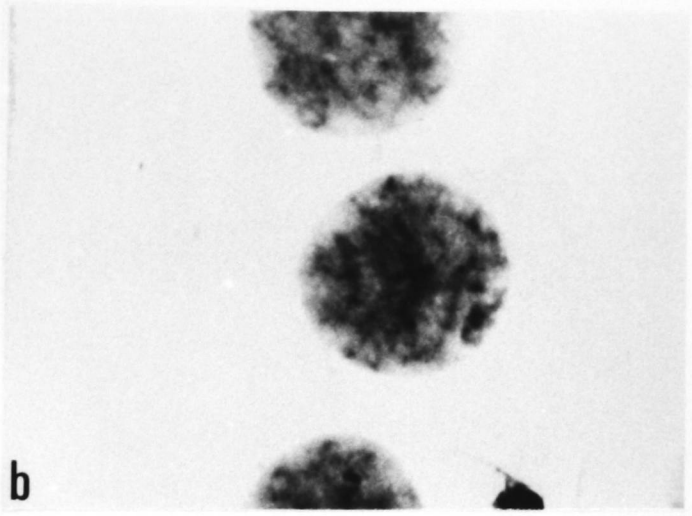
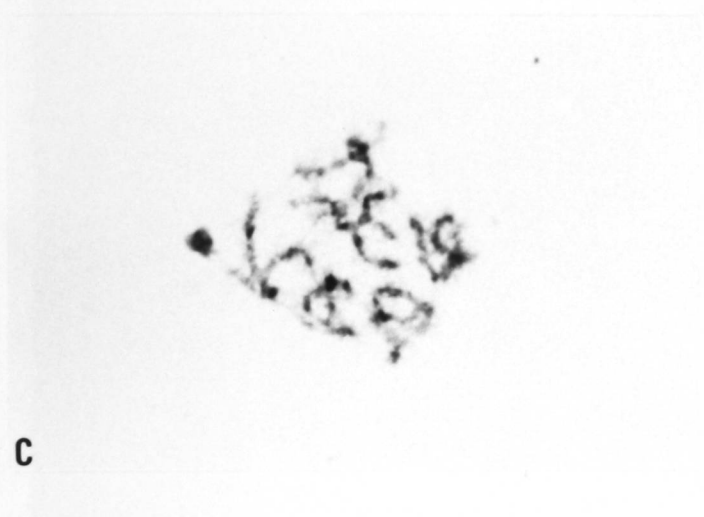
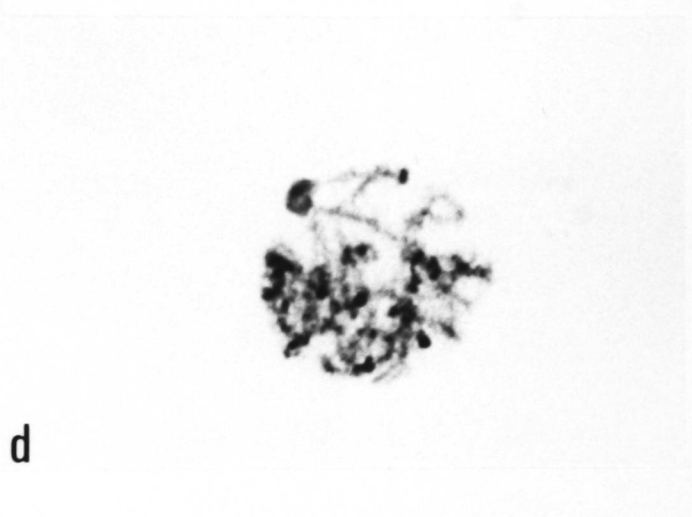


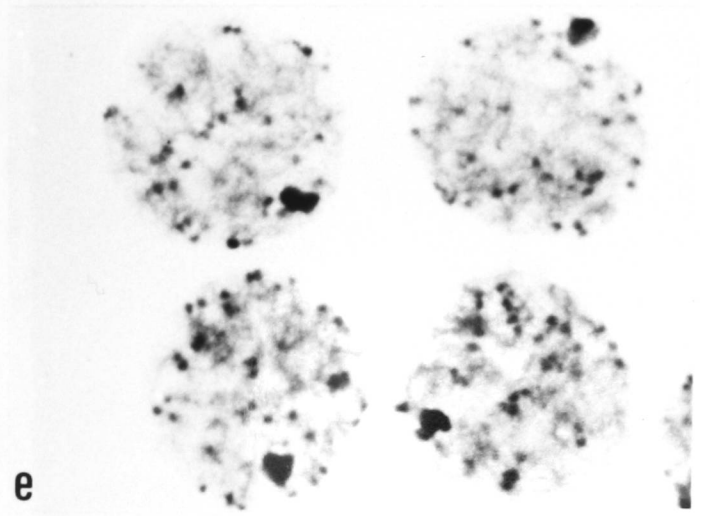
Fig. 4-3

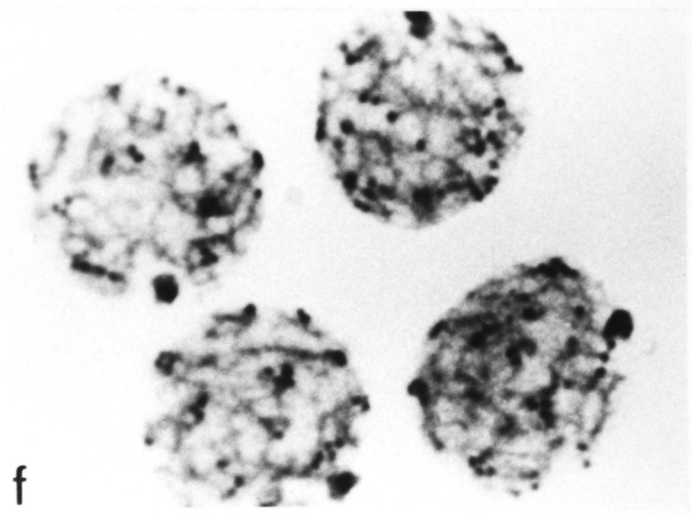


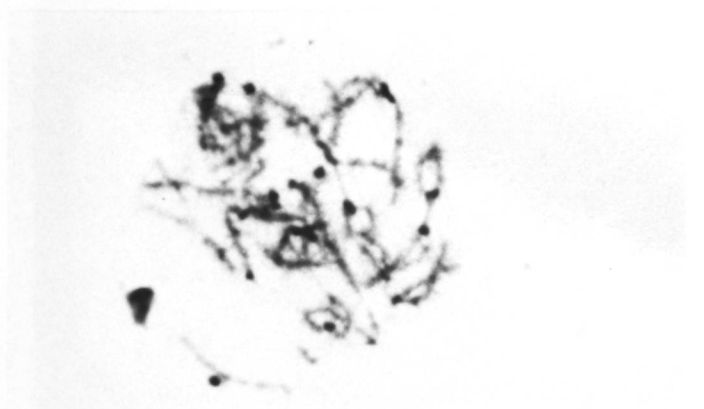












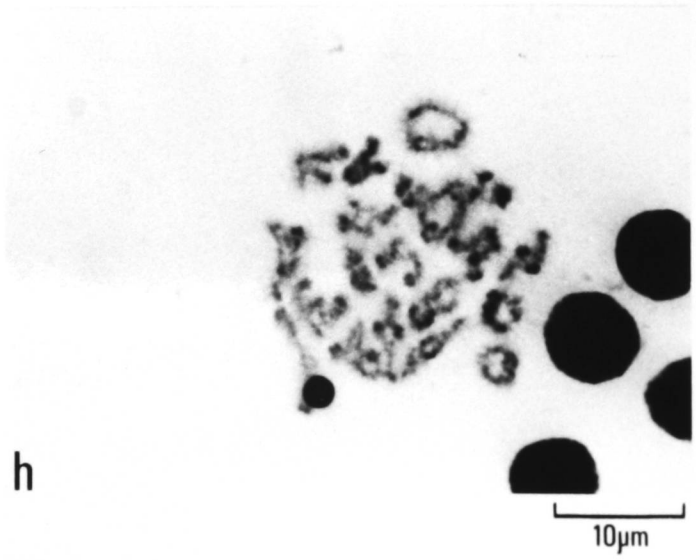
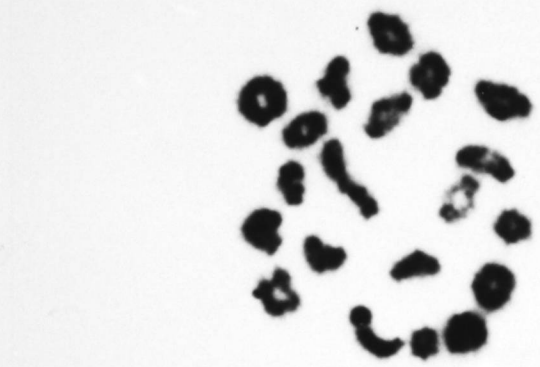


Fig.4-4



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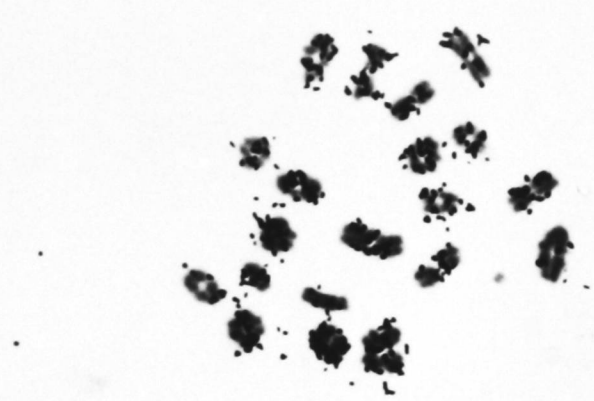

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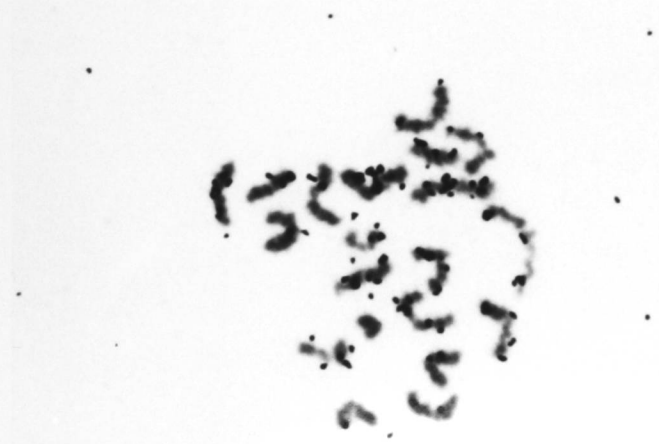

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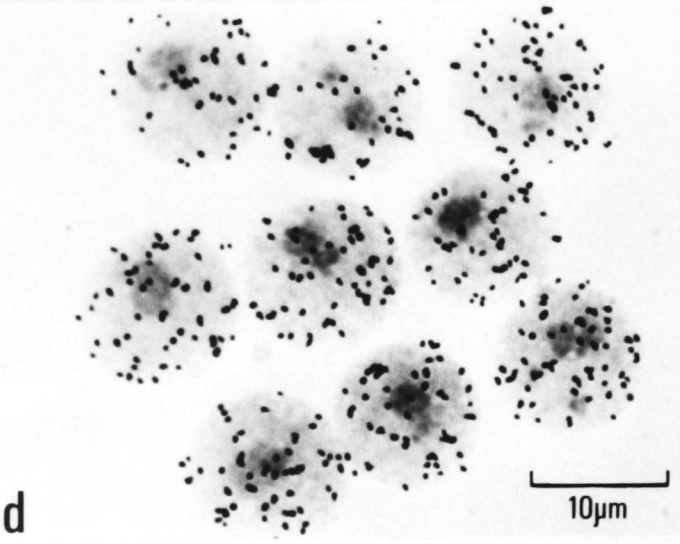

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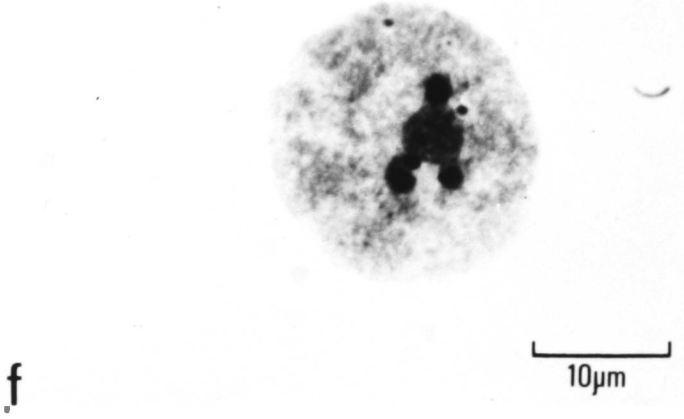

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Table 4.2 The Percentage Number of Cells showing Radioactive Label at Daily Intervals Subsequent to Incorporation of Tritiated Thymidine in Control Mice

Time Day(hrs)	No. of Cells	Spermatogonia								
		Type A	Intermediate	Type B	PMI	Leptotene	Zygotene	Pachytene/ Diplotene	Diakinesis/ MI	MII	Round Spermatids
0(2)	400	20.3	23.8	42.0	14.0						
1(24)	323	3.4	13.0	30.3	19.8	33.5					
2(48)	403	1.5	3.0	5.0	16.9	55.1	18.6				
3(72)	407			0.3	3.2	25.6	48.9	22.1			
4(96)	525					12.6	44.9	43.5			
5(120)	529					7.7	38.2	54.1			
6(144)	549						24.6	75.4			
7(168)	501						13.8	86.2			
8(192)	406							100			
9(216)	526							100			
10(240)	489							100			
11(264)	547							99.1	0.9		
12(288)	549							42.3	3.5	3.6	50.6
13(312)	158								2.5	2.5	95.0

stage making this the best treatment time. From days 5 to 11 the vast majority of labelled cells were in the pachytene and diplotene stages. The first labelled MI's were observed on day 11, although this represented less than 1% of all labelled cells. On the following day (day 12) however, over 50% of labelled cells had passed through MI and MII, and reached the round-spermatid (R-tid) stage. The daily sampling time used was not precise enough to pinpoint the exact lengths of MI and MII, hence further mice were set up and killed at two-hourly intervals between day 11 and day 11 plus 16 hours. Tables 4.3(a) and 4.3(b) show the times taken for grains to appear over MI and MII preparations respectively. It was not until 11 days 6 hours after the injection of tritiated thymidine that label appeared in the majority (94%) of MI spreads. MII spreads first showed grains after 11 days 6 hours, although the majority did not show label until 11 days 10 hours. Estimation of the intervals between introduction of label at S-phase (pre-leptotene) and the subsequent appearance of specific labelled cell types provides a means of establishing time intervals between specific stages in the spermatogenic sequence. The time taken for pre-leptotene, zygotene and MI cells to reach MII was, however, the primary consideration in this experiment. From the labelling studies (Tables 4.2, 4.3(a) and 4.3(b) these were established as:

Preleptotene → MII	11 days 4 hours
Zygotene → MII	8 days 10 hours
MI → MII	4 hours

Table 4.3(a) Time Taken for Labelled Cells to Reach Metaphase I

Time After Injection of Label Day - Hr	No. of Cells Analysed	% Labelled
11 - 2	50	12
11 - 4	50	36
11 - 6 *	50	94
11 - 8	50	93

*Time selected for treatment

Table 4.3(b) Time Taken for Labelled Cells to Reach Metaphase II

Time After Injection of Label Day - Hr	No. of Cells Analysed	% Labelled
11 - 2	34	0
11 - 4	33	0
11 - 6	50	26
11 - 8	50	48
11 - 10 *	50	66
11 - 12	50	64
11 - 14	37	52
11 - 16	50	58

*Time selected for sampling

4.3.1.2 The Effect of Chemicals on Spermatogenic Timing

The above time intervals were established for untreated animals. Table 4.4 shows the effect on these intervals following treatment with the various chemicals under test. 6MCP, 4CMB and Tween 80 treatments produced no delay in spermatogenesis. Similarly no delay was observed when the pre-leptotene and zygotene stages were treated with pFPA or phenylalanine. Treatment with pFPA at MI (killing 4 hours later) produced, however, only 36% of all MII's showing label, compared with approximately double this number for most other treatments and for controls. It would appear, therefore, that this treatment is producing a delay to spermatogenesis between MI and MII. A reduced number of labelled MII's was also found following treatment with phenylalanine at MI. Several of these cells (c.20%) showed a late-labelling pattern (Kofman-Alfaro and Chandley, 1970). There was, however, no significant difference in the number of labelled cells found after treatment at this time (MI) compared with the previous treatment time (zygotene).

Further mice in the pFPA group were set up to establish the length of time taken for the majority of spermatocytes treated in MI to reach MII. Table 4.5 shows that the majority of MII's were labelled 6 hours after treatment at MI with pFPA, thus indicating a delay of about 2 hours compared with controls. This was therefore chosen as the best time interval to sample MII's following pFPA treatment at MI. The overall treatment regime for all four chemicals under test is shown in Figure 4.5. For the aneuploidy studies therefore, injection-to-killing intervals for all chemicals and controls were as stated earlier (Section 4.3.1.1)

Table 4.4 The Effect of Various Compounds on the Time Taken for Labelled Cells to Reach Metaphase II (11 Days 10 Hours Post-Label)

Chemical Treatment	Time of Treatment after Label Incorporation Days - Hrs	No. of MII Cells Analysed	% Labelled
6 Mercaptopurine (150 mg/Kg)	0 - 6 (Pre-Leptotene)	78	66
	3 - 0 (Zygotene)	100	79
	11 - 6 (Metaphase I)	100	78
Phenylalanine (100 mg/Kg)	0 - 6 (Pre-Leptotene)	100	76
	3 - 0 (Zygotene)	100	62
	11 - 6 (Metaphase I)	100	58
p-Flurophenylalanine (100 mg/Kg)	0 - 6 (Pre-Leptotene)	100	71
	3 - 0 (Zygotene)	100	75
	*11 - 6 (Metaphase I)	100	36
4-Chloromethylbiphenyl (100 mg/Kg)	11 - 6 (Metaphase I)	100	68
Tween-80	11 - 6 (Metaphase I)	50	62

*Significantly fewer labelled cells than any other treatment

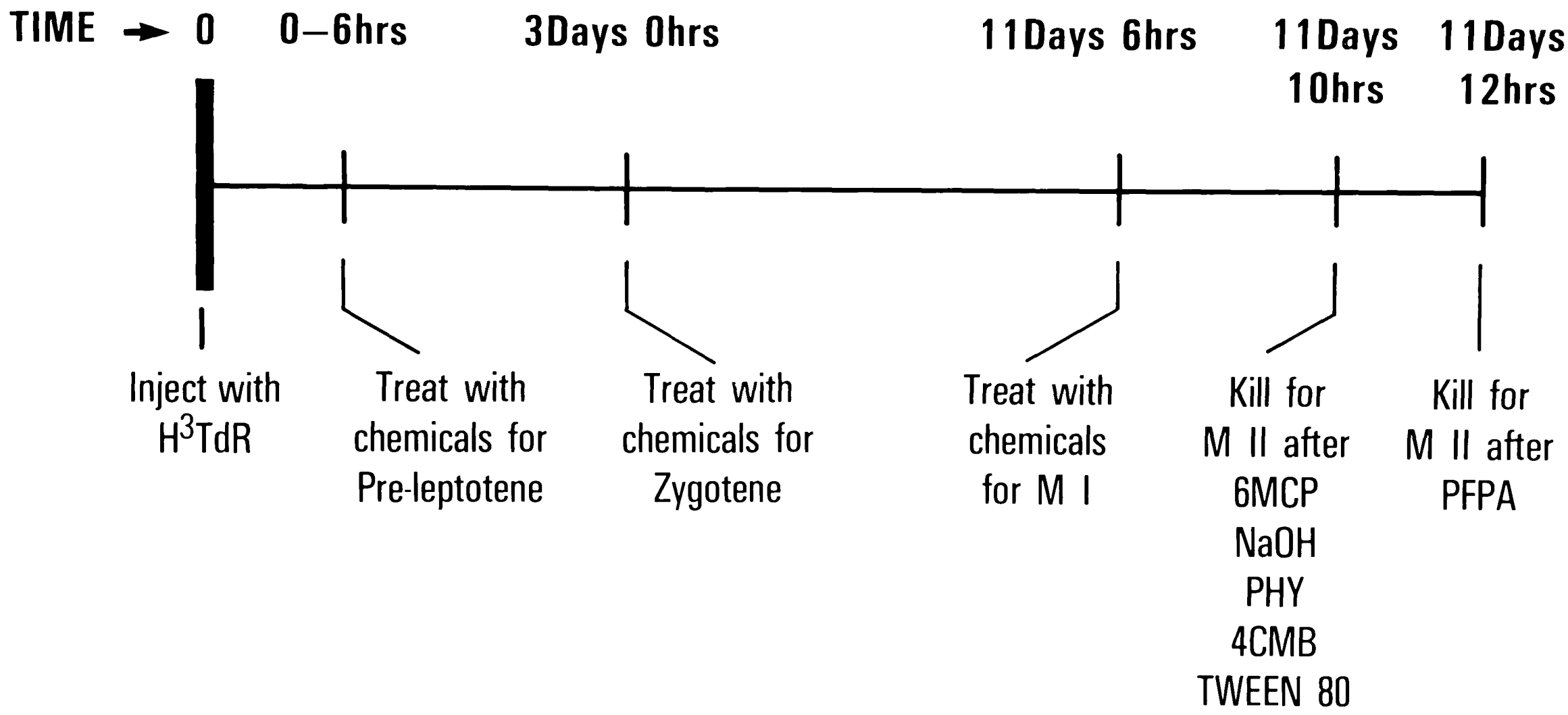
Table 4.5 Time Taken for Labelled Cells to Reach MII Following Treatment with pFPA at MI

Time after Injection of Label Days - Hrs	No. of MII Cells Analysed	% Labelled
11 - 10	100	36
11 - 11	100	49
11 - 12 *	100	64
11 - 13	100	66

*Time selected for sampling

Fig.4-5

TREATMENT REGIME FOR CHEMICAL INDUCTION OF ANEUPLOIDY IN MALES



except for pFPA treatment at MI. when 2 hours was added before killing.

4.3.1.3 The Chemical Induction of Aneuploidy

Typical male MII chromosome spreads are shown in Figure 4.6. The total scores obtained from such MII spreads following treatment with 6MCP, pFPA, phenylalanine and NaOH are given in Table 4.6. It is seen in all groups, including controls, that hyperploidy is far less frequent than hypoploidy. Many of the hypoploids are believed, however, to have arisen through chromosome loss due to cell breakage during slide preparation. The level of true aneuploidy, i.e. that due to non-disjunction alone, is better expressed therefore by doubling the number of disomic counts (n = 21). Chi-squared analyses of the numbers of 21's in the treated group totals of Table 4.6, compared with controls, are not significant at the 5% level. This is even the case following pFPA treatment where the percentage number of hyperploids is six times greater than in the control group.

Comparison of the hyperploid levels following stage-specific treatments with 6MCP, phenylalanine, and 4CMB with controls showed no significant differences (Table 4.7). pFPA treatment at MI however, did produce significantly greater levels of hyperploidy than with NaOH (control) treatment ($\chi^2 = 5.14$, d.f. = 1, $\therefore 0.05 > p > 0.01$). When compared with the Tween 80 MI control however, this difference was not significant. Comparison of MI treatment with pFPA and the NaOH control treatments at zygotene and pre-leptotene both proved non-significant, in fact one hyperploid

Fig.4-6

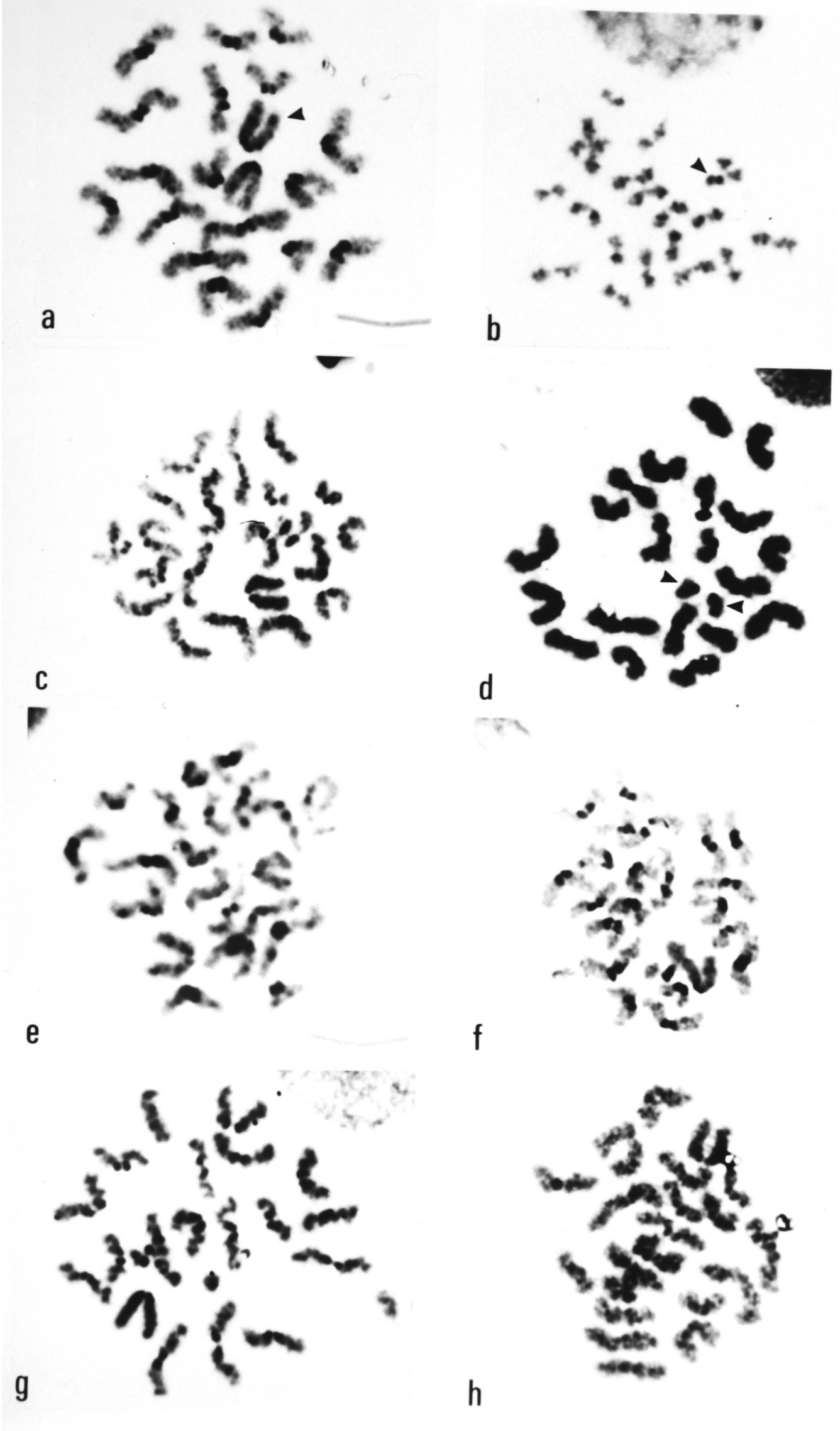


Table 4.6 The Effect of Chemical Treatment on Aneuploidy Induction in Spermatocytes

Treatment and Dose	Total MII's Scored	Chromosome number n = 17	18	19	20	21	% all Aneuploids	(% True* Aneuploids)
Control (NaOH)	1158	17	56	131	953	1	17.4	(0.2)
6MCP (150 mg/Kg)	1663	28	74	202	1351	8+	18.8	(1.0)
pFPA (100 mg/Kg)	1384	26	64	173	1114	7	19.5	(1.0)
Phenylalanine (100 mg/Kg)	1839	21	88	220	1506	4	18.1	(0.4)

*'True' aneuploidy frequency estimated by doubling the hyperploid (n=21) counts

+includes one n = 23

Table 4.7 The Effect of Stage-Specific Treatment with Various Chemicals on Aneuploidy Induction in Spermatocytes

Chemical Used	Stage Treated	Total Cells Scored	Chromosome number n = 17	18	19	20	21	% All Aneuploid	(% True* Aneuploids)
Control (NaOH)	Pre-Lept	316	3	8	34	270	1	14.6	(0.6)
	Zy	293	8	16	27	242	0	17.4	(0)
	MI	549	6	32	70	441	0	22.5	(0)
6MCP (150 mg/Kg)	Pre-Lept	565	8	24	63	467	3+	17.3	(1.0)
	Zy	503	11	22	65	402	3	20.1	(1.2)
	MI	595	9	28	74	482	2	19.0	(0.7)
pFPA (100 mg/Kg)	Pre-Lept	668	15	30	91	529	2	20.8	(0.6)
	Zy	494	8	20	56	408	2	17.4	(0.8)
	MI	222	3	14	26	177	3	20.3	(2.7)
Phenylalanine (100 mg/Kg)	Pre-Lept	815	11	41	97	665	1	18.4	(0.2)
	Zy	337	3	12	25	295	2	12.5	(1.2)
	MI	687	7	35	98	546	1	21.6	(0.3)
4CMB (100 mg/Kg)	MI	478	4	10	58	406	0	15.1	(0)
Tween 80	MI	358	3	10	43	301	0	15.9	(0)

*'True' aneuploidy frequency estimated by doubling the hyperploid (n = 21) counts

+Includes one n = 23

spermatocyte was observed in this latter control group. Neither of the other pFPA treatment times gave levels of hyperploidy significantly different from controls.

4.3.1.4 Chemical Induction of Chromosome Damage

The effect of stage specific treatment with various chemicals on chromosome anomalies other than aneuploidy in spermatocytes is shown in Table 4.8. The number of cells with broken chromatids was very low. Only 9 spermatocytes of 6900 scored showed breaks, of these 3 occurred following pFPA treatment at the pre-leptotene stage.

Twenty-five cells (0.36%) showed unpaired chromatids, i.e. cells with 19 dyads plus 2 univalents. Seven of these occurred following 4CMB treatment at MI, although four were found in the control group (NaOH) following treatment at pre-leptotene and three occurred in the Tween 80 group following MI treatment.

4.3.2 Aneuploidy Induction in Females

The effect of 4CMB on the number of oocytes recovered following superovulation is shown in Table 4.9. For treatment with 50 mg/Kg the mean number of eggs ovulated was reduced by 21.1% compared with controls, whilst treatment at a dose of 100 mg/Kg caused a reduction of 32.26% in the mean ovulation rate. The effect of the other chemicals on ovulation was not considered.

Figure 4.7 shows MII spreads from oocytes and Table 4.10 shows the effect of chemical treatment on dictyate and diakinesis/MI

Table 4.8 The Effect of Stage Specific Treatment with Various Chemicals on Chromosome Damage in Spermatocytes

Treatment	Stage Treated	Total MII Cells Scored	No. of Cells with Univalents	No. of Cells with Breaks
Control- (NaOH)	Pre-Lept	316	4	0
	Zy	293	1	0
	MI	569	1	0
6MCP (150 mg/Kg)	Pre-Lept	565	0	0
	Zy	503	3	1
	MI	595	1	0
pFPA (199 mg/Kg)	Pre-Lept	668	1	3
	Zy	494	1	2
	MI	222	0	0
Phenylalanine (100 mg/Kg)	Pre-Lept	815	2	2
	Zy	337	0	0
	MI	687	1	0
4CMB (100 mg/Kg)	MI	478	7	1
Tween 80	MI	358	3	0

Table 4.9 The Effects of 4CMB on Ovulation

Treatment	No. of Animals	Total Eggs Recovered	Mean No. Eggs Recovered	(Range)
Tween 80	24	1058	44.08	(10-69)
4CMB (50 mg/Kg)	15	522	34.80	(18-57)
4CMB (100 mg/Kg)	22	657	29.86	(18-57)

Fig. 4-7

Table 4.10 Aneuploidy Induction in Female Mice by Chemical Treatment

Treatment	Group (as in Figure 4.1)	No. of Animals	Total Eggs Analysed	Chromosome number n = 17	18	19	20
NaOH (Control)	B	9	96	1	8	12	75*(2)
	C	6	70	0	1	13	56
	D	8	66	0	4	13	49
6MCP (150 mg/Kg)	B	10	57	1	3	6	47
	C	6	78	0	4	13	61*(7)
	D	8	71	1	2	13	55
pFPA (100 mg/Kg)	B	7	78	2	7	7	62*(3)
	C	6	86	1	3	16	66
	D	6	51	0	2	6	43
Phenylalanine (100 mg/Kg)	B	8	87	1	5	12	69
	C	7	73	0	3	10	60
	D	8	82	3	6	11	62
Tween 80	A	11	109	5	10	23	71
4CMB (50 mg/Kg)	A	15	111	6	6	19	80
4CMB (100 mg/Kg)	A	15	99	9	12	18	60

*(Numbers of ovulated eggs still in MI)

oocytes in young and aged females when scored for numerical chromosome abnormalities at MII. In all cases, hypoploid oocytes were observed, their occurrence ranging from 34.9% in the Tween 80 treatment A group to 15.7% of the total in the pFPA treatment D group. Chi-squared analysis of the distributions of these two extremes proved non-significant ($\chi^2_6 = 7.3$ $p > 0.1$). No disomic complements were observed from the total of 1202 oocytes analysed. A minority of oocytes showed separated chromatids, i.e. 19 dyads plus 2 univalents. These were included in the n=20 chromosome counts. In some of the groups, eggs were ovulated which showed an MI rather than an MII chromosome configuration. It would appear that in these cases the first polar body had not been expelled. This was found for 6MCP treatment during the dictyate stage in young animals (C) and pFPA and NaOH (control) treatments at MI in aged animals (treatment B), the frequencies being 9.0%, 3.1% and 2.1% respectively. In all cases the oocytes analysed were ovulated by aged females, irrespective of the age at the time of treatment. This phenomenon was not observed in any of the other treatment groups.

4.4 DISCUSSION

4.4.1 Spermatogenic Stage Classification

The duration of spermatogenesis from pre-leptotene to the round spermatid stage, and more particularly the timing of the various intermediate stages, found in the present study agrees closely with those established by other authors for the male mouse (Oakberg, 1956; Kofman-Alfaro and Chandley, 1970). One report, however, has recently questioned the widely accepted

spermatogenic classification and timing of Oakberg (1956). Oud *et al* (1979), using hydroxyurea/triaziquone treatment to produce a restricted spermatocyte population which could be followed on a daily basis in order to make a sequential analysis of meiosis in the male mouse, have suggested that the pachytene stage is in fact shorter than was formerly believed. They suggest that there is a long diplotene stage, following pachytene, which lasts for about 3 days and which can be divided into three distinct periods - the "pre-diffuse", "diffuse" and "post-diffuse" diplotene stages respectively. According to Oakberg (1956) the diplotene stage lasted only 21 hours. The time taken for cells to reach MI from pre-meiotic interphase, according to Oud *et al.*(1979), is the same as for previous estimates, i.e. about 10½-11 days, but the lengths of the intermediate stages different with pachytene being shorter and diplotene longer. Work by Goetz, P. (personal communication), using surface-spreading and air-drying techniques on prophase spermatocytes of peri-pubertal male mice, to study the sequential development of germ cells in the first wave of meiosis, suggests that the very large cells seen in late prophase, and which Kofmann-Alfaro and Chandley (1970) had interpreted to be late pachytenes, are indeed diplotenes as stated by Oud *et al* (1979). According to Goetz, P. (personal communication) however, the diplotene stage lasts about 24 hours, more in line with Oakberg's (1956) timing. This point, although not directly relevant to the present aneuploidy induction work, since overall timings from preleptotene and zygotene to MII are not affected by it, does affect any precise classification of the pachytene and diplotene stages. Table 4.2 thus shows a classification in which the pachytene and diplotene stages are combined.

4.4.2 The Effect of Chemicals on Spermatogenic Timing

When the effects of the chemicals on spermatogenic timing were assessed, it was obvious that pFPA, when applied to spermatocytes at MI, caused a delay such that the majority of MII spreads did not appear labelled until 6 hours after MI compared with 4 hours in the case of controls and with other chemicals tested. Some of these spreads (10%), 4 hours after treatment, showed a late-labelling pattern. It does not seem likely, however, that pFPA treatment at this stage was killing cells as the overall proportion of MII's on the preparations remained the same as for other treatments. 6MCP had no effect on spermatogenic timing, a finding in agreement with Oakberg (1979). Fewer labelled MII's were seen at 4 hours after phenylalanine treatment, and a number were late-labelled indicating that these belonged to the vanguard of labelled cells coming through, although this shortfall was not significantly different from controls.

pFPA treatment has previously been shown to cause delays in mitosis in cultured cells. Sisken and Wilkes (1967) using time-lapse photography, found an increase in the duration of metaphase when cultured human amnion cells were given continuous treatment with 1.0 mM pFPA. This effect became obvious 1-2 hours after treatment, although all cells entering mitosis within the first 5 hours were capable of completing mitosis. That pFPA can be incorporated into proteins in place of phenylalanine, was first demonstrated over 20 years ago (Vaughan and Steinberg, 1960; Westhead and Boyer, 1961). It is not incorporated in place of tryosine, however, which it also resembles (Westhead and Boyer, 1961).

Sisken and Wilkes (1967) suggested that the delay caused by pFPA treatment was because of its incorporation into a cell-specific protein in place of phenylalanine. pFPA treatment was also shown to induce some instability and structural abnormalities in the mitotic spindle (Sisken *et al*, 1972). More than twenty years ago, Kerridge (1960) showed that the presence of pFPA did not seriously inhibit the regeneration of bacterial flagellae, but such flagellae were non-functional.

There is evidence in the literature to suggest that phenylalanine, when in excess, can also affect the constitution of tubulin, which may affect its function. For example, Barra *et al* (1973) demonstrated that phenylalanine will compete with tyrosine for a binding site at the carboxyl terminal end of the α chain of the tubulin dimer during the post-translational modification of this molecule. In the brain of new-born rats about half the molecules are modified post-translationally. Rodriguez and Borisy (1979) demonstrated that 96% of the added residues are tyrosine and only 4% are phenylalanine. After the induction of hyperphenylalaninemia, however, the proportion of tubulin molecules containing phenylalanine at the carboxy-terminal end increases up to eight-fold. They suggested that although the *in vitro* assembly of microtubules is unaffected by the substitution of phenylalanine for tyrosine, the configuration of that region of the polypeptide may be affected. Moreoever, the chemical difference between tyrosine and phenylalanine could affect the capacity of the protein or the resultant microtubles to interact with other cellular elements (Rodriguez and Borisy (1979).

In view of the incorporation of pFPA into proteins in place of phenylalanine (Vaughan and Steinberg, 1960) and the competition between phenylalanine and tyrosine for the carboxyl terminal binding site on tubulin, it could be argued that the delay observed during MI in the present study, and in mitotic metaphase by others, may be due to the same phenomenon. High levels of pFPA act like increased phenylalanine, and compete with tyrosine for the carboxyl-terminal binding site, thus affecting the function of any resulting microtubules. Phenylalanine, however, unlike pFPA, did not cause any appreciable delay between MI and MII in the present study. There was only a hint of a delay as seen from the higher number of late-labelled cells compared with the other chemical treatments. The fact that pFPA will incorporate into proteins in place of phenylalanine, but not tyrosine (Westhead and Boyer, 1961) suggests that pFPA is having a more specific effect than phenylalanine. Indeed, Sisken *et al* (1972) found metaphase spindles of pFPA-treated cells to be smaller than those from controls, indicating that this chemical is having a morphological effect. Whether this could be demonstrated for phenylalanine-treated cells with higher doses however, is not known.

4.4.3 The Effect of Chemicals on Aneuploidy Induction

A high frequency of hypohaploids (n=19), many of which may have arisen through chromosome loss because of cell breakage during preparation, was found in all treatment groups and controls. Disomic gametes (n=21) which are indicative of true non-disjunction

were, however, rare. Of those observed, all were in spermatocytes. No disomic oocytes were found, although this could be due, at least in part, to the lower numbers analysed. In fact with these small groups of about 80 scores, 5% hyperploidy would be necessary in treated groups to show a significant difference from the zero level in controls, using χ^2 analysis. This test was obviously insensitive.

In most cases the number of spermatocytes scored was five times the number of oocytes. From spermatocyte scores the level of hyperploidy following treatment at diakinesis/MI with pFPA, reached 1.35% compared with none found in the NaOH control. This difference was significant ($\chi^2 = 6.5$, d.f. = 1: $0.05 > p > 0.01$). It was not, however, significantly greater than the Tween 80 control level (which had a smaller sample size) nor was it significantly greater than the NaOH treatments given at pre-leptotene or zygotene.

Tates (1979) found that pFPA could induce a 10 x increase in XY non-disjunction in the Northern vole (*Microtus oeconomus*) when late spermatogonial stages and early spermatocytes (i.e. leptotenes, zygotenes and early pachytenes) were treated. Diploid spermatids occurred with raised frequency following treatment of zygotene and pachytene spermatocytes, the most advanced stages tested by Tates (1979). No significant increase in aneuploidy was found following pFPA treatment of pre-leptotene and zygotene in the present study, although the dose used by Tates (1979) (2 x 150 mg/Kg given over 24 hrs) was three times higher than that employed in the present experiments.

Again, as for the oocyte scores, the problem of test sensitivity must be considered. If sufficiently large numbers of

spermatocytes are scored in the control groups, for example 549 at MI in the present study, a 1% hyperploidy level in a treated group of 200 will be significantly higher than a zero level in controls. Where fewer controls are scored, however, the test sensitivity decreases. With only 150 scores in the control group for example, 2% hyperploidy in a similar size treated group would not prove significantly greater than a zero level in controls.

A further comparison of the pFPA-MI treatment group data was made with control data obtained some years ago by Beatty *et al* (1975) for male mice. These authors found 10 spermatocytes with 21 chromosomes from 5,200 MII cells analysed, giving a hyperploidy frequency of 0.19%. The findings for the MI treatment with pFPA in the present study, did differ significantly from this level of hyperploidy ($\chi^2 = 4.7$, d.f. = 1: $0.05 > p > 0.01$), although the comparison cannot be considered strictly valid as the two sets of data were gathered in different laboratories at different times. Nevertheless, it is interesting that the induction of non-disjunction at MI by pFPA correlates with the delay to spermatogenesis found at the same time. Approximately 50% fewer labelled MII spreads were seen 4 hours after pFPA treatment compared with controls, suggesting that more than one-third of all cells at MI were delayed by the chemical.

Aneuploidy induction by pFPA has been reported for lower organisms. Griffiths and Delange (1977) for example, found a six-fold increase in non-disjunction over control values when pFPA was tested on a *Neurospora* system. Bond and McMillan (1979) also

reported a strong positive effect for pFPA in *Sordaria brevicollis*. In this case a dose-response relationship was also demonstrated.

It is possible that differences could exist in the way pFPA acts on the spindle in the two types of organism (Bond and Chandley, 1983). Other substances which act on the spindle have also been shown to produce different results for aneuploidy induction in lower and higher eukaryotes. Colchicine, for example, although producing significantly increased levels of aneuploidy in the Chinese hamster (Sugawara and Mikamo, 1980) has been found to be ineffective at inducing metaphase arrest, polyploidy or aneuploidy in lower eukaryotes (Richards, 1938; Sansome and Bannon, 1946; Haber *et al*, 1972). Colchicine inhibits spindle polymerization by binding to tubulin (Margolis and Wilson, 1981), although not all microtubules in the cell are equally sensitive to its effects (Stebbings and Hyams, 1979). According to Bryan (1972), microtubules have three classes of binding site, one of which is occupied by colchicine and Colcemid. This site is different in lower eukaryote microtubles. Indeed Heath (1975) found that the assembly of microtubules in some fungi was not affected at all by Colcemid or colchicine. Although the magnitude of the effect may be different, the action of pFPA on lower and higher organisms may well be the same since an effect on spermatogenic timing and some increase in aneuploidy induction has been demonstrated in the present study and aneuploidy has also been induced by others (Bond and McMillan, 1979; Tates, 1979).

Indications that 6MCP may increase non-disjunction at the second meiotic division in the male mouse have been given.

Cacheiro and Generoso (1975) found 3 XYY males amongst 615 sterile F_1 sons of 6MCP-treated males. Tates (1979) however, did not find any 6MCP-induced aneuploidy in *Microtus oeconomus* for any of the prophase stages he tested. The most interesting observation with 6MCP in the present study was the increased number of oocytes blocked at MI in the aged females which had been treated at the dictyate stage when young. Two other treatments: pFPA and NaOH (control) treatment of aged females at diakinesis/MI, also produced oocytes blocked at MI, but only in the 6MCP treatment group did the difference reach significant levels ($\chi^2 = 5.5$; d.f. = 1; $0.05 > p > 0.01$). Watanabe *et al* (1977) observed similar MI blockage of oocytes following treatment at MI with cadmium. They suggested that such oocytes might produce triploidy were they to proceed to MII and form gametes. 6MCP is an analogue of adenine and hypoxanthine, and the basis for its biological activity is considered to be its interference with normal DNA synthesis (Holden *et al*, 1973). Since no DNA synthesis occurs during the dictyate stage, the S-phase of meiosis in females occurring at pre-meiotic interphase in the fetal oocyte (Peters *et al*, 1962), it would appear that the chemical is not acting in this way to block oocyte development. 6MCP was effective, nevertheless, at blocking oocytes at MI when females were treated at the dictyate stage. pFPA, on the other hand, did cause a delay between MI and MII in the male, These observations indicate a different mode of action for the two chemicals. The significance of these oocytes, blocked at MI, however, is somewhat obscure in view of the occurrence of similarly blocked oocytes in controls.

Reimers *et al* (1978) found that pregnant mice treated with 6MCP had second and third generation offspring that were sterile or had smaller litters with higher numbers of dead fetuses. Furthermore, patients treated with 6MCP acquire genetic damage, such as chromosome aberrations, in both somatic and germ cells (Elion *et al*, 1961; Leb *et al*, 1971). In view of the use of 6MCP to treat Crohn's disease, an inflammatory bowel disorder affecting young people still in their reproductive years, Steckman (1980) has pointed out that the long-term effects of 6MCP on human reproduction should be evaluated. From the present study it would appear that the treatment of mice with 6MCP, at the dose employed, does not produce increased levels of non-disjunction at anaphase I, although it may cause blockage of oocytes in MI. The observation of XYY progeny following 6MCP treatment, indicates non-disjunction at anaphase II (Cacheiro and Generoso, 1975). Such non-disjunction would however go undetected in the present test system. Further studies on early embryos would be needed to evaluate non-disjunction at anaphase II.

There was no previous evidence to suggest that 4CMB, a suspected carcinogen, could increase non-disjunction, and certainly none was found in the present study. It did, however, produce a reduction in the mean number of eggs ovulated, the effect increasing with dose. This could possibly be due to an effect of the chemical on the ovary causing fewer eggs to be ovulated, or alternatively degeneration of some oocytes may have occurred soon after ovulation. Counts of *corpora lutea* were not carried out and it was thus not possible to decide between the two alternatives. The former would seem the more likely, however, as degenerating eggs would almost certainly still be present, and be recoverable such a short time after ovulation.

The usefulness of mouse germ cells in the routine testing of chemical substances for aneuploidy induction will be considered in Chapter 5.

PART III

HUMAN ANEUPLOIDY - CONSIDERATIONS FOR THE FUTURE

CHAPTER 5

GENERAL CONCLUSIONS

In Part I of the thesis the problem of human aneuploidy, its frequency, its origins and the various hypotheses to account for it were outlined. In the second part, factors which may play a part in its induction were examined experimentally. In this, the third and final part of the thesis, the relevance of the experimental work to the problem of human aneuploidy will be considered and future prospects assessed.

From a review of the literature it would appear that two main problems concerning estimates of aneuploidy levels in man still exist. Firstly, the true level of aneuploidy at conception remains unknown. The karyotyping of embryos after *in vitro* fertilization will almost certainly provide the answer to this question in the near future, in fact three relevant studies have already been performed. Edwards (1977) examined chromosomes from 15 embryos and found them all to be "approximately diploid". Wramsby *et al* (1982) found that of three oocytes examined one had a haploid set of 23 or 24 chromosomes and Angell *et al* (1983) have recently reported that of 11 embryos examined for their ploidy, 2 appeared to be haploid, and of the 3 examined chromosomally, 2 showed evidence of non-disjunction of an autosome. In one, in addition to its haploid state, there was trisomy D, and in the other, which was of maternal origin, there was also monosomy 15. These findings are scant and further work is clearly necessary to establish levels of chromosome abnormality at conception. When more complete studies are performed, data should also emerge on the

parental origins and also the division of origin of the errors. In this respect more data are needed to trace the origin of aneuploidies in spontaneous abortions. Expansion of these data will undoubtedly help in understanding the aetiology of human aneuploidy. These data should show that, not only is it necessary to explain maternal age dependent aneuploidy (the focus of most attention), but also aneuploidy which is maternal-age independent and that which is paternally derived.

In the Preface to the thesis two main questions were posed. Firstly, what factors are responsible for, or associated with, maternal age-dependent aneuploidy? Secondly, can chemical substances induce aneuploidy in higher organisms?

In order to answer the first question two independent approaches were adopted. In the first of these reported in Chapter 2, the relationship between X-chromosome segregation, maternal age and aneuploidy were examined in the XO female mouse. This animal had previously been suggested as a good model for the pre-menopausal human female (Lyon and Hawker, 1973). Unfortunately, because of the complicating factor of X-chromosome segregation during meiosis in this mouse, problems were encountered in assessing the true aneuploidy level at all maternal ages. In view of this difficulty the suitability of the XO mouse as a model for the peri-menopausal human female in aneuploidy studies was questioned.

The second approach to understanding the factors responsible for, or associated with maternal age dependent aneuploidy was reported in Chapter 3. Use was made of the CBA mouse as a model for the ageing human female and here the results proved more successful. Not only was an increase in the aneuploidy observed with increasing

maternal age, as others have also found (Gosden, 1973; Martin *et al*, 1976; Max, 1977; Fabricant and Schneider, 1978), but, more significantly this occurred prematurely following unilateral ovariectomy. The results from this experiment suggest that abnormal segregation of meiotic chromosomes in ageing oocytes, is an epiphenomenon of physiological ageing, rather than dependent upon the chronological age of the oocyte or mother. Factors, including hormonal imbalance and irregular oestrous cycles, both of which are themselves interrelated, are associated with physiological ageing in the female and are thus implicated in aneuploidy induction. Whether hormonal imbalance *per se* contributes to aneuploidy induction, or whether oestrous cycle irregularities resulting from hormonal imbalance is more important, remains to be resolved. The hormone assays of serum from animals killed on the day of pro-oestrous as part of the experiment reported in Chapter 3 may provide data which will help to answer this question. Furthermore, the *in vitro* culture of oocytes in different hormone concentrations, as already performed by McGauhey (1977) to a limited extent, may also prove informative in this respect. Recent data obtained from cytological studies in the foetal mouse ovary (Speed and Chandley, in press 1983) and from studies of meiosis in the adult Chinese hamster (Sugawara and Mikamo, 1983) seem to indicate that the production-line hypothesis of oocyte development in the foetal ovary cannot fully explain the age-related aneuploidy increases found in these two species. Similarly the data from the unilateral ovariectomy experiment in this thesis cannot be explained on the basis of this model and so it would appear that some factor in the adult maternal environment must be sought.

In Chapter 4 the aneuploidy inducing potential of chemicals known to induce aneuploidy in lower organisms was assessed on both the male and female mouse. That the findings were largely negative in these aneuploidy induction tests is important and raises several questions. Firstly, are the chemicals getting into the gonads and actually affecting the vulnerable germ cell stages selected for exposure? Secondly, how valid is the test system? Finally, does the environmental exposure to chemicals play a significant part in human aneuploidy induction?

In answering the first question consideration must be given to the blood-testis barrier. This barrier provides a means by which harmful substances can be prevented from reaching the male germ cells. In rodents the primary barrier to substances penetrating from the interstitium is the myoid layer, junctions in which measure 200 Å. Penetration of this barrier will allow the exposure of spermatogonia and preleptotene spermatocytes. Further penetration to cells in the adluminal compartment is prevented by the Sertoli cells (Setchell and Waites, 1975). The delay to spermatogenesis observed when spermatocytes were treated with pFPA, indicates that this chemical is able to penetrate the barrier. Furthermore, it would appear that 6MCP is reaching all spermatogenic stages in view of the observation that 6MCP causes cell killing in spermatogonia (Oakberg, 1979) and dominant lethality in spermatogonia and early spermatocytes (Generoso *et al*, 1975). Whether 4CMB reached all spermatogenic stages is unclear, although it did reach the ovary as ovulation rates were affected (Brook, 1982). There was no evidence to suggest that the other chemicals used were not also reaching the female germ cells.

The second question raised by the findings in Chapter 4 concerns the validity of the test system. This has two aspects, (a) the stages exposed to the chemical, and (b) the mouse strain selected. From previous evidence it was thought that the stages selected for exposure in this study might be vulnerable to non-disjunction induction. Zygotene was selected as suitable in view of the pairing of homologous chromosomes which takes place at this stage, the rationale being that any substance affecting pairing might lead to the production of univalents which, if they underwent random segregation, could lead to aneuploidy. Similarly, the time of spindle formation might also turn out to be sensitive, if treated. Hansmann (1974) has suggested that different stages of meiosis may be sensitive to different chemicals. This being so, the requirement would be to expose all spermatogenic and oogenic stages to the chemicals, and in differently aged animals (for female tests), to detect those sensitive stages not otherwise considered. Quite clearly this is impractical. Hence the need for selection of certain specific stages on an *a priori* basis, where sensitivity might be expected. In terms of long-term effects of exposure to hazardous environmental compounds in man, the vulnerable stages would be the stem cell spermatogonia in males and the dictyate oocytes in females. These stages appear, however, from the mouse data on radiation induction in males (Ford *et al*, 1969; Russell and Montgomery, 1974), and in females (Tease, 1981, 1982a) to be insensitive to non-disjunction induction. Selection against aneuploid products among treated spermatogonia may occur (Searle, 1975; Russell, 1976). Treatment of the short pre-diakinesis stage was found to be sensitive to X-ray exposure by Tease (1982a) and this may perhaps be the stage to select for future testing.

Choice of strain could also be important in testing. Previous experiments have shown that the spontaneous level of non-disjunction in male mice is very low indeed (Beatty *et al*, 1975; Ohno *et al*, 1959). The Swiss mice used in the present study also showed no base-line aneuploidy, and certainly no age-effect in the females at all ages tested up to 40 weeks. Golbus (1981) also failed to find maternal-age effect in Swiss females tested up to 60 weeks. An age-effect for CBA females has been found (Gosden, 1973; Fabricant and Schneider, 1978), as already mentioned, and certainly the CBA strain appears to give a higher frequency of aneuploidy overall than many other strains. However, it does not necessarily follow that a higher baseline of spontaneous non-disjunction would pre-dispose towards increased induction by chemicals. Current data in the mouse show that X-irradiation does not enhance levels of aneuploidy over and above those due to maternal ageing (Tease, 1982a). It would seem, therefore, that little can be done, by way of stage or strain alteration, to amplify the aneuploidy inducing effect of the chemicals.

The final question concerning the relevance of exposure to chemicals or environmental pollutants for aneuploidy induction in man, appears to be the most crucial. Apart from colchicine, no chemical has yet been shown to produce significant levels of aneuploidy in mammals, although in this case negative results were found for fungal test systems (see Bond and Chandley, 1983). pFPA treatment in the present study, only induced significant levels of aneuploidy when administered during MI in males. Little aneuploidy was produced at other stages tested in males and females were negative at all stages tested. On the other hand marked effects have been observed in fungi treated with pFPA (Griffiths and Delange,

1977; Bond and McMillan, 1979). Such results make it obvious that caution should be exercised when attempts are made to extrapolate from results obtained in fungi to their potential effects in man. The great need is for much more testing to be carried out in mammalian systems using compounds which have been found to be strongly-positive aneuploidy inducers in lower organisms. It would appear from both the present study and other mammalian tests that despite incidental reports, environmental chemicals play little role in aneuploidy induction in man. Compared to the overwhelming effect of maternal ageing on human aneuploidy induction the effects of environmental hazards remain to be clearly demonstrated.

The present thesis has been concerned with identifying factors responsible for the induction of non-disjunction. Despite the fact that the level of aneuploidy at conception could be as high as 20% (see Chapter 1-4) the actual level of aneuploidy amongst the newborn population is reduced to 0.31%. The majority are lost through spontaneous abortion and therefore do not present a social or financial burden, unlike Down's patients who are often institutionalized. Nevertheless, the emotional distress to any mother suffering a lost pregnancy, whether it be spontaneous or induced, has to be considered, even though it may be impossible to assess. If every pregnant female could be offered 'safe' amniocentesis - that is one producing no risk of a spontaneous abortion - with the offer of an induced abortion to those mothers carrying a chromosomally abnormal fetus, the incidence of liveborn aneuploidy would obviously be reduced. The costs incurred, however, mean that at present this is not a practical proposition. Earlier detection of an aneuploid conception may be possible, in future, using cytogenetic analysis or molecular biology techniques on chorion

biopsies (Old *et al*, 1982; Gosden *et al*, 1982) taken during the first timester. This would reduce the period of worry for any particular female, but there would still be no reduction in the overall number of aneuploid embryos conceived.

The most sensible approach would be to try and identify females who are 'at risk' of conceiving trisomic embryos. Even then, those females conceiving paternally derived trisomic embryos would go unidentified. Nevertheless, the identification of any 'at risk' females would prove beneficial. This would include those with premature ovarian ageing (e.g. those having undergone unilateral ovariectomy and possibly those with auto-immune disorders) or even those with particular α_1 antitrypsin sub-types. Women suffering repeated abortion or those coming from families with a history of Down's syndrome would also be included. General 'at risk' groups may also be identified, for example all women approaching the menopause, and particularly those women with irregular menstrual cycles whether young or old, or even those women who have recently stopped using the contraceptive pill (Read, 1982). By increased public awareness, those females 'at risk' of producing an aneuploid fetus could be advised to take greater contraceptive care, and those 'at risk' who wish to become pregnant could receive better pre-natal counselling.

"The elusive cause of Down's syndrome" (Editorial, Lancet, 21st May 1983) which could perhaps be better expressed as the elusive cause of non-disjunction, remains a subject for discussion (Polani, 1983). It is undoubtedly a subject which would benefit

from more hard experimental data and fewer hypotheses! Nevertheless aneuploidy still presents a challenging, if often frustrating problem, worthy of further investigation.

BIBLIOGRAPHY

Aarskog, D. and Fagerhol, M.K. (1970)
Protease Inhibitor (Pi) Phenotypes in Chromosome Aberrations. Journal of Medical Genetics, 7, 367-370.

Adamopoulos, D.A., Loraine, J.A. and Dove, G.A. (1971)
Endocrinological studies in women approaching menopause. Journal of Obstetrics and Gynaecology of the British Commonwealth, 78, 62-79.

Alberman, E.D. (1981)
The Abortus as a predictor of future trisomy 21 pregnancies. In: Trisomy 21 (Down Syndrome) Research Perspectives. Eds. de la Cruz, F.F., and Park, S.G., pp.69-76 University Park Press, Baltimore, U.S.A.

Alberman, E.D. and Creasy, M.R. (1977)
Frequency of chromosomal abnormalities in miscarriages and perinatal deaths. Journal of Medical Genetics, 14, 313-315.

Alberman, E., Polani, P.E., Fraser-Roberts, J.A., Spicer, C.C., Elliott, M., Armstrong, E. and Dhadial, R.K. (1972)
Parental X-irradiation and chromosome constitution in their spontaneously aborted foetuses. Annals of Human Genetics 36, 185-194.

Angell, R.R., Aitken, R.J., van Look, P.F.A., Lumsden, M.A. and Templeton, A.A. (1983)
Chromosome abnormalities in human embryos after *in vitro* fertilization. Nature, 303, 336-338.

Arai, H. (1920)
On the cause of the hypertrophy of the surviving ovary after semi-spaying (albino rat) and on the number of ova in it. American Journal of Anatomy, 28, 59-79.

Arnaud, P.H., Burdash, N.M., Wilson, G.B. and Fudenberg, H.H. (1976)
Alpha-1-antitrypsin (Pi) types in Down's syndrome. Clinical Genetics 10, 239-243.

Asdell, S.A. (1924)
Some effects of unilateral ovariectomy in rabbits. British Journal of Experimental Biology, 1, 473-486.

Austin, C.R. (1967)
Chromosome deterioration in ageing eggs of the rabbit. Nature, 213, 1018-1019.

Ayme, S. and Lippman-Hand (1982)
Maternal age effect in aneuploidy: Does altered embryonic selection play a role? American Journal of Human Genetics 34, 558-565.

Baker, T.G., Challoner, S. and Burgoyne, P.S. (1980)
The number of oocytes and the rate of atresia in unilaterally ovariectomized mice up to 8 months after surgery. Journal of Reproduction and Fertility, 60, 449-456.

Barlow, P. and Vosa, C.G. (1970)
The Y Chromosome in human spermatozoa. Nature, 226, 961-962.

Barlow, D.H., Macnaughton, M.C., Mowat, J. and Coutts, J.R.T. (1981)
Hormone profiles in the menopause. In: Functional Morphology of the Human Ovary. Ed. J. R. T. Coutts, pp.223-234 (MTP Press Ltd., Lancaster, England).

Barra, H.S , Arce, C.A., Rodriguez, J.A. and Caputto, R. (1973)
Incorporation of phenylalanine as a single unit into rat brain protein: Reciprocal inhibition by phenylalanine and tyrosine of their respective incorporations. Journal of Neurochemistry, 21, 1241-1251.

Bass, H.N., Crandall, B.F. and Sparkes, R.S. (1973)
Probable trisomy 22 identified by fluorescent and trypsin-giemsa banding. Annals Genetique, 16, 189-192.

Bast, J.D. and Greenwald, G.S. (1977)
Acute and chronic elevations in serum levels of FSH after unilateral ovariectomy in the cyclic hamster. Endocrinology, 100 , 955-966.

Bauld, R., Sutherland, G.R. and Bain, D. (1974)
Chromosome studies in investigation of stillbirths and neonatal deaths. Archives of Disease in Childhood, 49, 782-788.

Beatty, R.A. (1977)
F-bodies as Y-chromosome markers in mature human sperm heads. Cytogenetics and Cell Genetics, 18, 33-49.

Beatty, R.A. (1978)
Problems in estimating non-disjunction rate from the F-bodies in two-headed human spermatozoa. Human Heredity, 28, 335-340.

Beatty, R.A., Lim, M.C. and Coulter, V.J. (1975)
A quantitative study of the second meiotic metaphase in male mice (*mus musculus*). Cytogenetics and Cell Genetics, 15, 256-275.

Belling, J. (1925)
The origin of chromosomal mutations in *Uvularia*.
Journal of Genetics, 15, 245-266.

Bennet, M. (1982)
Nucleotypic basis of the spatial ordering of chromosomes in Eukaryotes and the implications of the order for Genome evolution and phenotypic variation. In: Genome Evolution. Eds. Dover, G.A. and Flavell, R.B., pp.239-261, Academic Press.

Biggers, J.D., Finn, C.A. and McLaren, A.J. (1962)
Long term reproductive performance of female mice. I. Effect of removing one ovary. Journal of Reproduction and Fertility, 3, 303-312.

Blandau, R.J. and Young, W.C. (1939)
The effects of delayed fertilization on the development of the guinea-pig ovum. American Journal of Anatomy, 64, 303-329.

Blumenthal, I. and Variend, M.S. (1972)
Maternal urinary oestriol excretion and fetal chromosome abnormalities. Lancet, 2, 1084.

Bobrow, M., Madan, K. and Pearson, P.L. (1972)
Staining of some specific regions of human chromosomes, particularly the secondary constriction of No. 9. Nature New Biology, 238, 122-124.

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

Bond, D.J. and McMillan, L. (1979)
Meiotic Aneuploidy: Its origins and induction following chemical treatment in Sordaria brevicolis.
Environmental Health Perspectives, 31, 67-74.

Boué, J. and Boué, A. (1973)
Anomalies chromosomiques dans les avortements spontanes.
In: Les Accidents Chromosomique de la Reproduction.
Ed. A. Boué and C. Thibault, pp.29-55, Paris: INSERM.

Boué, J., Boué, A. and Lazar, P. (1975)
Retrospective and prospective epidemiological studies of 1500 Karyotyped spontaneous human abortions.
Teratology, 12, 11-26.

Bracken, M.B., Holford, T.R., White, C. and Kelsey, J.L. (1978)
Role of oral contraception in congenital malformations of offspring. International Journal of Epidemiology, 7, 309-317.

Braw, R.H. and Tsafriri, A. (1980)
Effect of PMSG on follicular atresia in the immature rat ovary. Journal of Reproduction and Fertility, 59, 267-272.

Bridges, C.B. (1913)
Non-disjunction of the sex-chromosomes of Drosophila. Journal of Experimental Zoology, 15, 587-606.

Brook, J.D. (1982)
The effect of 4CMB on germ cells of the mouse. Mutation Research, 100, 305-308.

Bryan, J. (1972)
Vinblastine and microtubules, 3: Definition of 3 classes of binding-sites in isolated microtubule crystals. Biochemistry, 11, 2611-2616.

Burgoyne, P.S. and Baker, T.G. (1981)
Oocyte depletion in XO mice and their XX sibs from 12 to 200 days *post partum*. Journal of Reproduction and Fertility, 61, 207-212.

Butcher, R.L. (1975)
The role of intrauterine environment and intrafollicular aging of the oocyte on implantation rates and development. In: Aging Gametes. Int. Symp., Seattle, 1973, pp.201-218 (Karger, Basel).

Butcher, R.L., Blue, J.D. and Fugo, N.W. (1969)
Overripeness and the mammalian ova III. Fetal Development at midgestation and at term. Fertility and Sterility, 20, 223-231.

Butcher, R.L., Collins, W.E. and Fugo, N.W. (1974)
Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17β throughout the 4-day estrous cycle of the rat. Endocrinology, 94, 1704-1708.

Butcher, R.L. and Fugo, N.W. (1967)
Overripeness and the mammalian ova. II. Delayed ovulation and chromosome anomalies. Fertility and Sterility, 18, 297-302.

Butcher, R.L. and Pope, R.S. (1979)
Role of estrogen during prolonged estrous cycles of the rat on subsequent embryonic death or development. Biology of Reproduction, 21, 491-495.

Cacheiro, N.L.A. and Generoso, W.M. (1975)
Cytological studies of sterility in sons of mice treated at spermatogonial or early spermatocyte stages with mutagenic chemicals. Mouse News Letter, 53, p.52.

Cannings, C. and Cannings, M.R. (1968)
Mongolism, delayed fertilization and human sexual behaviour. Nature, 218, 481.

Carothers, A.D. (1983)
Evidence that altered embryonic selection contributes to maternal age effect in aneuploidy: A spurious conclusion attributable to Simpson's paradox? American Journal of Human Genetics. In press.

Carothers, A.D., Collyer, S., de Mey, R. and Frackiewicz, A. (1978)
Parental age and birth order in the aetiology of some sex-chromosome aneuploidies. Annals of Human Genetics, 41, 277-287.

Carr, D.H. (1967)
Chromosomes after oral contraceptives. Lancet, 2, 830-831.

Carr, D.H. (1971)
Chromosomes and Abortion. Advances in Human Genetics, 2, 201-257.

Carr, D.H. and Walker, J.E. (1961)
Carbol fuchsin as a stain for human chromosomes. Stain Technology, 36, 233-236.

Casperson, T., Farber, S., Foley, G.E., Kudynowski, J., Modest, E.J., Simonsson, E., Wagh, U. and Zech, L. (1968)
Chemical differentiation along metaphase chromosomes. Experimental Cell Research, 49, 219-222.

Caspersson, T., Zech, L., Johansson, C. and Modest, E.J. (1970)
Identification of human chromosomes by DNA binding fluorescent agents. Chromosoma 30, 215-227.

Cattanach, B.M. (1962)
XO mice. Genetical Research, Cambridge, 3, 487-490.

Chebotar, W.A. (1978)
The increase in the frequency of spontaneous chromosome aberrations in mice with increasing age. Genetika, 14, 551-553.

Clarke, C.M., Edwards, J.H. and Smallpiece, U. (1961)
21 Trisomy/normal mosaicism in an intelligent child with some mongoloid characters. Lancet, 1, 1028-1030.

Collman, R.D. and Stoller, A. (1962)
A survey of mongoloid births in Victoria, Australia, 1942-1957. American Journal of Public Health, 52, 813-829.

Coté, G.B. (1973)
On the origin of 46,XX and 47,XXY males, 46,XY females and the position of the Xg locus. Annals of Human Genetics, 37, 21-30.

Court-Brown, W.M., Law, P. and Smith, P.G. (1969)
Sex chromosome aneuploidy and parental age. Annals of Human Genetics, London, 33, 1-14.

Creasy, M.R. and Crolla, J.A. (1974)
Prenatal mortality of Trisomy 21 (Down's Syndrome). Lancet, 1, 473-474.

Creasy, M.R., Crolla, J.A. and Alberman, E.D. (1976)
A cytogenetic study of human spontaneous abortions using banding techniques. Human Genetics, 31, 177-196.

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

Deckers, J.F.M., Van de Kroon, P.H.W. and Douglas, L.T.H. (1981)
Some characteristics of the XO mouse (*Mus musculus* L) II. Reproduction: fertility and gametic segregation. Genetica, 57, 2-11.

Donahue, R.P. (1972)
The relation of oocyte maturation to ovulation in mammals. In: Oogenesis. Eds. Biggers and Schuetz, p.413, University Park Press, Baltimore.

Döring, G.K. (1969)
The incidence of anovular cycles in women. Journal of Reproduction and Fertility Supplement, 6, 77-81.

Down, J.H.L. (1866)
Observation on an ethnic classification of idiots. London Hospital Report, 3, 259-262.

Edwards, J.H., Harnden, D.G., Cameron, A.H., Crosse, V.M. and Wolff, O.H. (1960)
A new trisomic syndrome. Lancet, 1, 787-789.

Edwards, R.G. (1977)
In: Scientific Foundations in Obstetrics and Gynaecology. Eds. Phillipp, E.E., Barnes, J. and Newton, M., pp. 175-252, Heinemann Medical, London.

Elion, G.B., Callahan, S., Beider, S., Rundles, R.W. and Laszlo, J. (1961)
A summary of investigations with 6-[(1-methyl-4-nitro-5-imidazole)thio]purine. Cancer Chemotherapy Reports, 14, 93-98.

Emanuel, I., Sever, L.E., Milham, S. and Thuline, H.C. (1972)
Accelerated ageing in young mothers of children with Down's Syndrome. Lancet, 1, 361-363.

England, P.C., Skinner, L.G., Cottrell, K.M. and Sellwood, R.A. (1974)
Serum Oestradiol-17β in normal women. British Journal of Cancer, 29, 462-469.

Erhardt, C.L. (1963)
Pregnancy losses in New York City,1960. American Journal of Public Health, 53, 1337-1352.

Erickson, J.D. (1978)
Down Syndrome, paternal age, maternal age and birth order. Annals of Human Genetics, 41, 289-298.

Erickson, J.D. (1979)
Paternal age and Down Syndrome. American Journal of Human Genetics, 31, 489-497.

Evans, E.P., Breckton, G. and Ford, C.E. (1964)
An air drying method for meiotic preparations from mammalian testes. Cytogenetics, 3, 289-294.

Evans, H.J. (1967)
The nucleolus, virus infection and trisomy in man. Nature, 214, 361-363.

Fabricant, J.D. and Schneider, E.L. (1978)
Studies of the genetic and immunologic components of the maternal age effect. Developmental Biology, 66, 337-343.

Faddy, M.J., Gosden, R.G. and Edwards, R.G. (1983)
Ovarian follicle dynamics in mice: a comparative study of three inbred strains and an F_1 hybrid. Journal of Endocrinology, 96, 23-33.

Falconer, D.S. (1973)
Replicated selection for body weight in mice. Genetical Research, Cambridge, 22, 291-321.

Fechheimer, N.S. and Beatty, R.A. (1974)
Chromosomal abnormalities and sex ratio in rabbit blastocysts. Journal of Reproduction and Fertility, 37, 331-341.

Feingold, M. and Atkins, L. (1973)
A case of Trisomy 9. Journal of Medical Genetics, 10, 184-187.

Ferguson-Smith, M.A., Mack, W.S., Ellis, P.M., Dickinson, M., Sanger, R. and Race, R.R. (1964)
Parental age and the source of the X-chromosome in XXY Klinefelter syndrome. Lancet, 1, 46.

Fialkow, P.J. (1966)
Autoimmunity and chromosomal aberrations. American Journal of Human Genetics, 18, 93-108.

Fineman, R.M., Kidd, K.K., Johnson, A.M. and Breg, W.R. (1976)
Increased frequency of heterozygotes for α_1 antitrypsin variants in individuals with either sex chromosome mosaicism or trisomy 21. Nature, 260, 320-321.

Foote, R.H. (1975)
The Gametogenic function of the aging ovary in the mammal. In: Aging Gametes. Int. Symp., Seattle, 1973, pp.179-200, Karger, Basel.

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 Wild, A.E., pp.285-304, Cambridge University Press.

Ford, C.E. (1981)
Nondisjunction. In: Trisomy 21. An International Symposium. Eds. Burgio, G.R., Fraccaro, M., Tiepolo, L. and Wolf, U. Springer-Verlag, Berlin Heidelberg, 103-143.

Ford, C.E. and Evans, E.P. (1973)
Non-expression of genome unbalance in haplophase and early diplophase of the mouse and incidence of karyotypic abnormality in post-implantation embryos. In: Les Accidents Chromosomiques de la Reproduction. Eds. Boue, A. and Thibault, C. (INSERM, Paris).

Ford, C.E., Jones, K.W., Polani, P.E., De Almeida, J.C. and Briggs, J.H. (1959)
A sex-chromosome anomaly in a case of gonadal dysgenesis (Turner's Syndrome). Lancet, 1, 709-710.

Ford, C.E., Searle, A.G., Evans, E.P. and West, B.J. (1969)
Differential transmission of translocations induced in spermatogonia of mice by irradiation. Cytogenetics, 8, 447-470.

Fraser, J. and Mitchell, A. (1876)
Kalmuk idiocy: Report of a case with autopsy. Notes on sixty-two cases. Journal of Mental Science, 22, 169.

French, F.E. and Bierman, J.M. (1962)
Probabilities of fetal mortality. Public Health Report, 77, 835-847.

Fugo, N.W. and Butcher, R.L. (1966)
Overripeness and the mammalian ova. I. Overripeness and early embryonic development. Fertility and Sterility, 17, 804-814.

Fugo, N.W. and Butcher, R. L. (1971)
Effects of prolonged estrous cycles on reproduction in aged rats. Fertility and Sterility, 22, 98-101.

Furuhjelm, M. (1966)
Urinary excretion of hormones during the climacteric. Acta Obstetrica et Gynaecologica Scandanavica 45, 352-364.

Generoso, W.M., Preston, R.J. and Brewen, J.G. (1975)
6 Mercaptopurine, an inducer of cytogenetic and dominant lethal effects in premeiotic and early meiotic germ cells of male mice. Mutation Research, 28, 437-447.

Geraedts, J.P.M. and Pearson, P.L. (1973)
Specific staining of the human No. 1 chromosome in spermatozoa. Humangenetik, 20, 171-173.

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

Golbus, M.S. (1981)
The influence of strain, maternal age, and method of maturation on mouse oocyte aneuploidy. Cytogenetics and Cell Genetics, 31, 84-90.

Goodlin, R.C. (1965)
Non-disjunction and maternal age in the mouse. Journal of Reproduction and Fertility, 9, 355-356.

Gosden, J.R., Mitchell, A.R., Gosden, C.M., Rodeck, C.H. and Morsman, J.M. (1982)
Direct vision chorion biopsy and chromosome-specific DNA probes for determination of fetal sex in first trimester prenatal diagnosis. Lancet, 2, 1416-1419.

Gosden, R.G. (1973)
Chromosomal anomalies of preimplantation mouse embryos in relation to maternal age. Journal of Reproduction and Fertility, 35, 351-354.

Gosden, R.G. (1979)
Effects of age and parity on the breeding potential of mice with one or two ovaries. Journal of Reproduction and Fertility, 57, 477-487.

Gosden, R.G., Laing, S.C., Felicio, L.S., Nelson, J.F. and Finch, C.E. (1983)
Imminent oocyte exhaustion and reduced follicular recruitment mark the transition to acyclicity in aging C57BL/6J mice. Biology of Reproduction, 28, 255-260.

Greenwald, G.S. (1974)
Role of follicle-stimulating hormone and luteinizing hormone in follicular development and ovulation. In: Handbook of Physiology, Section 7, Vol. IV, Part 2. Eds. R. O. Greep and E. B. Astwood. Williams and Wilkins Co., Baltimore, pp.293-323.

Griffiths, A.J.F. (1979)
Neurospora prototroph selection system for studying aneuploidy production. Environmental Health Perspectives, 31, 75-80.

Griffiths, A.J.F. and Delange, A.M. (1977)
ρ-Fluorophenylalanine increases meiotic non-disjunction in Neurospora Test system. Mutation Research, 46, 345-354.

Gropp, A. (1982)
Value of an animal model for trisomy. Virchows Archives (Pathology and Anatomy), 395, 117-131.

Gropp, A., Kolbus, U. and Giers, D. (1975)
Systematic approach to the study of trisomy in the mouse. II. Cytogenetics and Cell Genetics, 14, 42-62.

Guanti, G. and Di Loreto, M. (1980)
α-1-antitrypsin quantitative and qualitative (Pi phenotyping) characterization in Down Syndrome subjects and their parents. American Journal of Human Genetics, 32, 174-178.

Haber, J.E., Peloquin, J.G., Halvorson, H.O. and Borsiy, G.G. (1972)
Colcemid inhibition of cell growth and the characterization of a colcemid binding activity in *Saccaromyces cerevisiae*. Journal of Cell Biology, 55, 355-367.

Hansmann, I. (1974)
Chromosome aberrations in metaphase II - oocytes, stage sensitivity in the mouse oogenesis to amethopterin and cyclophosphamide. Mutation Research, 22, 175-191.

Hansmann, I. and El Nahass, E. (1979)
Incidence of non-disjunction in mouse oocytes. Cytogenetics and Cell Genetics, 24, 115-121.

Harlap, S., Shiono, P., Pellegrin, F., Golbus, M., Bachman, R., Mann, J., Schmidt, L. and Lewis, J.P. (1979)
Chromosome abnormalities in oral contraceptive breakthrough pregnancies. Lancet, 1, 1342-1343.

Hassold, T., Chen, N., Funkhauser, J., Jooss, T., Manuel, B., Matsuura, J., Matsuyama, A., Wilson, C., Yamane, J.A. and Jacobs, P.A. (1980a)
A cytogenetic study of 1000 spontaneous abortions. Annals of Human Genetics, 44, 151-164.

Hassold, T., Jacobs, P., Kline, J., Stein, Z., and Warburton, D. (1980)
Effect of maternal age on autosomal trisomies. Annals of Human Genetics, 44, 29-36.

Hassold, T. and Matsuyama, A. (1979)
Origin of trisomies in human spontaneous abortions. Human Genetics, 46, 285-294.

Heath, I.B. (1975)
Colchicine and colcemid binding components of fungus *Saprolegnia ferax*. Protoplasma, 85, 177-192.

Henderson, S.A. and Edwards, R.G. (1968)
Chiasma frequency and maternal age in mammals. Nature, 281, 22-28.

Hertig, A.T. (1967)
Human trophoblast; normal and abnormal. A plea for the study of the normal so as to understand the abnormal. American Journal of Clinical Pathology, 47, 249-268.

Hertig, A.T., Rock, J. and Adams, E.C. (1956)
A description of 34 human ova within the first 17 days of development. American Journal of Anatomy, 98, 435-493.

Hirshfield, A.N. (1982)
Follicular recruitment in long-term hemicastrate rats. Biology of Reproduction, 27, 48-53.

Hodgen, G.D., Goodman, A.I., O'Connor, A. and Johnson, D.K. (1977)
Menopause in rhesus monkeys: Model for study of disorders in the human climacteric. American Journal of Obstetrics and Gynaecology, 127, 581-584.

Holden, H.E., Ray, V.A., Wahrenburg, G.M. and Heleneski, J.D. (1973)
Mutagenicity studies wit9 6-mercaptopurine I. Cytogenetic activity *in vivo*. Mutation Research, 20, 257-263.

Hook, E.B. (1981)
Down Syndrome: frequency in human populations and factors pertinent to variation in rates, p.3. In: Trisomy 21 (Down Syndrome): Research Perspectives. Eds. de la Cruzz, F.F. and Gerald, P.S. University Park Press, Baltimore.

Hook, E.B. and Cross, P.K. (1982)
Paternal age and Down's Syndrome genotypes diagnosed prenatally: No association in New York State data. Human Genetics, 62, 167-174.

Iffy, L. (1963)
Time of conception in pathological gestations. Proceedings of the Royal Society of Medicine, 56, 1098-1100.

Irvine, W.J., Chan, M.M.W., Scarth, L., Kolb, F.O., Hartog, M., Bayliss, R.I.S. and Drury, M. (1968)
Immunological aspects of premature ovarian failure associated with idiopathic Addison's disease.
Lancet, 2, 883-887.

Jacobs, P.A. (1972)
Chromosome mutations: frequency at birth in humans.
Humangenetik, 16, 137-140.

Jacobs, P.A., Baikie, A.G., Court-Brown, W.M. and Strong, J.A. (1959)
The somatic chromosomes in mongolism. Lancet, 1, 710.

Jacobs, P.A. and Hassold, T.J. (1980)
The origin of chromosome abnormalities in spontaneous abortions. In: Embryonic and Fetal Death. Eds. Porter, I.H. and Hook, E.B. pp.289-298, Academic Press, New York.

Jacobs, P.A., Melville, M. and Brittain, R.P. (1965)
Aggressive behaviour, mental subnormality and the XYY male.
Nature (London), 208, 1351-1352.

Jacobs, P.A., Melville, M., Ratcliffe, S., Keay, A.J. and Syme, J. (1974)
A cytogenetic survey of 11,680 newborn infants.
Annals of Human Genetics 37, 359-376.

Jacobs, P.A. and Morton, N.E. (1977)
Origin of human trisomic and polyploids. Human Heredity, 27, 59-72.

Jagiello, G. and Lin, J.S. (1972)
Oral contraceptive compounds and mammalian oocyte meiosis.
American Journal of Obstetrics and Gynaecology, 120, 390-406.

Jagiello, G. and Lin, J.S. (1973)
An assessment of the effects of mercury on the meiosis of mouse ova. Mutation Research, 17, 93-99.

James, W.H. (1983)
Letter to the Editor: Parental coital rates and Down Syndrome. American Journal of Medical Genetics, 14, 783-784.

Jenkins, R.L. (1933)
Etiology of mongolism. American Journal of the Diseases of Children, 45, 506-519.

Jones, E.C. and Krohn, P.L. (1960)
The effect of unilateral ovariectomy on the reproductive lifespan of mice. Journal of Endocrinology, 20, 129-134.

Jones, E.C. and Krohn, P.L. (1961)
The relationships between age, numbers of oocytes and fertility in virgin and multiparous mice. Journal of Endocrinology, 21, 469-495.

Jongbloet, P.H., Frants, R.R. and Hamers, A.J. (1981)
Parental α_1-antitrypsin (PI) types and meiotic non-disjunction in the aetiology of Down Syndrome. Clinical Genetics, 20, 304-309.

Jørgensen, P.I. and Trolle, D. (1972)
Low urinary oestriol excretion during pregnancy in women giving birth to infants with Down's Syndrome. Lancet, 2, 782-784.

Kajii, T., Ohama, K. and Mikamo, K. (1978)
Anatomic and cytogenetic studies on 944 induced abortuses. Human Genetics, 43, 247-258.

Kajii, T., Okama, K., Niikawa, N., Ferrier, A. and Avirachan, S. (1973)
Banding analysis of abnormal karyotypes in spontaneous abortions. American Journal of Human Genetics, 25, 539-547.

Kaufman, M.H. (1972)
Non-random segregation during mammalian oogenesis. Nature, 238, 465-466.

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

Kendall, M.G. and Stuart, A. (1961)
The advanced theory of statistics. Vol. 2, 92-95, Charles Griffin and Co., London.

Kerridge, D. (1960)
The effect of inhibitors on the formation of flagella by *Salmonella typhimurium*. Journal of General Microbiology, 23, 519-538.

Klinefelter, H.F., Jr., Reifenstein, E.C., Jr., and Albright, F. (1942)
Syndrome characterized by gynecomastia, aspermatogenesis without aleydigism, and increased secretion of follicle-stimulating hormone (gynecomastia). Journal of Clinical Endocrinology and Metabolism, 2, 615-627.

Kofman-Alfaro, S. and Chandley, A.C. (1970)
Meiosis in the male mouse. An autoradiographic investigation. Chromosoma, 31, 404-420.

Koulischer, L. and Gillerot (1980)
Down's Syndrome in Wallonia (South Belgium), 1971-1978: Cytogenetics and Incidence. Human Genetics, 54, 243-250.

Kuleshov, N.P. (1976)
Chromosome anomalies of infants dying during the perinatal period and premature newborn. Humangenetik, 31, 151-160.

Laing, S. (1983)
M. Phil. Thesis: University of Edinburgh, Teviot Place, Edinburgh.

Lauristen, J.G., Jonasson, J., Therkelsen, A., Lass, F., Lindsten, J. and Bruun-Petersen, G. (1972)
Studies on spontaneous abortions. Fluorescence analysis of abnormal karyotypes. Hereditas, 71, 160-163.

Leb, D.E., Weisskopf, B. and Kanovitz, B.S. (1971)
Chromosome aberrations in the child of a kidney transplant recipient. Archives of Internal Medicine, 128, 441-444.

Lejeune, J., Gautier, and Turpin, R. (1959)
Le mongolism, premier example d'aberration autosomique humaine. C. R. Acadamie Sciences, 248, 1721-1722.

Leonard, A. and Schroder, H. (1968)
Incidence of XO mice after X-irradiation of spermatogonia. Molecular and General Genetics, 101, 116-119.

Léridon, H. and Boué, J. (1971)
La mortalité intrauterine d'origine chromosomique. Population, 26, 113-138.

Licznerski, G. and Lindsten, J. (1972)
Trisomy 21 in man due to maternal non-disjunction during the first meiotic division. Hereditas, 70, 153-154.

Lloas, P. (1961)
Mitotic haploidization by treatment of *Aspergillus niger* diploid with ρ-fluorophenylalanine. Nature, 190, 744.

Lowry, R.B., Jones, D.C. and Renwick, D.H. (1976)
Down's Syndrome in British Columbia, 1952-1973: incidence and mean maternal age. Teratology, 14, 29-34.

Luthardt, F.W. (1976)
Cytogenetic analysis of oocytes and early preimplantation embryos from XO mice. Developmental Biology, 54, 73-81.

Luthardt, F.W., Palmer, C.G. and Yu, P-L. (1973)
Chiasma and univalent frequencies in aging female mice. Cytogenetics and Cell Genetics, 12, 68-69.

Lyon, M.F. and Hawker, S.G. (1973)
Reproductive lifespan in irradiated and unirradiated chromosomally XO mice. Genetical Research, 21, 185-194.

Lyon, M.F., Ward, H.C. and Simpson, G.M. (1976)
A genetic method for measuring non-disjunction in mice with Robersonian translocations. Genetical Research, Cambridge, 26, 283-295.

McGauhey, R.W. (1977)
The culture of pig oocytes in minimal medium, and the influence of progesterone and estradiol-17β on meiotic maturation. Endocrinology, 100, 39-45.

McLaren, A. (1966)
Regulation of ovulation rate after removal of one ovary in mice. Proceedings of the Royal Society B, 166, 316-340.

Machin, G.A. and Crolla, J.A. (1974)
Chromosome constitution of 500 infants dying during the perinatal period. Humangenetik, 23, 183-198.

Magensis, R.E., Hecht, F. and Milham, S. (1968)
Trisomy 13 (D) syndrome: studies on parental age, sex ratio and survival. Journal of Pediatrics, 73, 222-228.

Mandl, A.M. and Zuckerman, S. (1951)
Numbers of normal and atretic oocytes in unilaterally spayed rats. Journal of Endocrinology, 7, 112-119.

Mantel, N. and Stark, C.N. (1967)
Paternal age and Down's Syndrome. American Journal of Mental Deficiency, 71, 1025-1027.

Margolis, R.L. and Wilson, L. (1981)
Microtubule treadmills - possible molecular machinery. Nature, 293, 705-711.

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

Martin, R.H., Lin, C.C., Balkan, W. and Burns, K. (1982)
Direct chromosomal analysis of human spermatozoa: Preliminary results from 18 normal men. American Journal of Human Genetics, 34, 459-468.

Matsunaga, E., Tonomura, A., Oishi, H. and Kikuchi, Y. (1978)
Reexamination of paternal age effect in Down's Syndrome. Human Genetics, 40, 259-268.

Maudlin, I. and Fraser, L.R. (1977)
The effect of PMSG dose on the incidence of chromosomal anomalies in mouse embryos fertilized *in vitro*. Journal of Reproduction and Fertility, 50, 275-280.

Maudlin, I. and Fraser, L.R. (1978)
Maternal age and the incidence of aneuploidy of first-cleavage mouse embryos. Journal of Reproduction and Fertility, 54, 423-426.

Mavor, J.W. (1921)
On the elimination of the X-chromosome from the egg of *Drosophila melanogaster* by X-rays. Science, 54, 277-279.

Mavor, J.W. (1922)
An effect of X-rays on inheritance. Albany Medical Annals, May Issue, 209-220.

Mavor, J.W. (1924)
The production of non-disjunction by X-rays. Journal of Experimental Zoology, 39, 381-432.

Max, C. (1977)
Cytological investigation of embryos in low-dose X-irradiated young and old female inbred mice. Hereditas, 85, 199-206.

Mikamo, K. (1968)
Mechanism of non-disjunction of meiotic chromosomes and of degeneration of maturation spindles in eggs affected by intrafollicular overripeness. Experimentia, 24, 75-78.

Mikamo, K. and Hamaguchi, H. (1975)
Chromosomal disorder caused by preovulatory overripeness of oocytes. In: Aging Gametes. Ed. R. J. Blandau, pp. 72-97, S. Karger, Basel/New York.

Mikkelsen, M., Hallberg, A. and Poulsen, H. (1976)
Maternal and paternal origin of extra chromosome 21 in trisomy 21. Human Genetics, 32, 17-21.

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

Miller, J.F., Williamson, E., Glue, J., Gordon, Y.B., Grudzinskas, J.G. and Sykes, A. (1980)
Fetal loss after implantation. Lancet, 2, 554-556.

Milstein-Moscati, I. and Becak, W. (1983)
Letter to the Editor: Occurrence of Down Syndrome and Human sexual behaviour. American Journal of Medical Genetics, 14, 785-786.

Morris, T. (1968)
The XO and OY chromosome constitutions in the mouse. Genetical Research, Cambridge, 12, 125-137.

Mulcahy, M.T. (1978)
Down Syndrome and parental coital rate. Lancet, 2, 895.

Nelson, J.F., Felicio, L.S. and Randall, P.K., Sims, C. and Finch, C.E. (1982)
A longitudinal study of estrous cyclicity in aging C57BL/6J mice: I. Cycle frequency, length and vaginal cytology. Biology of Reproduction, 27, 327-339.

Nordensen, I. (1979)
Population studies in Northern Sweden IX Incidence of Down's Syndrome by time, region and maternal age. Hereditas, 91, 257-262.

Oakberg, E.F. (1956)
Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. American Journal of Anatomy, 99, 507-516.

Oakberg, E.F. (1979)
Response of spermatogonia and kinetics of spermatogenesis to 6-mercaptopurine. Environmental Mutagenesis, 1, 126 (Abstract).

Ohno, S., Kaplan, W.D. and Kinosita, R. (1959)
Do XY and O-sperm occur in *mus musculus?* Experimental Cell Research, 18, 382-384.

Old, J.M., Ward, R.H.T., Petrou, M., Karagozlu, F., Modell, B. and Weatherall, D.J. (1982)
First-trimester fetal diagnosis for haemoglobin-opathies: three cases. Lancet, 2, 1413-1416.

Øster, J. (1953)
In: Mongolisme, a clinicogenealogical investigation comprising 526 mongols living in Sealand and neighbouring islands in Denmark, pp.96-122 (Danish Science Press, Copenhagen).

Oud, J.L., de Jong, J.H. and de Rooij, D.G. (1979)
A sequential analysis of meiosis in the male mouse using a restricted spermatocyte population by Hydroxyurea/ Triaziquone Treatment. Chromosoma, 71, 237-248.

Papanicolaou, A.D., Loraine, J.A., Dove, G.A. and London, N.B. (1969)
Hormone excretion patterns in perimenopausal women. Journal of Obstetrics and Gynaecology of the British Commonwealth, 76, 308-316.

Parker, D.R. and Busby, N. (1973)
Observations concerning the effects of radiation on the segregation of chromosomes. Mutation Research, 18, 33-46.

Patau, K., Smith, D.W., Therman, E., Inhorn, S.L. and Wagner, H.P. (1960)
Multiple congenital anomaly by an extra autosome. Lancet, 1, 790-793.

Pawlowitzki, I.H. and Pearson, P.L. (1972)
Chromosomal aneuploidy in human spermatozoa. Humangenetik, 16, 119-122.

Pearson, P.L., Geraedts, J.P.M., van der Ploeg, M. and Pawlowitzki, H. (1975)
Chromosome studies on human male gametes. Biological Journal of the Linnenaen Society (Supplement 1) 7, 279-289.

Penrose, L.S. (1933)
Relative effects of paternal and maternal age in mongolism. Journal of Genetics, 27, 219-224.

Penrose, L.S. (1934)
The relative aetiological importance of birth order and maternal age in mongolism. Proceedings of the Royal Society B, 115, 431-443.

Penrose, L.S. (1961)
Mongolism. British Medical Bulletin, 17, 184-189.

Penrose, L.S. (1965)
Mongolism as a problem in human biology. In: The Early Conceptus, Normal and Abnormal. (Symposium held at Queen's College, Dundee, September 1964). Ed. W. W. Park, pp.94-97, Dundee, University of St. Andrews.

Penrose, L.S. and Berg, J.M. (1968)
Mongolism and duration of marriage. Nature, 218, 300.

Penrose, L.S. and Smith, G. F. (1966)
Down's Anomaly (J and A Churchill, London).

Peppler, R.D. (1971)
Effects of unilateral ovariectomy on follicular development and ovulation in cycling, aged rats. American Journal of Anatomy, 132, 423-428.

Peppler, R.D. and Greenwald, G.S. (1970)
Influence of unilateral ovariectomy on follicular development in cycling rats. American Journal of Anatomy, 127, 9-14.

Peters, H., Levy, E. and Crone, M. (1962)
Deoxyribonucleic acid synthesis in oocytes of mouse embryos. Nature, 195, 915-916.

Pincus, G., Romanoff, L.P. and Carlo, J. (1954)
The excretion of urinary steroids by men and women of various ages. Journal of Gerontology, 9, 113-132.

Polani, P.E. (1983)
The elusive cause of Down's Syndrome. Lancet, 1, 1340.

Polani, P.E., Briggs, C.E., Ford, C.E., Clarke, C.M. and Berg, J.M. (1960)
A mongol girl with 46 chromosomes. Lancet, 1, 721-724.

Polani, P.E. and Jagiello, G.M. (1976)
Chiasmata, meiotic univalents, and age in relation to aneuploid imbalance in mice. Cytogenetics and Cell Genetics, 16, 505-529.

Race, R.R. and Sanger, R. (1975)
Blood Groups in Man. 6th ed., Blackwell, Oxford.

Ratcliffe, S.G. Axworthy, D. and Ginsborg, A. (1979)
The Edinburgh study of growth and development in children with sex chromosome abnormalities. Birth Defects, 15, 243-260.

Read, S.G. (1982)
The distribution of Down's Syndrome. Journal of Mental Deficiency Research, 26, 215-227.

Reimers, T.J. and Sluss, P.M. (1978)
6-mercaptopurine treatment of pregnant mice: effects on second and third generations. Science, 201, 65-66.

Riccardi, V.M. (1977)
Trisomy 8: An international study of 70 patients. Birth Defects: Original Article Series, Volume XIII, Number 3C, 171-184.

Richards, O.W. (1938)
Colchicine stimulation of yeast growth fails to reveal mitosis. Journal of Bacteriology, 36, 187-195.

Roberts, C.J. and Lowe, C.R. (1975)
Where have all the conceptions gone? Lancet, 1, 498-499.

Rodman, T.C. (1971)
Chromatid disjunction in unfertilized ageing oocytes. Nature, 233, 191-193.

Rodriguez, J.A. and Borisy, G.G. (1979)
Experimental Phenyketonuria: Replacement of Carboxyl Terminal Tyrosine by Phenylalanine in Infant Rat Brain Tubulin. Science, 206, 463-465.

Rohrborn, G., Kühn, O., Hansmann, I. and Thon, K. (1971)
Induced chromosome aberrations in early embryogenesis of mice. Humangenetik, 11, 316-322.

Roth, D.B. (1963)
The frequency of spontaneous abortion. International Journal of Fertility, 8, 431-434.

Rudak, E., Jacobs, P.A., Yanagimachi, R. (1978)
Direct analysis of the chromosome constitution of human spermatozoa. Nature, 274, 911-913.

Rundle, A.T., Coppen, A. and Cowie, V. (1961)
Steroid excretion in mothers of mongols. Lancet, 2, 846-848.

Russell, L.B. (1976)
Numerical sex-chromosome anomalies in mammals: Their spontaneous occurrence and use in mutagenesis studies. In: Chemical Mutagens, ed. A. Hollaender. Vol. 4, 55-91. Plenum Publishing (New York).

Russell, L.B. (1979)
Meiotic nondisjunction in the mouse: methodology for genetic testing and comparison with other methods. Environmental Health Perspectives, 31, 113-128.

Russell, L.B. and Montgomery, C.S. (1974)
The incidence of sex-chromosome anomalies following irradiation of mouse spermatogonia with single or fractionated doses of X-rays. Mutation Research, 25, 367-376.

Sandberg, A.A., Koepf, G.F., Ishihara, T. and Hauschka, T.S. (1961)
An XYY human male. Lancet, 2, 488-489.

Sanger, R., Tippett, P. and Gavin, J. (1971)
Xg groups and sex abnormalities in people of North-European ancestry. Journal of Medical Genetics, 8, 417-426.

Sanger, R., Tippett, P., Gavin, J., Teesdale, P. and Daniels, G.L. (1977)
Xg groups and sex abnormalities in people of northern European ancestry: an addendum. Journal of Medical Genetics, 14, 210-213.

Sansome, E.R. and Bannon, L. (1946)
Colchicine ineffective in inducing polyploidy in *Penicillium notatum*. Lancet, 2, 828-829.

Savontaus, M.L. (1975)
Relationship between effects of X-rays on non-disjunction and crossing-over in *Drosophila melanogaster*. Hereditas, 80, 195-204.

Schnedl, W. (1973)
Analysis of the human karyotype by recent banding techniques. Archives of Genetics, 46, 65-98.

Searle, A.G. (1975)
Radiation-induced chromosome damage and the assessment of genetic risk. In: Modern Trends in Human Genetics, Vol. 2 (ed. A. E. H. Emery), pp.83-110, Butterworths, London and Boston.

Setchell, B.P. and Waites, G.M.H. (1975)
The blood-testis barrier. In: Handbook of Physiology, Sect. 7, Endocrinology, Vol. 5, Male Reproductive System, pp.143-172. Pub. American Physiological Society.

Sharman, A. (1962)
The menopause. In: The Ovary, Vol. 1, ed. Zuckerman, Mandl and Eckstein, Academic Press, New York, p.539.

Sherman, B.M., West, J.H. and Korenman, S.G. (1976)
The menopausal transition: Analysis of LH, FSH, Estradiol and progesterone concentrations during menstrual cycles of older women. Journal of Clinical Endocrinology and Metabolism, 42, 629-636.

Sigler, A.T., Cohen, B.H., Lilienfeld, A.M., Westlake, J.E. and Hetznecker, W.H. (1967)
Reproductive and marital experience of parents of children with Down's Syndrome (mongolism).
Journal of Pediatrics, 70, 608-614.

Sisken, J.E., Stevens, E.E. and Donnelly, G.M. (1972)
Effects of p-DL-Fluorophenylalanine on mitosis and the mitotic spindle. Experimental Cell Research, 73, 25-32.

Sisken, J.E. and Wilkes, E. (1967)
The effects of p-DL-Fluorophenylalanine on chromosome movement and cytokinesis of human amnion cells in culture.
Chromosoma, 44, 91-98.

Smith, G.F. and Berg, J.M. (1976)
Down's Anomaly (2nd ed.) Churchill Livingstone, Edinburgh London and New York.

Sopelak, V.M. and Butcher, R.L. (1982)
Decreased amount of ovarian tissue and maternal age effect embryonic development in old rats. Biology of Reproduction, 27, 449-455.

Speed, R.M. (1977)
The effects of ageing on the meiotic chromosomes of male and female mice. Chromosoma, 64, 241-254.

Speed, R.M. and Chandley, A.C. (1981)
The response of germ cells of the mouse to the induction of non-disjunction by X-rays. Mutation Research, 84, 409-418.

Speed, R.M. and Chandley, A.C. (1983)
Meiosis in the foetal mouse ovary. II Oocyte development and age-related aneuploidy. Does a production line exist? Chromosoma. In press.

Stebbings, H. and Hyams, J.S. (1979)
Cell Motility - (Integrated Themes in Biology). Longman, London.

Steckman, M.L. (1980)
Treatment of Crohns disease with 6-mercaptopurine: What effects on fertility? The New England Journal of Medicine, 303, p.817.

Stene, J., Fischer, G., Stene, E., Mikkelsen, M. and Petersen, E. (1977)
Paternal age effects in Down's Syndrome. Annals of Human Genetics, 40, 229-306.

Stene, J. and Stene, E. (1978)
On data and methods in investigations on parental age effects, comments on a paper by J. D. Erickson. Annals of Human Genetics, 41, 465-468.

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

Sugawara, S. and Mikamo, K. (1983)
Absence of correlation between changed chiasma configuration and meiotic nondisjunction in the aged female Chinese hamster. Cytogenetics and Cell Genetics, 35, 34-40.

Sumner, A.T. (1972)
A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research, 75, 304-306.

Sumner, A.T. and Robinson, J.A. (1976)
A difference in dry mass between the heads of X- and Y-bearing human spermatozoa. Journal of Reproduction and Fertility, 48, 9-15.

Sutherland, G.R., Carter, R.F., Bauld, R., Smith, I.I. and Bain, A.D. (1978)
Chromosome studies at the paediatric necropsy. Annals of Human Genetics, 42, 173-181.

Sved, J.A. and Sandler, L. (1981)
Relation of maternal age effect in Down Syndrome to nondisjunction. In: Trisomy 21 (Down Syndrome) Research Perspectives. Ed. de la Cruz, F.F. and Gerald, P.S. University Park Press, Baltimore, U.S.A.

Szemere, G. and Chandley, A.C. (1975)
Trisomy and triploidy induced by X-irradiation of mouse spermatocytes. Mutation Research, 33, 229-238.

Szemere, G. and Marczinovits, I. (1977)
EMS and MMS-induced meiotic non-disjunction in the male mouse. In: Progress in Genetic Toxicology. Ed. D. Scott, B. A. Bridges and F. H. Sobels, pp.215-222. Elsevier/North Holland Biomedical Press.

Täckholm, G. (1922)
Zytologische Studien uber die Gattung *Rosa*. Acta Hort Berg. 7.

Tarkowski, A.K. (1966)
An air-drying method for chromosome preparations from mouse eggs. Cytogenetics, 5, 394-400.

Tates, A.D. (1979)
Microtus oeconomus (Rodentia), a useful mammal for studying the induction of sex-chromosome nondisjunction and diploid gametes in male germ cells. Environmental Health Perspectives, 31, 151-159.

Taylor, A.I. (1968)
Autosomal trisomy syndromes: a detailed study of 27 cases of Edwards syndrome and 27 cases of Patau's Syndrome. Journal of Medical Genetics, 5, 227-252.

Tease, C. (1981)
Chromosome non-disjunction in female mice after X-irradiation. Mouse News Letter, 65, 22.

Tease, C. (1982a)
Similar dose-related chromosome non-disjunction in young and old female mice after X-irradiation. Mutation Research, 95, 287-296.

Tease, (1982b)
Radiation-induced chromosome non-disjunction in oocytes stimulated by different doses of superovulating hormones. Mutation Research, 105, 95-100.

Therkelsen, A.J., Grunnet, N., Hjort, T., Myhre Jensen, O., Jonasson, J., Lauristen, J.G., Lindsten, J. and Bruun Petersen, G. (1973)
Studies on spontaneous abortions. In: Chromosomal Errors in Relation to Reproductive Failure, pp.81-93. Ed. Boué, A. and Thibault. INSERM, Paris.

Threlkeld, S.F.H. and Stoltz, J.M. (1970)
A genetic analysis of non-disjunction and mitotic recombination in *Neurospora crassa*. Genetical Research, 16, 29-35.

Thung, P.J. (1961)
Ageing changes in the ovary. In: Structural Aspects of Ageing. Ed. G. H. Bourne, pp.109-142, Pittman Medical Publishing (London).

Thung, P.J., Boot, L.M. and Muhlbock, O. (1956)
Senile changes in the oestrous cycle and in ovarian structure in some inbred strains of mice. Acta Endocrinologia, 23, 8-32.

Treloar, A.E., Boynton, R.E., Behn, B.G. and Brown, B.W. (1967)
Variation of the human menstrual cycle through reproductive life. International Journal of Fertility, 12, 77-126.

Turner, H.H. (1938)
A syndrome of infantilism, congenital webbed neck, and cubitus valgus. Endocrinology, 23, 566-574.

Uchida, I.A. (1970)
Epidemiology of mongolism: The Monitoba study. In: Down's Syndrome (Mongolism) (ed. V. Apgar), Annals of the New York Academy of Sciences, 171, 361-369.

Uchida, I.A. (1979)
Radiation-induced non-disjunction. Environmental Health Perspectives, 31, 13-17.

Uchida, I.A. and Lee, C.P.V. (1974)
Radiation-induced nondisjunction in mouse oocytes. Nature, 250, 601-602.

Vallotton, M.B. and Forbes, A.P. (1969)
Premature menopause in autoimmune disease. Lancet, 1, 156-157.

Vaughan, M. and Steinberg, D. (1960)
Biosynthetic incorporation of fluorophenylalanine into crystalline proteins. Biochemical and Biophysical Acta, 40, 230-236.

Vickers, A.D. (1969)
Delayed fertilization and chromosomal anomalies in mouse embryos. Journal of Reproduction and Fertility, 20, 69-76.

Warburton, D. and Fraser, F.C. (1964)
Spontaneous abortion risks in man: data from reproductive histories collected in a medical genetics unit. American Journal of Human Genetics, 16, 1-25.

Warburton, D., Stene, Z., Kline, J. and Susser, M. (1980)
Chromosome abnormalities in spontaneous abortion: Data from the New York city study. In: Embryonic and Fetal Death. Eds. Porter, I.H. and Hook, E.B., pp.261-287, Academic Press, New York.

Watanabe, T. and Endo, A. (1982)
Chromosome Analysis of preimplantation embryos after cadmium treatment of oocytes at Meiosis I. Environmental Mutogenesis, 4, 563-567.

Watanabe, T., Shimada, T. and Endo, A. (1977)
Mutagenic effects of cadmium on the oocyte chromosomes of mice. Japanese Journal of Hygiene, 32, 472-481.

Watanabe, T., Shimada, T., and Endo, A. (1979)
Mutagenic effects of cadmium on mammalian oocyte chromosomes. Mutation Research, 67, 349-356.

Welschen, R., Dullaart, J. and de Jong, F.H. (1978)
Interrelationships between circulating levels of Estradiol-17β, Progesterone, FSH and LH immediately after unilateral ovariectomy in the cyclic rat. Biology of Reproduction, 18, 421-427.

Westhead, E.W. and Boyer, P.D. (1961)
The incorporation of p-Fluorophenylalanine into some rabbit enzymes and other proteins. Biochemical and Biophysical Acta, 54, 145-156.

Wramsby, H., Hansson, A. and Leidholm (1982)
In: World Conference on Embryo Transfer, *in vitro* Fertilization and Instrumental Insemination. Keil (1980) Eds. Hafez, E.S.E. and Semm, K., pp.263-271 (Liss, New York).

Yamamoto, M., Shimada, T., Endo, A. and Watanabe, G. (1973)
Effects of low-dose X-irradiation on the chromosomal non-disjunction in aged mice. Nature New Biology, 244, 206-208.

Yamamoto, M. and Watanabe, G. (1978)
See Kajii *et al* (1978).

Yanagimachi, R., Yanagimachi, H. and Rogers, B.J. (1976)
The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. Biology of Reproduction, 15, 471-476.

Zech, L. (1969)
Investigation of metaphase chromosomes with DNA-binding fluorochromes. Experimental Cell Research, 58, 463.

APPENDIX: PUBLICATIONS RESULTING FROM THIS THESIS

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THE EFFECT OF 4CMB ON GERM CELLS OF THE MOUSE

J.D. BROOK

MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Edinburgh EH4 2XU (Scotland)

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SUMMARY

The effect of 4CMB on meiosis in the mouse was studied using both male and female test systems.

Females were superovulated and treated with 4CMB at metaphase I and oocytes sampled at metaphase II. Similarly with males, chromosome analysis was made at metaphase II for spermatocytes treated at metaphase I.

No increase in the frequency of structural or numerical chromosome abnormalities was noted for treated mice as compared to controls.

Previous studies have shown that germ cells of both the male and female mouse provide suitable material for mutagenicity testing (Szemere and Chandley, 1975; Hansmann, 1974). Both chromosomal structural damage and aneuploidy can be used as end points.

In the present study the effect of 4CMB on germ cells of the mouse has been studied. For both males and females the stage treated was metaphase I and the spermatocytes and oocytes respectively were sampled at metaphase II.

METHODS

For both oocyte and spermatocyte preparations, mice of the strain Swiss (Schofield) were used.

Female mice, aged 8–12 weeks, were superovulated using 1.5 IU pregnant mares serum (PMS) and 1.0 IU human chorionic gonadotrophin (HCG) 48 h later. 3 h after HCG treatment the mice were injected i.p. with 4CMB at concentrations of 50 and 100 mg/kg dissolved in 0.05% 'Tween 80'. 0.05% Tween 80 alone was used for injection of controls. The stage treated corresponded to metaphase I and the sampling time to metaphase II – 15 h after injection of HCG. Mice were killed by

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cervical dislocation, the oocytes removed from the ampullae of the fallopian tubes and counted. (It has been shown previously that these doses of hormones do not affect the incidence of non-disjunction during the first meiotic division [Hansmann and El-Nahass, 1979].)

Oocytes obtained by superovulation, as described above, were fixed using the technique of Tarkowski (1966). They were C-banded and analysed at metaphase II with regard to structural and numerical abnormalities.

Male mice, aged 8–12 weeks, were treated with 4CMB dissolved in 0.05% Tween 80 at a dose of 100 mg/kg and 0.05% Tween 80 alone was again injected into controls. 4 h later the mice were killed by cervical dislocation and the testes removed. At this time interval, cells treated in metaphase I had progressed to metaphase II, a fact established earlier by us for mice of this strain using tritium autoradiography. Meiotic preparations were made using the technique of Evans et al. (1964), staining and chromosome analysis being carried out as described above for oocytes.

RESULTS AND DISCUSSION

Table 1 shows that at the highest dose of 4CMB there was a significant decrease in the number of eggs recovered. This could possibly be due to an effect of the chemical on the ovary causing fewer eggs to be ovulated or alternatively degeneration of some oocytes may have occurred soon after ovulation. Counts of corpora lutea were, however, not carried out and it was thus not possible to decide between these two alternatives.

In Table 2 the results of the chromosome analyses carried out on metaphase II oocytes are given. No disomic eggs ($n=21$) were found in any group indicating the 4CMB had no effect on disjunction at anaphase I in the oocyte. Eggs with less than 20 chromosomes were equally frequent in both control and treated groups and it is concluded therefore that these were due to chromosome loss during preparation.

Results of the chromosome analysis carried out on spermatocytes are given in Table 3. No increase in the frequency of aneuploidy was found in 4CMB-treated mice as compared to controls. As in females, preparations with less than 20 chromo-

TABLE 1

THE EFFECT OF 4CMB ON OVULATION

Treatment	Number of animals	Total eggs recovered	Mean	Range
Tween 80	24	1058	44.08	10–69
4CMB, 50 mg/kg	15	522	34.80	18–57
4CMB, 100 mg/kg	22	657	29.86	10–57

Using t test 50 mg/kg 4CMB gave $P>0.05$; 100 mg/kg 4CMB gave $P<0.001$.

TABLE 2

RESULTS OF CHROMOSOME ANALYSIS CARRIED OUT AT METAPHASE II ON OOCYTES OF TREATED AND CONTROL MICE

Treatment	Number of animals	Total eggs analyzed	Normal (%)	Chromosome count			
				20	19	18	<18
Tween 80	11	109	65.1	71	23	10	5
4CMB, 50 mg/kg	15	111	72.1	80	19	6	6
4CMB, 100 mg/kg	15	99	60.6	60	18	12	9

TABLE 3

RESULTS OF CHROMOSOME ANALYSIS CARRIED OUT AT METAPHASE II ON SPERMATOCYTES OF TREATED AND CONTROL MICE

Treatment	Number of animals	Total spermatocytes analyzed	Normal (%)	Chromosome count			
				20	19	18	<18
Tween 80	4	358	84.1	301	43	12	1
4CMB 100 mg/kg	4	478	84.9	406	58	14	–

somes were equally frequent in both control and treated groups, again suggesting that they were due to chromosome loss during the making of the preparations.

In this study no difference in aneuploidy induction between control and treated germ cells has been observed for either male or female germ cells at the doses tested and at the stages treated.

Higher doses were originally tried but these proved lethal to the mice. The highest dose used (100 mg/kg) was therefore selected to produce optimum effects on disjunction without causing death of the animals. Initially an attempt was also made to look at the effects of 4CMB on disjunction at anaphase I in oocytes following treatment of embryonic oogonia. The pregnant mothers, however, proved particularly sensitive to the compound with death resulting from doses as low as 20 mg/kg. This test therefore had to be abandoned.

The analysis carried out in this study was confined to the treatment of metaphase I in both oocytes and spermatocytes. Had other germ-cell stages been treated, sensitivity to the compound may perhaps have been revealed. Szemere and Chandley (1975), for example, found pre-leptotene to be the most sensitive stage to aneuploidy induction following X-irradiation of mouse spermatocytes. Hansmann (1974) has pointed out that the *most* sensitive and *least* sensitive stages for one agent may not necessarily be the same for all other agents. A wide variety of tests would therefore be required to reveal all possible effects, a task which was beyond the scope of this present enquiry.

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REFERENCES

Evans, E.P., G. Breckon and C.E. Ford (1964) An air-drying method for meiotic preparations from mammalian testes, Cytogenetics, 3, 289–294.

Hansmann, I. (1974) Chromosome aberrations in metaphase II oocytes stage sensitivity in the mouse oogenesis to amethopterin and cyclophosphamide, Mutation Res., 22, 175–191.

Hansmann, I., and E. El-Nahass (1979) Incidence of non-disjunction in mouse oocytes, Cytogenet. Cell Genet., 24, 115–121.

Szemere, G., and A.C. Chandley (1975) Trisomy and triploidy induced by X-irradiation of mouse spermatocytes, Mutation Res., 33, 229–238.

Tarkowski, A.K. (1966) An air-drying method for chromosome preparations from mouse eggs, Cytogenetics, 5, 394–400.

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X-chromosome segregation, maternal age and aneuploidy in the *XO* mouse

By J. D. BROOK

MRC Clinical and Population Cytogenetics Unit, Western General Hospital, Crewe Road South, Edinburgh, EH4 2XU

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SUMMARY

Cytogenetic studies have ascertained that the segregation of the *X*-chromosome, during the first meiotic division of the oocyte in *XO* mice, occurs at random, contrary to the finding of some earlier authors. The ratio of nullo-*X* to *X*-bearing oocytes at ovulation does not change with maternal age. The usefulness of the *XO* mouse as a model for aneuploidy production in women (Lyon & Hawker, 1973) is discussed.

INTRODUCTION

Unlike *XO* women, *XO* mice are fertile, albeit subject to reproductive impairment (Lyon & Hawker, 1973).

Ever since a breeding stock of *XO* mice was established by Cattanach (1962), using the sex-linked gene tabby, as marker, controversy has existed in the literature concerning the question of segregation of the single *X*-chromosome at meiosis in the *XO* oocyte. From his own breeding data, Cattanach (1962) observed that although litter size was near normal for this particular stock, a shortfall of some 30–37 % in *XO* compared to *XX* offspring occurred from *XO* mothers. He was unable to determine the reason for the reduction in *XO* progeny, but as one possibility, suggested that preferential loss of the chromosome sets lacking an *X* chromosome to the polar bodies in the meiotic divisions of the ova might have occurred. The alternative was that death of the missing classes during embryonic development had taken place. To account for the higher than expected litter size in *XO* mothers, he did not, however, discount the possibility of early loss of inviable embryos, compensated by the development of all individuals of the viable classes, some of which would have been lost in larger normal litters as a result of overcrowding in the uterus.

In a subsequent study, Morris (1968) examined reproductive performance and embryonic mortality in a large series of *XO* and *XX* females. One series of pregnant females of both genotypes was dissected after 15 days gestation and another series after $3\frac{1}{2}$ days. From his findings, he concluded that there could be both an abnormally low segregation of nullo-*X* gametes in *XO* females *and* a reduction in

viability of *XO* foetuses during the early stages of gestation. This lower viability of *XO*'s *in utero* contrasted with their seemingly normal viability after birth. Strong circumstantial evidence was also found for the death of all *OY* zygotes before implantation.

Direct cytological information on the segregation of the *X* chromosome has since been obtained by several groups of authors analysing chromosomal complements in the metaphase II oocytes ovulated by *XO* females. The results however, are conflicting (see Russell, 1976 for review). According to Evans and Ford (unpublished data), segregation of the *X* to egg or polar body is random. The data of Kaufman (1972) and Luthardt (1976), however, suggest that it is non-random, with the *X*-bearing chromosome sets being preferentially included in the egg nucleus.

A further complicating factor is the claim made by Deckers *et al.* (1981), from breeding data on *XO* mice, that the phenomenon of non-random segregation is maternal-age related. These authors found a greater recovery of *XO* progeny relative to *XX* as the age of the mother (or litter number) advanced.

The present study was initiated in a further attempt to clarify some of these issues. Breeding data on a large series of *XO* mice at a range of ages have been coupled with a cytological analysis of ovulated metaphase II oocytes. The question of whether or not segregation of the single *X*-chromosome is random has been re-investigated. Also, evidence for changing relationship between *X*-segregation and age has been sought. The question of whether the *XO* mouse constitutes a good model for the human pre-menopausal female, in terms of chromosomal nondisjunction as suggested by Lyon & Hawker (1973), is also considered.

METHODS AND MATERIALS

(i) *Animals used*

The colony of *XO* mice used was set up from mice kindly supplied by Dr Mary Lyon, MRC Radiobiology Unit, Harwell, England. The sex-linked gene, Tabby, was used as marker. Normal-coated *XO* females mated to Tabby males produced three types of phenotypically distinct offspring; normal-coated males (+/*Y*), greasy-coated females (Ta/O) and striped females (Ta/+). The Ta/O and +/*Y* offspring were subsequently used as breeders to regenerate +/O and Ta/*Y* animals, with striped females (Ta/+) again being produced. In this way all offspring could be identified from their coat colours. Brother-sister matings were avoided. The stock was checked occasionally, by blood karyotyping, to ensure that all supposed phenotypic *XO* females were in fact of the *XO* genotype.

Offspring were classified at weaning and female breeders used until they reached 36 weeks of age, after which time they were killed and used for oocyte chromosome analysis.

(ii) *Oocyte collection and chromosome preparation*

The female mice used for chromosome analysis in metaphase II oocytes constituted six groups, divided according to age and phenotype. There were two

XO groups i.e. +/O and Ta/O with Ta/+ sibs serving as controls. Analysis was carried out over two different ages within each group i.e. 8–20 weeks (young) and 30–40 weeks (old) (Lyon & Hawker, 1973 have shown that in *XO* mice, both age-related ovarian changes and reduced reproductive performance are detectable by 28 weeks).

Table 1. *Birth data from XO mice*

	Total animals			Animals at weaning			
Mating type	Pairs	Litters	At birth	*XY*	*XO*	*XX*	Dead
Ta/O♀X+/Y♂	50	177	624	188 (30·1%)	113 (18·1%)	212 (34·0%)	111 (17·8%)
+/O♀XTa/Y♂	48	248	940	339 (36·1%)	166 (17·7%)	314 (33·4%)	121 (12·9%)

Figures in parentheses represent percentages of total births.

Each female was superovulated using 2·5 i.u. pregnant mares serum (PMS) and 2·0 i.u. human chorionic gonadotrophin (HCG) given 48 h later. The oocytes were sampled 15 h after HCG injection at a time corresponding to metaphase II. Hansmann and El Nahass (1979) have previously shown that these hormone doses do not affect the incidence of non-disjunction during the first meiotic division in the mouse oocyte. Mice were killed by cervical dislocation, the oocytes being removed from the ampullae of the fallopian tubes and fixed by the method of Tarkowski (1966). The preparations were C-banded according to the method of Sumner (1972) and chromosome counts made.

RESULTS

(i) *Birth data*

Birth data were obtained from both Ta/O and +/O mothers (Table 1). Analysis showed that there was a significant difference between the two breeding groups ($\chi^2_3 = 9{\cdot}73$; $P < 0{\cdot}05$), this being due to a higher level of death among offspring from Ta/O mothers than +/O mothers between birth and weaning. Ta/O females are generally less robust than +/O females and appear to be less competent as mothers. The data at weaning, showing increased death of offspring from Ta/O mothers compared with +/O mothers, support the findings of Cattanach (1962), Morris (1968) and Deckers *et al.* (1981), although the difference between the two types of mother is lower in the present study than has been found by these other authors.

There was no significant difference in the distribution of *XY*: *XX*: *XO* offspring at weaning from the two types of *XO* mother. Neither was there any difference in the birth ratio of *XX*: *XO* offspring between the two maternal genotypes, the proportion being 1·88:1 for Ta/O mothers and 1·89:1 for +/O mothers. Both ratios were lower than those found by earlier investigators (Table 2) indicating that comparatively more *XO* progeny were born to *XO* mothers in our stocks.

(ii) *Chromosome counts*

A total of 379 metaphase II (MII) counts were made from 82 *XO* females and 179 counts from 28 *XX* females. The results have been grouped in Tables 3–5 according to maternal age and genotype. Cells giving counts of less than 17 were few in number, tending to be from poor quality preparations: They were discounted as unreliable.

Table 2. *Ratios of XO to XX offspring at weaning*

	+/O Mothers		Ta/O Mothers	
Author	No. of ♀♀	Ratio Ta/O:Ta/+	No. of ♀♀	Ratio +/O:Ta/+
Cattanach (1962)	661	1:2·74	276	1:3·30
Morris (1968)	966	1:2·37	926	1:2·67
Russell (1976)	118	1:2·17	192	1:3·23
Deckers *et al.* (1981)	362	1:2·45	119	1:2·84
Brook (Present Study)	480	1:1·89	325	1:1·88
Léonard & Schröder (1968)	—	—	2029	1:1·97

Table 3. *Chromosome counts from MII preparations from Ta/+ mice.*

	Chromosome number n =					
	17	18	19	20	21	Total
Young (8–20 weeks)	1	4	13	87	0	105
Old (30–40 weeks)	1	9	11	51	0	72
Total	2	13	24	138	0	177

A break-down of the chromosome counts from the *XX* (Ta/+) females is given in Table 3. This shows a proportion (22·03 %) having counts below the expected $n = 20$ number. It is assumed that the vast majority of these hypomodal counts are attributable to artefactual loss of chromosomes during slide preparation.

Chromosome counts from the two genotypically different groups of *XO* mice (Ta/O and +/O) are given in Tables 4*a* and *b* respectively. These showed no significant differences ($\chi^2_{12} = 9{\cdot}94$; $P > 0{\cdot}5$) and the counts for the two genotypes were thus pooled (Table 5). From Table 5 it would appear, at first glance that segregation of the *X* chromosome, to egg or polar body, in *XO* females, is occurring entirely at random; equal numbers of $n = 19$ and $n = 20$ being recorded. From a consideration of the data obtained in *XX* females, showing a 22 % level of cell breakage and chromosome loss due to preparative technique, it is by no means justifiable, however, to reach such a straightforward conclusion. If artefactual loss of a single chromosome occurred, it would result in oocytes with 20 chromosomes being spuriously classified as having only 19 thus helping to inflate the $n = 19$ total. At the same time some oocytes with 19 chromosomes would be spuriously classified as having only 18. The net result would be to deplete the number of counts in the

20-chromosome category whilst leaving the number in the 19-chromosome category **approximately** the same. A correction factor is thus clearly necessary in order to **arrive at a true** figure for the ratio of nullo-*X* to *X*-bearing ova at ovulation. This **has been devised** in the following way, taking into account the possibility that each chromosome count has arisen by a two-step process involving firstly, the segregation

Table 4. *Chromosome counts from MII preparations from XO mice*

	Chromosome number n =					
(*a*) Ta/O	17	18	19	20	21	Total
Young (8–20 weeks)	1	13	29	39	0	82
Old (30–40 weeks)	1	11	46	44	1	103
Total	2	24	75	83	1	185
	Chromosome number n =					
(*b*) +/O	17	18	19	20	21	Total
Young (8–20 weeks)	3	11	42	39	0	95
Old (30–40 weeks)	3	11	44	39	2	99
Total	6	22	86	78	2	194

Table 5. *Chromosome counts from MII preparations from Ta/O and +/O mice combined*

	Chromosome number n =					
	17	18	19	20	21	Total
Young (8–20 weeks)	4	24	71	78	0	177
Old (30–40 weeks)	4	22	90	83	3	202
Total	8	46	161	161	3	379

of chromosomes at anaphase I and secondly, possible breakage and loss of a chromosome (or chromosomes) by technical artefact. If it is assumed (1) that all those cells with less than 20 chromosomes in the control group (Ta/+) have arisen through breakage, and (2) that the probability of oocyte breakage is the same in both *XO* and *XX* mice, then, it is possible to estimate the proportion of all spreads from *XO* mice with 19 or less chromosomes which have arisen through breakage as follows:

If p_0 represents the proportion of unbroken cells in the control group (Table 3), p_1 the proportion losing 1 chromsome and $1-p_0-p_1$ the proportion losing more than 1 chromosome, then the following values can be assigned to each group:

$$p_0 = 0{\cdot}7797,$$

$$p_1 = 0{\cdot}1356,$$

$$1-p_0-p_1 = 0{\cdot}0847,$$

$$1-p_0 = 0{\cdot}2203.$$

For the *XO* oocyte spreads, the number found in the 20-chromosome group ($n = 20$) is made up of the actual number ovulated with 20 chromosomes (prior to breakage) multiplied by the proportion of unbroken spreads. Similarly the number of counts in the 19-chromosome ($n = 19$) group comprises the number of non-broken 19-chromosome-bearing spreads plus the number ovulated with 20

Table 6. *Segregation ratios found by various authors, before and after correction*

	Original findings			Corrected findings		
Author	20	≦ 19	% X-bearing	20	≦ 19	% X-bearing
Evans and Ford (unpublished)	61	59	50·8	—	—	—
Kaufman (1972)	65	40	61·9	76	29	72·4
Luthardt (1976)	52	37	58·4	61	28	68·3
Brook (present study)	164	215	43·3	205	171	54·6

chromosomes which have subsequently lost 1 chromosome. The number with 18 (or less) chromosomes ($n = 18$) is made up of the number ovulated with 20 chromosomes which subsequently lose more than one chromosome plus the number ovulated with 19 losing one or more chromosome subsequently.

This can be expressed algebraically as shown below:

$$n20 = Mp_0$$

$$n19 = Mp_1 + (N - M)p_0$$

$$n18 = M(1 - p_0 - p_1) + (N - M)(1 - p_0)$$

Where N = total number of oocyte preparations scored, and M = the number in the $n = 20$ group prior to breakage.

These equations can be used to estimate M, most conveniently by the modified minimum chi-square method (Kendall and Stuart, 1961).

For the *XO* females the segregation ratio is 205:171 for oocytes with counts of 20 and 19 chromosomes respectively (Table 6). This 1·2:1 ratio does not differ significantly from the 1:1 ratio expected if random segregation is occurring ($\chi_1^2 = 3{\cdot}07 : 0{\cdot}1 > P > 0{\cdot}05$).

To simplify the calculation, the effect of non-disjunction was ignored, as there were only three disomic eggs with counts of $n = 21$. The effect of chromosome gain on the segregation ratio would be in the opposite direction to that of chromosome loss, thus slightly decreasing the 1·2:1 ratio, taking it even closer to a 1:1 ratio.

Table 6 also shows a comparison with data obtained by Kaufman (1972) and Luthardt (1976). These authors did not introduce a correction factor into their results to allow for artefactual breakage. Their data have, however, been subjected to our correction model allowing for their own levels of control breakage. When this is done the data show an even greater bias towards non-random segregation than when the uncorrected figures are considered. The discrepancy between their data and those obtained in the present study will be dealt with in the Discussion.

It is not possible to adjust the data of Evans and Ford (unpublished) to allow for breakage as no control data were given by these authors.

For the stock of mice use in the present study, the ratio of *X*-bearing to nullo-*X* eggs at ovulation (1·2:1) differs from that found at weaning, the ratio of *XX* to *XO* offspring at that time being 1·88:1. Assuming there to be an equal chance of

Table 7. *Genotype of offspring weaned from +/O mothers in terms of litter order*

Litter	No. of mothers	Total	Ta/O	Ta/+	+/Y	$\frac{\text{Ta/+ + +/Y}}{\text{Total}} \times 100$
1	48	186	39	71	76	79·03 ± 2·99
2	45	139	26	50	63	81·29 ± 3·33
3	39	123	25	43	55	79·67 ± 3·61
4	35	110	28	39	43	74·55 ± 3·60
5	28	98	14	44	40	85·71 ± 3·51
6	23	82	21	33	28	74·39 ± 4·84
7	14	42	5	20	17	88·10 ± 5·01

fertilization of *X*-bearing and nullo-*X* eggs, it would thus appear, from the altered ratios, that 36·2 % of *XO* mice die between fertilization and weaning. Cattanach (1962) has shown that *XO* offspring have as good a chance of survival between birth and weaning as do *XX* offspring, and it can thus be assumed that the 36·2 % death of *XO*'s occurs during gestation.

(iii) *XO segregation and maternal age*

In view of the claim made by Deckers *et al.* (1981), that a greater number of *XO* offspring are born to mothers of advanced age, the cytological data were considered, not only in relation to genotype, but also to maternal age. The data presented in Tables 3 and 4 show no significant differences however, either for Ta/O or +/O mothers, in distribution of chromosome counts in the young group compared with the old. Tables 7 and 8 moreover, show the numbers of offspring of each genotype weaned from +/O and Ta/O mothers respectively, in terms of litter order. χ^2 tests for heterogeneity, between the two sets of breeders showed no change in the relative proportions of offspring with litter order – so the two sets of data can be combined. Regression analysis on the combined data shows there to be no significant change in the proportion of progeny born to older mothers ($t = 0{\cdot}356$: $P > 0{\cdot}1$). This finding, together with the cytological evidence, gives no indication in our stock of a changing pattern of *X*-segregation with age of the mother. This constrasts with claims made by Deckers *et al.* (1981) for an increasing recovery of *XO* progeny with increasing maternal age. (see Discussion).

(iv) *Aneuploidy*

As can be seen from Table 5, three disomic eggs ($n = 21$) were found in the old age group of *XO* females compared with none in young *XO* or in control *XX* females (Table 3). These disomic eggs are assumed to have arisen by non-disjunction

in the X -bearing oocytes, and constitute 3/86 (3·5 %) of the total eggs assumed to be X-bearing. Their frequency was not significantly greater, however, than in the other two groups of female (young XO and control XX). If the assumption is made that a similar level of non-disjunction occurs among nullo-X eggs, (the hyperploid ($n = 20$) products however being hidden among the normal X-bearing

Table 8. *Genotype of offspring weaned from Ta/O mothers in terms of litter order*

Litter	No. of mothers	Total	+/O	Ta/+	Ta/Y	$\frac{\text{Ta/+ + +/Y}}{\text{Total}} \times 100$
1	52	186	32	62	92	82·80 ± 2·75
2	41	110	25	43	42	77·27 ± 4·01
3	34	95	21	39	35	77·89 ± 4·25
4	21	72	17	32	23	76·39 ± 4·71
5	15	45	7	20	18	84·44 ± 5·47
6	9	33	9	13	11	72·73 ± 7·73
7	4	13	2	5	6	84·62 ± 9·90

($n = 20$) totals), a projected figure of 7 out of 202 hyperploid counts for old XO mothers would be obtained. This enlarged figure is again not significantly different from the zero level of aneuploidy of young XO and control XX females. It is also expected that for each non-disjunctional event producing a disomic egg, there would be a comparable X-bearing nullisomic ($n = 19$) egg produced. These would be hidden in the naturally occurring nullo-X bearing total. Similarly, non-identifiable double nullisomics ($n = 18$) may be produced by non-disjunction in nullo-X oocytes but these could not be distinguished from oocytes which had lost chromosomes through breakage. If the overall level of aneuploidy were thus derived by doubling again, there would then be 14/202 or a 7 % frequency for the aged females and this would be statistically significant ($P < 0·05$). The assumption is made in the above calculation that for every non-disjunctional event, producing a disomic egg, a corresponding event would produce a nullisomic. This, of course, is the conventional view of aneuploidy production by non-disjunction. Recent data of Maudlin & Fraser (1978) indicate, however, that the trisomy might arise in ageing female mice without equivalent monosomy. How this could come about is not stated, but if it were to be true, these calculations would not, of course, be valid.

DISCUSSION

In view of contradictions in the literature concerning the XO mouse, the present sudy was set up in an attempt to answer three basic questions. Firstly, does the segregation of the X-chromosome, during the first meiotic division of the oocyte, occur entirely at random? Secondly, if the X-chromosome is preferentially incorporated into either egg or polar body, does this change with maternal age? Thirdly, does the XO mouse constitute a good model for the pre-menopausal human female in terms of maternally age-related aneuploidy?

The ratio of *XX* to *XO* offspring at weaning (1·88:1) in the present study is considerably lower than the ratios observed by others (Cattanach, 1962; Morris, 1968; Russell, 1976). Similarly, the ratio of ovulated *X*-bearing to nullo-*X* eggs (1·18:1) is lower than has been found in previous cytological studies. In fact, unlike the studies of Kaufman (1972) and Luthardt (1976), the corrected figures in the present study are consistent with a 1:1 segregation ratio, in agreement with Evans and Ford (pers. comm). The large difference between the results of this study, and those of Kaufman (1972) and Luthardt (1976), cannot be easily reconciled. When both previous sets of data are corrected for breakage however, (see Table 6) the proportion of *X*-bearing gametes becomes so high that, to be reconciled with the birth data from our own and other studies, it would be necessary to postulate *preferential survival* of *XO*'s during gestation. This clearly is not the case. The present study indicates a 36·2 % loss of *XO* progeny during gestation, and others have shown that there is excess death for *XO* litters during early gestation, as compared with *XX*'s (Morris, 1968 and Russell, 1976). The early loss of OY embryos accounts for part of this but loss of a considerable proportion of *XO*'s prior to day 12 post-conception also seems to occur (Russell, 1976; Luthardt, 1976). As pointed out by Russell (1976), the further from randomness one postulates the segregation of the *X* chromosome to be, the lower need be the prenatal loss of *XO* embryos. To reconcile his findings, Morris (1968) concluded that there was preferential segregation of the *X*-bearing set of chromosomes into the gamete *and* death of some *XO*'s during the early stages of gestation. However, Evans and Ford (pers. comm) on re-analysing Morris's data, subsequently suggested that they could be interpretated as showing a 1:1 segregation ratio, and even an increased production of nullo-*X*, as compared to *X*-bearing gametes.

It would appear from these contradictory results that the cytological studies are unsatisfactory because of the problem of breakage and chromosome loss. Obviously it would be ideal if it were possible to identify the *X*-chromosome in the oocyte and then eggs could be simply scored as *X*-bearing or nullo-*X*. Nevertheless, it seems unlikely that the different results obtained by various authors can be explained on the basis of differing amounts of breakage encountered in each different study. One possibility is that there is a drive mechanism, which is responsible for the excess production of *X*-bearing gametes but which varies in strength from one stock to another. Genetic background may be important. Thus, in the present study, there may be little, if any, preferential loss of the chromosome set lacking an *X* to the polar body, whereas in others – such as those used by Kaufman (1972) and Luthardt (1976) the drive mechanism may be stronger. It would, however, seem unlikely that the amount of death of *XO* progeny during gestation should differ significantly in other stocks from the 36·2 % found in the present study.

The second point which arises out of this study concerns the question of whether preferential segregation of the *X* to the egg changes with maternal age. Since the data in the present study give no indication of any such change, they are at variance with those of Deckers *et al.* (1981). Both the birth data and the MII counts found in the present study show no reduction in the transmission of *X*-bearing

gametes with age in *XO* mothers. Regression analysis on *X*-segregation data in successive litters of Deckers *et al.* (1981), however, showed a significant negative slope, indicating change with maternal age. Similar treatment of our data gave no such significant result, with the slope in fact being slightly positive. As a χ^2 test for heterogeneity proved negative, regression analysis of the combined data was performed producing a non-significant – even though slightly negative, slope. This would suggest that the two sets of data are homogenous but the anomalous result of Deckers *et al.* (1981) is due to their small sample size.

Finally, on the question of aneuploidy in ageing *XO* mice, and because of the similarity to the human female, in that fertility ends through depletion of oocytes, Lyon & Hawker (1973) suggested that *XO* mice may pass through a period of irregular oestrous cycles towards the end of their reproductive life, during which time hormonal imbalance may occur thus leading to aneuploidy. Consequently, *XO* mice could provide a useful model for the situation in human premenopausal females, where non-disjunction occurs with a high frequency leading to the birth of abnormal children. It is not known whether *XO* mice pass through a period of irregular cyclicity towards the end of their reproductive life although work now in progress, in this laboratory, will, hopefully, show this to be the case. Studies in other strains of mice that, before cycling ceases, a period of irregular cyclicity occurs (Thung *et al.* 1956; Thung, 1961; Brook and Gosden, unpublished data). Although an increase in disomic oocytes was observed with increasing age in the *XO* mice used in the present study, this alone was not found to be statistically significant. When *X*-bearing nullisomics and non-disjunction in nullo-*X* eggs were considered, this figure did, however, become significant: The usefulness of the *XO* mouse as an appropriate model for human aneuploidy and the maternal age effect is, however, questionable in view of these complications arising out of the estimation of the true aneuploidy frequency.

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REFERENCES

Cattanach, B. M. (1962). *XO* mice. *Genetical Research.* **3**, 487–490.

Deckers, J. F. M., Van de Kroon, P. H. W. & Douglas, L. T. H. (1981). Some characteristics of the *XO* mouse (*Mus musculus* L) II. Reproduction: fertility and gametic segregation. *Genetica* **57**, 2–11.

Hansmann, I. & El Nahass, E. (1979). Incidence of non-disjunction in mouse oocytes. *Cytogenetics & Cell Genetics* **24**, 115–121.

Kaufman, M. H. (1972). Non-random segregation during mammalian oogenesis. *Nature* **238**, 465–466.

Kendall, M. G. & Stuart, A. (1961). *The Advanced Theory of Statistics*, vol. 2, pp. 92–95. London: Charles Griffin.

Luthardt, F. W. (1976). Cytogenetic analysis of oocytes and early preimplantation embryos from *XO* mice. *Developmental Biology* **54**, 73–81.

Lyon, M. F. & Hawker, S. G. (1973). Reproductive lifespan in irradiated and unirradiated chromosomally *XO* mice. *Genetical Research;* **21**, 185–194.

MAUDLIN, I., & FRASER, L. R. (1978). Maternal age and the incidence of aneuploidy of first-cleavage mouse embryos. *Journal of Reproduction and Fertility* **54**, 423–426.

MORRIS, T. (1968). The *XO* and *OY* chromosome constitutions in the mouse. *Genetical Research* **12**, 125–137.

RUSSELL, L. B. (1976). Numerical sex-chromosome anomalies in mammals: Their spontaneous occurrence and use in mutogenesis studies. In *Chemical Mutagens*, vol. 4 (Ed. A. Hollaender), pp. 55–91. New York: Plenum Publishing.

SUMNER, A. T. (1972). A simple technique for demonstrating centromeric heterochromatin. *Experimental Cell Research* **75**, 304–306.

TARKOWSKI, A. K. (1966). An air-drying method for chromosome preparations from mouse eggs. *Cytogenetics* **5**, 394–400.

THUNG, P. J. (1961). Ageing changes in the ovary. In *Structural Aspects of Ageing* (ed. G. H. Bourne), pp. 109–142, London: Pitman Medical Publishing.

THUNG, P. J., BOOT, L. M. & MUHLBECK, O. (1956). Senile changes in the oestrous cycle and in ovarian structure in some inbred strains of mice. *Acta Endocrinologica* **23**, 8–32.