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Aneuploidy

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Aneuploidy

CONTENTS

SUMMARY

Aneuploidy plays a significant role in adverse human health conditions including birth defects, pregnancy wastage and cancer.

Although there is clear evidence of chemically induced aneuploidy in experimental systems to date there are insufficient data to determine with certainty if chemically-induced aneuploidy contributes to human disease. However, since there is no reason to assume that chemically induced aneuploidy will not occur in human beings, it is prudent to address the aneugenic potential of chemicals in the safety assessment process.

A wide range of methods has been described for the detection of chemically induced aneuploidy including subcellular systems, tests with fungi, plants and Drosophila as well as *in vitro* mammalian systems and *in vivo* mammalian somatic and germ cell assays. However none of these methods are sufficiently validated or widely used for routine screening.

Underlying the efforts to develop aneuploidy specific assays is the presumption that current genetic toxicology tests do not detect chemicals that have aneuploidy-inducing potential. To address this we have critically evaluated data from standard genetic toxicology assays for 16 known or suspected aneugens.
The conclusions from the review are:

- At present there are only nine chemicals that can be classified as definitive aneugens as determined by positive results in *in vivo* rodent assays.

- As expected, the majority of definitive and suspected aneugens are negative in the bacterial mutation assay.

- The majority of definitive aneugens evaluated induced polyploidy *in vitro*. With few exceptions, they also induced structural chromosome aberrations *in vitro*.

- All of the definitive aneugens that have been sufficiently tested induced micronuclei in rodent bone marrow cells *in vivo*. A number of these chemicals also induced structural chromosome aberrations *in vivo*.

- There is no evidence for a unique germ cell aneugen, that is a chemical that induces aneuploidy in germ cells and not in somatic cells.

Based on these conclusions the following recommendations are made:

1. for screening purposes, a standard genotoxicity test battery (including an *in vitro* cytogenetic assay with an assessment of polyploidy) should be performed;

2. in the absence of polyploidy induction *in vitro* no further evaluation of aneuploidy-inducing potential is needed;

3. if polyploidy is observed *in vitro* follow-up testing to investigate further the aneuploidy-inducing potential should be conducted.

   Such follow-up testing will generally start with the conduct of a standard *in vivo* somatic cell micronucleus assay:

   - if the *in vivo* somatic cell micronucleus assay is negative no further testing of aneuploidy-inducing potential is needed.

   - if the *in vivo* somatic cell micronucleus assay is positive further information on mechanisms of micronucleus induction can be obtained by using kinetochore/centromeric staining *in vitro* and/or *in vivo*.

   - an assessment of potential germ cell aneuploidy activity may then be considered.

4. aneuploidy induction which does not involve the direct interaction of a chemical or its metabolite with DNA is expected to have a threshold. This must be considered in the risk assessment of such chemicals.

1. BACKGROUND

Aneuploidy refers to a change in chromosome number from the normal diploid or haploid number for the species; changes to an exact multiple of the haploid number (eg 3n, 4n) is termed polyploidy. Aneuploidy may arise either spontaneously or by chemical induction of chromosome loss or gain during cell division in germ and somatic cells. Addition of one chromosome leads to trisomy, loss of one results in monosomy. All trisomies and most monosomies are believed to be generated by nondisjunction, that is the failure of sister chromatids in mitosis or of paired chromosomes in meiosis to migrate to opposite poles at cell division. Monosomies may also result from chromosome lagging during cell division and their subsequent loss.

Aneuploidy in germ cells is a significant contributor to genetic disease in man (e.g. Down's syndrome). Furthermore, approximately 30% of all spontaneous abortions and approximately 6% of all infant deaths result from aneuploidy (Dellarco *et al*, 1986). Aneuploidy in somatic cells is associated with the development of several cancers (Fearon and Vogelstein, 1990; Cavenee *et al*, 1991).

Currently there is no convincing evidence for a chemical inducing adverse effects in man due to its aneugenic potential. Several chemicals are known to induce aneuploidy *in vitro* or in animals *in vivo*. Chemicals which induce aneuploidy are known as aneugens (aneuploidogens). Examples of *in vitro* and/or *in vivo* aneugens include such diverse chemicals as colchicine, vinblastine, benomyl, chloral hydrate, diethylstilboestrol, griseofulvin, hydroquinone and thiabendazole.

Many diverse methods exist for the detection of aneuploidy, including subcellular systems, plant and fungal assays, *in vitro* mammalian assays and assays in somatic and germ cells of whole animals. To date none of the tests available for the detection of aneugens have been sufficiently validated for routine use. Furthermore, the understanding of underlying mechanisms of chemically-induced aneuploidy and the relevance of aneugens to man is limited. Therefore there is no regulatory requirement for assays specifically for the detection of aneuploidy to date. As a result of the 4th Environmental Research and Development Programme of the EEC, Parry and Sors (1993) proposed a preliminary recommendation for a screening strategy for aneugenic chemicals. It was acknowledged that this strategy was based on limited data and on assays which are not fully validated. We therefore re-evaluated existing data and methods and recommend approaches that allow the detection of aneugens and evaluate their relevance.

The purpose of this report is to:

- describe the role of aneuploidy in human disease with special consideration of chemically-induced aneuploidy,
- evaluate methods for the detection of aneugens,
- recommend a set of procedures for testing chemicals for their aneugenic potential and,

- consider hazard identification and recommend a suitable process for risk characterisation of aneugens.

2. ROLE OF ANEUPLOIDY IN DISEASE

This section focuses on the role of aneuploidy in various diseases in man and animals. The role of aneugenic chemicals on these processes is addressed in Chapter 3.

2.1 HUMAN DATA

Aneuploidy is a frequent cause of mental retardation, congenital malformations and pregnancy wastage in human beings. In addition numerical chromosomal changes are also frequently observed in various stages of carcinogenesis in man.

2.1.1 Congenital and Reproductive Effects

Aneuploidy in Germ Cells and Zygotes

Studies of human germ cells demonstrate that chromosome abnormalities are common. As a result a large proportion of human conceptions are chromosomally abnormal. Current data indicate that approximately 1-2% of human sperm are hyperploid (Hassold *et al*, 1996). A compilation of 6 studies using fluorescence *in situ* hybridisation (FISH) staining of human sperm indicates a slightly higher incidence, where the frequency of disomy is approximately 2% (Coonen *et al*, 1991: Holmes and Martin, 1993: Martin *et al*, 1993; Hassold *et al*, 1996).The mean frequencies of hyperploid oocytes in human beings from 9 studies was 9.2% (range 1.5-17%) (reviewed in Martin *et al*, 1991). These oocyte values were obtained from *in vitro* fertilisation patients and therefore may over represent the true incidence.

Aneuploidy in Early Embryos and Spontaneous Abortions

Between 30% and 70% of spontaneous abortions are chromosomally abnormal (Table 1) and consequently aneuploidy is the major known cause of reproductive loss in man.

Trisomies for all chromosomes except chromosome 1 have been identified. However, there is considerable variation in frequency among individual trisomies with trisomy 16 accounting for 20 - 30% of all trisomies in abortions (Eiben *et al*, 1990). These differences in incidence are presumably attributable to variation among chromosomes in the frequency of nondisjunction, or differential selection against individual trisomies. Autosomal monosomies are not found in spontaneous abortions, presumably reflecting their early termination. However, monosomy of the X-chromosome, the constitution associated with Turner's syndrome in live-births, is quite common and accounts for approximately 5-10% of spontaneous abortions.

Table 1 - Frequency and Type of Chromosome Anomalies in Human Spontaneous Abortions

		% of Spontaneous Abortions							
Study	No. of Abortions Karyotyped	Triploid	Tetraploid	Trisomic	45,XO	Structural Anomalies	Other	Abnormal (total %)	Reference
Cultured amniotic fluid cells	941	4.0	1.3	16.3	7.2	1.2	0.5	30.5	Creasy *et al*, 1976
	402	5.2	1.7	32.6	10.5	2.7	0.8	53.5	Kajii *et al*, 1980
	967	5.4	2.0	18.0	5.0	0.9	1.0	32.3	Warburton *et al*, 1980
	1,498	12.2	3.8	33.0	9.4	2.3	0.7	61.5	Boué *et al*, 1985
	2,919	6.9	2.5	29.1	9.1	2.4	0.7	50.5	Hassold, 1986
Chorionic villi:	750	6.3	4.7	31.5	5.3	2.4	0.5	70.5	Eiben *et al*, 1990

Aneuploidy in New-born Children

Approximately 5% of still-born infants are chromosomally abnormal. Of live-births, approximately 0.25-0.3% have a numerical chromosome abnormality; of these, most have a significant physical and/or mental impairment. Consequently aneuploidy is a major cause of mental retardation. The most frequent abnormality is trisomy 21 (Down's syndrome) with an incidence of about 0.1% (Table 2).

Table 2 - Incidence of the Aneuploidies in Live-born Children

Type of Aneuploidy	Chromosome Affected	Frequency	Reference
Trisomy	13 (Patan's Syndrome)	1/12,000-24,000	Mehes and Bajnocky, 1990
	18 (Edwards' Syndrome)	1/6,000-9,000	Mehes and Bajnocky, 1990
	21 (Down's Syndrome)	1/700- 1,000	Serra and Neri, 1990 Stoll *et al*, 1990 Mehes and Bajnocky, 1990
	XXX	1//975	Bond and Chandley, 1983
	XXY (Klinefelter's Syndrome)	1/930	Bond and Chandley, 1983
	XYY	1/975	Bond and Chandley, 1983
Monosomy	X (Turner's Syndrome)	1/2,500-5,000	Mehes and Bajnocky, 1990

Similar incidences of aneuploidy at birth have been reported in various surveys of newborns from different geographical areas (Table 3).

About 95% of the Down's syndrome children receive their extra chromosome from their mother (Gaulden, 1992); the difference in frequency of nondisjunction between oocytes and spermatocytes is not well understood. The only factor definitely associated with trisomy 21 is increased maternal age.

If physiological changes are a significant factor in the induction of aneuploidy it can be inferred that endogenous factors may be of greater importance than chemicals in causing aneuploidy in human beings. The mechanism(s) for the maternal age effect are not clear at this time. One recent theory proposes that aneuploid oocytes arise from a number of events, beginning with hormonal imbalance, which results in a less than optimal microvasculature around the maturing and mature follicles (Gaulden, 1992). This reduces the volume of blood flow through the area leading to an oxygen deficit and concomitantly to an increase of carbon dioxide and anaerobic products in the follicle. This in turn causes a decrease in the intra cellular pH of the oocyte that diminishes the size of the spindle, with consequent displacement and nondisjunction of a chromosome.

2.1.2 Role of Aneuploidy in Human Carcinogenesis

Tumour Incidence in Individuals with Numerical Chromosome Abnormalities

An increased risk of tumour development has been observed with certain congenital aneuploidies. For example, individuals with Down's syndrome have a greater risk of leukaemia (Porter and Paul, 1974). Increased incidence of breast tumour and gonadoblastoma are associated with Klinefelter's syndrome (Simpson and Photopus, 1976) and tumours of neural crest origin are frequently encountered in Turner's syndrome (Wertelecki *et al*, 1970). Neuroblastoma and teratomas are observed in individuals with trisomy 13 (Dische and Gardner, 1978) and Wilm's tumour and neurogenic neoplasia with trisomy 18 (Karayalcin *et al*, 1981).

Non-random Chromosome Changes in Human Cancer

Boveri (1914) first discussed the importance of numerical chromosome imbalance in the development of malignant tumours. In fact, non-random numerical changes have been observed in a number of leukaemias and human cancers (Table 4). Most information on chromosome changes associated with tumour formation originates from lymphoma/leukaemia type diseases but due to improved techniques more data are now available on the chromosomal constitution of solid tumours. The majority of the investigations on solid tumours indicate complex karyotypes with remarkable instability which make it very difficult to identify (early) karyotypic changes (Sandberg, 1980; Sandberg and Turc-Carel, 1987; Hecht, 1988).

Table 3 - Incidence of Numerical Chromosome aberrations in some studies of newborn infants (after Young *et al*, 1984[1])

Location	Sex Chromosome Aneuploidies			Autosomal Trisomies					
	Males	**Females**	**Total**	**+13**	**+18**	**+21**	**Total**	**Structural Abnormalities**	**Sample size**
Edinburgh (UK)	0.29%	0.18%	0.26%	0.0%	0.02%	0.15%	0.16% (n=19)	0.23% (n=27)	11,680
Arhus (DK)	0.24%	0.20%	0.22%	0.01%	0.01%	0.14%	0.16% (n=18)	0.43% (n=48)	11,148
London (Canada)	0.47%	0.0%	0.24%	0.0%	0.0%	0.10%	0.10% (n=2)	0.14% (n=3)	2,081
Winnepeg (Canada)	0.17%	0.09%	0.13%	0.01%	0.02%	0.10%	0.13% (n=18)	0.19% (n=27)	13,939
Boston (US)	0.17%	–	–	0.0%	0.0%	0.14%	0.13% (n=19)	0.03% (n=41)	13,751
New Haven (US)	0.32%	0.18%	0.25%	0.02%	0.02%	0.07%	0.11% (n=5)	0.14% (n=6)	4,353
Moscow (Russia)	0.12%	0.12%	0.25%	0.0%	0.0%	0.16%	0.16% (n=4)	0.32% (n=8)	2,500
TOTAL	0.22%	0.15%	0.20% (n=118)	0.01%	0.01%	0.13%	0.14% (n=85)	0.27% (n=160)	59,452

[1] All percentages corrected to two decimal places

The specific role of aneuploidy in the early stages of the carcinogenesis process is seen by analysis of the genes involved in the process. One of the main classes of genes involved in carcinogenesis are tumour suppressor genes; both copies must be lost to remove the "suppression" effect. This has been shown to occur primarily through mutation of one copy of the normal gene followed by loss of the other gene through chromosomal nondisjunction. This has been described in detail for the Rb gene involved in retinoblastoma (Cavenee *et al*, 1991) and Wilm's tumour (Koufus *et al*, 1985). For other tumour suppressor genes such as the candidate tumour suppressor genes APC (Adenomatous Polyposis Coli), DCC (Deleted in Colon Carcinoma) and MCC (Mutated in Colorectal Cancer) found in colorectal carcinoma (Fearon and Vogelstein, 1990; Finlay, 1993) the second copy of the suppressor gene is lost by nondisjunction, recombination, deletion or gene mutation. These findings indicate that aneuploidy-induction is an early, primary event in some forms of cancer. Another class of recessive genes involved in carcinogenesis that may be activated by chromosome loss are the mutator genes and are thought to be mismatch repair genes.

Table 4 - Association between Aneuploidy and some Human Cancers.

Disorder	Frequently occurring aneuploidy	References
Acute lymphoblastic leukaemia (ALL)	+21	Oshimura *et al* , 1977
Acute non-lymphocytic leukaemia (ANL)	-X or -Y [accompanied by t(8;21 +8, +21)], -5, -7,	Sandberg, 1980; Mitelman and Levan, 1981; Mitelman *et al*, 1981
Chronic lymphocytic leukaemia (CLL)	+12	Morita *et al*, 1981; Han *et al*, 1983
Chronic myelocytic leukaemia (acute phase) (CML)	+8, +17, +19, +Ph[1]	Sandberg, 1980; Ishikara *et al*, 1983
Bladder Carcinoma	Trisomy 7, Monosomy 9	Sandberg and Turc-Carel, 1987
Colon Cancer	Monosomy 5,18	Fearon and Vogelstein , 1990
Large bowel Carcinoma	Trisomy 7, Trisomy 12	Sandberg and Turc-Carel, 1987
Malignant Melanoma	Trisomy 7 Monosomy 22	Sandberg and Turc-Carel, 1987
Uterine Carcinoma	numerical changes of 1	Sandberg and Turc-Carel, 1987

In contrast, activation of the third main class of genes involved in the carcinogenesis process, protooncogenes, is achieved by point mutation, small insertions or deletions and juxtaposition to other chromosome sequences but is unlikely to be induced to a significant extent by whole chromosome changes.

There is evidence that aneuploidy is also associated with later stages of the carcinogenesis process. An example of this is illustrated by chronic myeloid leukaemia. In this disease the initiation event is the translocation producing the Ph^1 chromosome and progression into the blastic phase appears to be correlated with the induction of specific hyperdiploidy changes (Sandberg, 1980).

2.2 ANIMAL DATA

2.2.1 Congenital and Reproductive Effects

Aneuploidy in Germ Cells

Since large surveys evaluating the incidence of aneuploidy in germ cells or at birth in laboratory and domestic animals are rarely performed, the contribution of aneuploidy to reproductive effects in animals other than man is not well documented. Sex chromosome aneuploidy has been reported in a variety of mammals and, as in man, is generally associated with infertility. Information on autosomal aneuploidies is more sparse. Some trisomies, derived from Robertsonian translocation in mice, do survive beyond birth but there are no reports of spontaneously arising autosomal trisomies.

In male mice the background frequency of hyperploid secondary spermatocytes is approximately 0-0.8% (Bond and Chandley, 1983). This is lower than the frequency of hyperploid human sperm of approximately 2% using FISH technology (Coonen *et al*, 1991, Holmes and Martin, 1993, Martin *et al*, 1993, Hassold *et al*, 1996) or 3-4% from karyotypes of sperm in the hamster egg *in vitro* fertilization assay (Martin *et al*, 1991).

Frequencies of hyperploid oocytes in mice of 0-6% have been reported although all recent studies have a frequency of less than 1% (reviewed in Mailhes and Marchetti, 1994). In contrast the mean frequencies of hyperploid oocytes in human beings from 9 studies was 9.2% (range 1.5-17%) (reviewed in Martin *et al*, 1991). Although these values were obtained from *in vitro* fertilization patients, and therefore may over represent the true incidence, they also indicate that rodent females may have a lower incidence of aneuploid oocytes than human beings.

Aneuploidy in Early Embryos and New Born

The incidence of aneuploidy in early embryos of mice have been reported to be 0.7% to 1.9% (summarised in Bond and Chandley, 1983). The frequency of aneuploidy at birth in mice is difficult to determine due to the possibility of cannibalism and the lack of comprehensive surveys. However the frequency appears to be significantly less than 0.1% (Bond and Chandley, 1983) which is lower than in man.

The dramatic increase of aneuploidy with age (expressed as a percentage of life-span) in human beings does not occur in mice, although some studies have shown a weak age related increase in certain strains (summarised in Bond and Chandley, 1983).

2.2.2 Role of Aneuploidy in Animal Carcinogenesis

There are two main animal models that have been used to study the role of specific structural and numerical chromosome changes in the multi-step process of carcinogenesis. These are the skin tumorigenesis model in mice and the liver hepatocarcinogenesis model in mice and rats.

Skin tumorigenesis in mouse

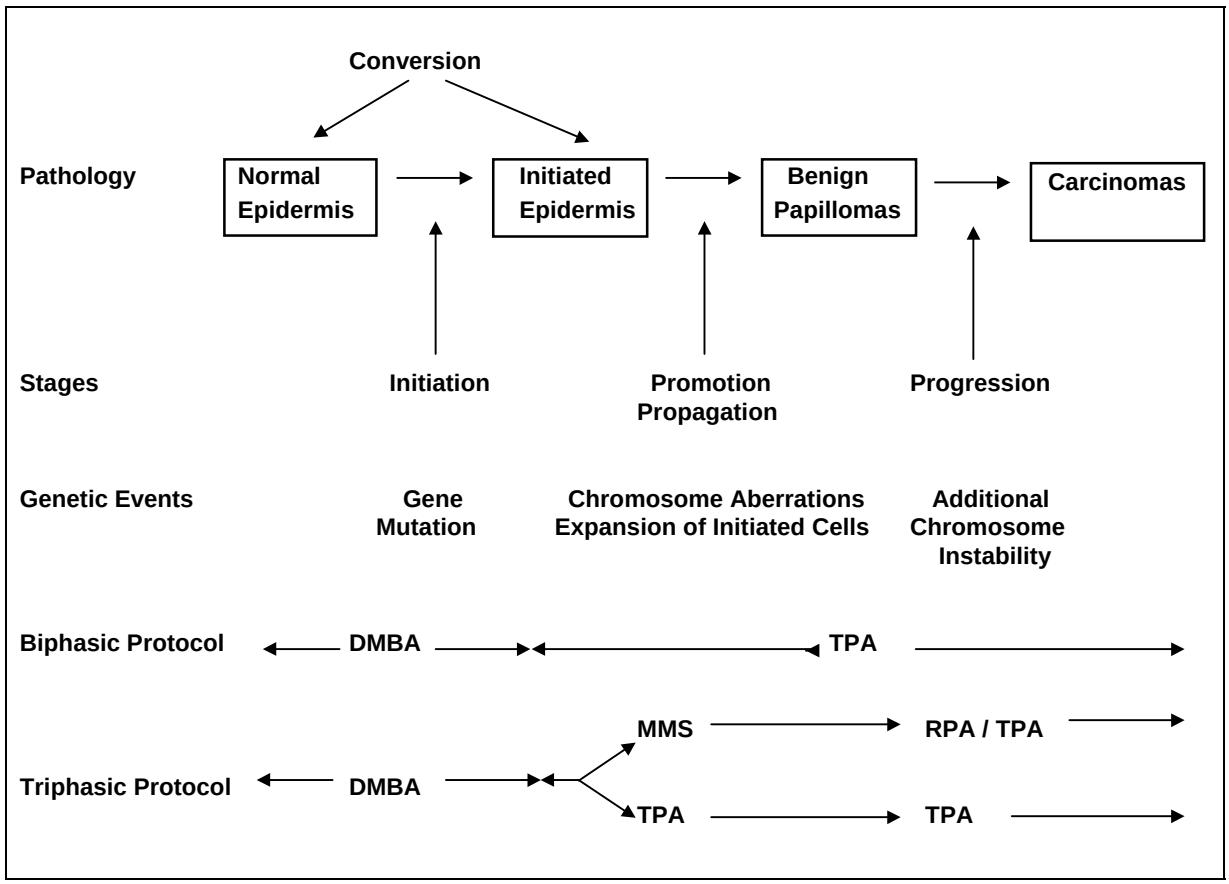
The carcinogenic process in skin is functionally subdivided into at least 3 distinct stages: initiation, promotion, and progression. The promotion phase can be further subdivided into two steps: conversion and propagation. Experimental models use either two or three treatments to model these different stages of carcinogenesis (Figure 1).

Briefly, the initiation process is irreversible and is thought to involve gene mutational events. In skin tumorigenesis models in Sencar mice, initiation is usually induced by dimethylbenz[a]anthracene (DMBA). In general, initiation is followed by promotion (Slaga *et al*, 1982; Marks *et al*, 1988). The promotion process involves the clonal expansion of initiated cells and can be induced by hyperplasia inducing agents such as the tumour promoter phorbol-12-myristate-13-acetate (TPA) or phorbol-12-retinoate-13-acetate (RPA) (Slaga *et al*, 1982). Finally, progression involves additional genotypic alterations that result in the conversion of benign lesions to malignancy.

Recently, chromosomal rearrangements were reported in mouse skin treated with cancer inducing protocols (DMBA-TPA; DMBA-methylmethanesulphonate-RPA) prior to formation of papillomas (Haesen *et al*, 1993). This demonstrates the occurrence of chromosomal changes early in the promotion step.

The first indication for a role of aneuploidy in skin tumorigenesis in Sencar mice came from Conti *et al* (1986) who observed that early papillomas induced by treatment with DMBA followed by TPA, contained aneuploid (hyperploid) cells and that their number increased with the duration of the treatment. The percentage of aneuploid metaphases in late papillomas reached the level of aneuploidy found in squamous cell carcinomas. Later on, the same group (Aldaz *et al*, 1988) induced papillomas in athymic mice by grafting immortalised keratinocytes (obtained by transfection with v-Hras). They showed that transfection was sufficient to achieve benign lesions but that malignant conversion is dependent on aneuploidisation.

Figure 1 - Cellular Events in Induction of Experimental Carcinogenesis and Experimental Protocols used to Study Skin Carcinogenesis



Aneuploidy observed during the carcinogenic process does not appear to occur at random. Aldaz *et al* (1989) reported a non-random trisomy of chromosomes 6 and 7 in pre-malignant papillomas in mouse skin. The southern blot hybridisation experiments of Bianchi *et al* (1990) further showed that trisomy of chromosome 7 is present in 85% of the squamous cell carcinomas induced by the DMBA-TPA protocol in Sencar mice. Mutations induced (by DMBA), in the Ha-ras oncogene, located in the mouse on chromosome 7, and further over-expression of the mutated oncogene product by trisomy, may lead to malignant transformation. This was confirmed by the finding that a significant association exists between the over expression of the mutated Ha-ras product and the malignancy of the tumours (Buchmann *et al*, 1991). Additionally, loss of heterozygosity was also observed for locus il-3 on chromosome 11, which shows homologies with the human chromosome 17 where the tumour suppressor gene p53 is localised (Burns *et al*, 1991). The involvement of the p53 gene in mouse tumorigenesis was confirmed by Ruggeri *et al* (1991) who detected alterations of the gene in tumours induced by the DMBA-TPA protocol. They suggested that p53 might be associated with late events in tumour progression.

It is not clear whether aneuploidy in skin tumour is a direct or indirect consequence of chemical treatment.

Rat Liver Carcinogenesis

The experimental protocols for studying hepatocarcinogenesis in rats are similar to that described for skin tumorigenesis in mice. Chromosome breakage, aneuploidy and other mutational events occur during the preneoplastic stages of hepatocarcinogenesis (Van Goethem *et al*, 1993, 1996). Additionally, it has been shown by a number of investigators that early neoplastic lesions in the liver involve specific changes in chromosome 1 (Kirsch-Volders *et al*, 1987; Holecek *et al*, 1989; Herens *et al*, 1992). In the rat, the Ha-ras oncogene is localised on the long arm of chromosome 1 and the c-myc oncogene on chromosome 7. The specific alterations occurring in gamma glutamyl transpeptidase (γ-GT) positive foci were mapped by Sargent *et al* (1992) to 1q33 to 1q43 close to the Ha-ras gene (q41-42). These data indicate that structural and/or numerical chromosomal changes are necessary to express the recessive mutation introduced by the initiation step. The observation that foci with diploid hepatocytes are more prone to malignant transformation than tetraploid ones confirm these findings (Deleener *et al*, 1987; Kirsch-Volders *et al*, 1987; Haesen *et al*, 1988; Castelain *et al*, 1989; Sargent *et al*, 1989; for review: Schwarze *et al*, 1991).

Hepatocarcinogenesis in Other Rodents

In metaphases of preneoplastic mouse liver nodules induced by a single injection of diethylnitrosamine DEN, trisomies/polysomies were observed for chromosome 11 and even more frequently for chromosome 19 (Danielsen *et al*, 1991); however, to date no oncogenes have been mapped on the latter chromosome. As far as the Ha-ras gene is concerned, a point mutation at codon 61 is frequently observed in the mouse liver tumours induced by DEN and none in phenobarbitone-induced tumours (Rumsby *et al*, 1991) although no aneuploidy for chromosome 7 was reported.

In the early stages of liver carcinogenesis in Chinese hamsters preferential involvement of chromosome 6 was observed and the derived cell lines were shown to be aneuploid (Swindell and Ocjey, 1983).

Other animal models of carcinogenesis

Several other animal models have been described, e.g. mammary carcinomas, thymic lymphomas, neuroectodermal tumours, gastro-intestinal tumours, renal mesenchymal tumours and oral carcinogenesis. Aneuploidy was shown to be an early marker of pre-malignancy in the rat model of oral carcinogenesis (Crane *et al*, 1989). Mutational activation of oncogenes, DNA-adduct formation and micronucleus induction have been reported in the other systems, but few specific studies on aneuploidy-induction are available (for review, Guerrero and Pellicer, 1987).

2.3 EVALUATION

There is sufficient evidence to indicate the relevance of aneuploidy for human health. Certain types of aneuploidy have been well characterised in man. Aneuploidy results in spontaneous abortions and still births. Of those aneuploid infants that survive, Down's Syndrome (trisomy 21) is the most common. Overall the effect of aneuploidy in terms of reproductive loss and birth defects in man is significant. Rodent studies indicate that aneuploidy is less common in the germ cells of rats and mice compared to that of man.

There is evidence for the relevance of aneuploidy in tumourigenesis in animals. In some cases aneuploidy appears to be a critical event in the carcinogenesis process, e.g. the loss of chromosomes leading to expression of tumour suppressor genes. In other cases the role of aneuploidy in the process is less clear as it is not known whether it is a cause or consequence of other genetic changes occurring in the process such as mutation of cell cycle control genes.

Technical difficulties in identifying and karyotyping preneoplastic and neoplastic cells have limited the amount of available data on solid tumours. The recent development of interphase cytogenetics with gene probing by fluorescence *in situ* hybridisation (FISH) will improve this situation. The development of chromosome specific probes for rat and mouse is in progress and should become commercially available in the near future. The use of animal models is a rapidly developing field of research in investigating the role of aneuploidy in tumour initiation and progression. Further work is required to investigate whether aneuploidy arises as a result of conversion or progression and conversely whether known aneugens are able to induce promotion and progression.

3. EVIDENCE THAT CHEMICALS CAN INDUCE ANEUPLOIDY

3.1 HUMAN DATA

3.1.1 Exposures Associated with Germ Cell Effects

To date, effects of chemical exposure on the human germ cell line have been limited to population studies on spontaneous abortions or congenital abnormalities. Such studies are often complicated by difficulties in accounting for confounding factors (single defined exposures have rarely been studied), availability of appropriate controls and low statistical power due to small sample sizes. Recent advances in the use of FISH in human sperm allow more powerful determinations of the incidence of aneuploidy in sperm from normal fertile men which can be used to study the effects of chemical exposures. However, such approaches cannot be used in the female due to the lack of availability of oocytes from normal females. This deficiency is severely limiting the development of the understanding of the process as most aneuploidy in the human conceptus arises in the female germ line.

Environmental Exposure

One factor indicating that environment does not have a major effect on aneuploidy is the similar incidence of aneuploidy found at birth in various surveys of newborns in different geographical areas (Table 3 in Chapter 2).

A variety of exposures have been investigated for their influence on the incidence of aneuploidy in germ cells. Some of these are reviewed below.

Drugs

Watanabe (1979) described the association of the use of a variety of medications, including antipyretics, analgesics and antibiotics with a number of abnormalities. A statistically significant high risk for trisomy, triploidy and tetraploidy was reported to be related to exposure during the perifertilisation period, that is within three weeks of the last menstrual period. Both triploid and tetraploid conceptions arise at the time of fertilisation and could be associated with exposure in the perifertilisation period. In contrast, trisomy generally originates from errors at maternal meiosis I. Consequently the precipitating event can occur *in utero* or during the resting phase which continues until ovulation and therefore perifertilisation exposure may induce the trisomy.

Oral contraceptives

The association of oral contraceptive use with triploidy among spontaneous abortions was reported by Butcher and Page (1981) but not by others (Lauritsen, 1975; Alberman *et al*, 1976; Baird and Wilcox, 1985). Although it has been suggested that exposure in the perifertilisation period may be related to trisomy 21 births (Harlap and Barras, 1984), the majority of studies of spontaneous abortions and in live-births show no such association.

Radiation

Studies on the effects of radiation on the incidence of numerical chromosome aberrations have indicated the key parameters that need to be evaluated either diagnostically or therapeutically. Trisomy was examined following radiation exposure of the Hiroshima, Nagasaki and Chernobyl populations. Comparison between studies is difficult because of the different methods of exposure assessment. There is no consistent evidence for a positive association between radiation and trisomy (ICPEMC, 1986) nor any suggestion that differences between studies are associated with variations in dose. The absence of effects on trisomy incidence in the Hiroshima and Nagasaki populations may have been biased by the fact that high-dose radiation might increase intra-uterine death (Wald *et al*, 1970). The evidence for a possible association of trisomy 21 and the radioactive fall out following the Chernobyl accident is not clear. Two studies in Germany (Sperling *et al*, 1987, 1991) and one in Scotland (Ramsey *et al*, 1991) reported an increased incidence of trisomy (combined prenatal and postnatal diagnosis). In Sweden, Finland, Hungary and France no clustering of Down's syndrome was seen (Källen, 1988; Czeizel, 1989; Stoll *et al*, 1990; Harjulehto-Mervaala *et al*, 1992). Also a study by EUROCAT based on 22 registers from 12 European countries did not reveal significant variations in the incidence of Down's syndrome (De Walls *et al*, 1990).

Increased aneuploidy in peripheral lymphocytes has been recorded in two studies following accidental ionising radiation (Natarajan *et al*, 1991, 1994).

Smoking

The relationship between smoking and several reproductive outcomes including trisomy has been described. Kline *et al* (1983) suggested that cigarette smoking either before or at the time of the last menstrual period is associated with trisomy in spontaneous abortions and this association varies with maternal age. The study concluded that trisomic abortion is positively associated with smoking in women over the age of 30 years.

The observations in spontaneous abortions described above are in contrast with those in the new-born. In the collaborative perinatal project reported by Kline *et al* (1983) the risk of Down's syndrome was lower

among women who smoked during pregnancy. Similarly, Cross and Hook (1984) reported that smoking within one year of conception was less common among mothers of Down's syndrome offspring than among mothers of both normal and other malformed offspring.

The apparent adverse effects of smoking in women under 30 years of age could be explained if smoking had an effect on the chance of intra-uterine death of trisomic concepts or on the processes that influence the likelihood of trisomy formation. The findings from older women on the incidence of trisomy in spontaneous abortions are in conflict: a protective effect of smoking in newborn but not in abortions. The mechanism of increased intra-uterine loss could explain the data in newborn but leaves a question mark concerning the data for spontaneous abortions.

Spermicides

An association of spermicide use with tetraploidy and hyperploidy was reported (Strobino *et al*, 1985). However the finding was based on small numbers: 4 of the 21 abortuses, (19%) showed such errors while only 3.9% was expected. Subsequent studies present a consistent picture of no association between spermicide use and trisomy (Jick *et al*, 1981; Shapiro *et al*, 1982; Huggins *et al*, 1982; Polednak *et al*, 1982; Mills *et al*, 1982; Bracken and Vita, 1983; Cordero and Layde, 1983; Strobino *et al*, 1985.)

Trichlorfon

Recently a cluster of congenital abnormalities has been reported from Hungary (Czeizel *et al*, 1993). Apart from the high incidence of several teratogenic effects (11 of 15 live births were affected by congenital abnormalities), four cases of trisomy 21 (two being homozygous twins) and an increased incidence of twins indicated an effect on the fidelity of oocyte meiosis and ovulation. The origin of the aneugenic effect in two cases was indicated to be due to failure of the second maternal meiotic division (this is indicative for a xenobiotic influence, as most 'spontaneous' errors occur at maternal meiosis I). Possible causative factors such as familial inherited disorders, consanguinity or exposure to known teratogenic agents could be excluded. The most likely source of exposure appeared to be fish treated with Flibol (40 % Trichlorfon) for ectoparasites. It was estimated that the trichlorfon concentration in the fish could have been as high as 100 mg/kg. All mothers who gave birth to children with congenital abnormalities had eaten contaminated fish. The non-specific effects in the children (Down's syndrome, twins, multifactorial abnormalities) argues against a single mechanism. The possibility that trichlorfon is responsible for the cases of trisomy is being followed-up (Parry, personal communication).

Assorted occupational exposures

There have been a few epidemiological studies of paternal occupation and Down's syndrome. A study of almost 1,600 cases of Down's syndrome detected at birth and reported to the malformation monitoring

programme of England and Wales for the period 1974-79 found increased proportional malformation ratios for glass and ceramic workers, labourers, clerical workers, armed forces, postmen and teachers (OPCS monitor, 1982). The results of this study should be viewed with caution because no external comparison group was used for the analysis and there was no adjustment for maternal age. Also the findings were not consistent over the two time periods, 1974-76 and 1977-79).

A case-control study of 128 Down's syndrome cases and 128 matched controls (all born in Baltimore during the period 1945-68) did not find an association with seven broad paternal occupational groups (professionals, other professionals, managers, clerical and sales, craftsmen, repairmen and foremen, operatives, service workers and labourers) (Cohen *et al*, 1977). An earlier observation by these authors of an association with father's military service was confirmed.

Another study (103 case-control pairs from upstate New York) found an excess of Down's syndrome cases where father's occupation was recorded as soldier (Polednak and Janerich, 1983). A similar study failed to find an increased risk for Down's syndrome (212 cases) for either veteran fathers or Vietnam veteran fathers (Erickson *et al*, 1984).

A case-control study of paternal exposure as a risk factor for Down's syndrome was conducted in British Columbia (Olshan *et al*, 1989). From the Health Surveillance Registry, 1,008 cases of live-born Down's syndrome were identified for the period of 1952-73. Two controls were matched with each case using the birth files from British Columbia and paternal occupation obtained from the birth's notice. Elevated maternal age-adjusted relative risks of Down's syndrome were found for fathers employed as janitors, mechanics, farm managers/workers, material moving equipment operators, sheet metal workers, iron workers, metal workers, saw mill workers and food processors. However, there are several limitations to this study in that the cases studied were live-born and if a substance only acts on foetal death the association would have been missed, and a large number of statistical comparisons were made and some significant findings may have been due to chance. Another drawback with this study relates to possible confounding effects of other factors such as maternal occupation, other parental exposure and household exposure.

3.1.2 Exposure Associated with Somatic Cells Effects (Carcinogenicity)

There are three established human carcinogens, asbestos, cadmium and cadmium compounds, and diethylstilboestrol, which have been shown to induce aneuploidy in *in vitro* or *in vivo* experimental systems. It is important to note however, that the role of aneuploidy induction in the mechanism of carcinogenesis of these chemicals is unknown. Asbestos is classified as a group 1 human carcinogen inducing mesotheliomas and bronchogenic carcinomas (IARC, 1987). Cadmium and cadmium compounds are also classifed as group 1 human carcinogens (IARC 1993), however individuals exposed to these compounds have increased frequencies of both structural and numerical chromosome

aberrations. Diethylstilboestrol, is classified as an IARC Group 1 human carcinogen, inducing adenocarcinomas in the vagina and cervix (IARC, 1987).

For other known or suspect aneugens, there is less evidence regarding human carcinogenicity and only a few studies where aneuploidy was measured in somatic cells of exposed individuals. The results for some of these are described below.

Benomyl/Carbendazim

The International Programme on Chemical Safety (IPCS, 1993a,b) concluded that it is unlikely that benomyl or its active metabolite carbendazim is carcinogenic for human beings. Examination of lymphocytes from peripheral blood obtained from agricultural sprayers exposed to benomyl showed no differences in structural chromosome aberrations, but numerical aberrrations were higher than in controls. It was noted in the IPCS review (1993a) that the sprayers had a higher level of numerical aberrations than controls even before benomyl exposure.

Diazepam

Diazepam is listed by IARC as having inadequate evidence of carcinogenicity to man (IARC, 1987; IARC, 1996) and a recent study of benzodiazepines, including diazepam, indicates that they do not influence the risk of cancer in man (Rosenberg *et al*, 1995). Van Bao *et al* (1992) reported the cytogenetic effects in peripheral lymphocytes of diazepam in 25 individuals who attempted suicide with the drug. There was no increase in chromosomal aberrations (chromatid-type, chromosome-type and micronuclei) in cultured cells from these individuals. However, an increase in the frequency of hypoploidy was seen immediately after poisoning. This effect disappeared quickly as lymphocytes taken 3 and 30 days later did not show aneuploidy. Unless aneuploidic lymphocytes have a shorter half-life than normal lymphocytes this observation casts doubt on the validity of the study as the time period for the depletion of the aneuploid cells is not consistent with the estimated life span of peripheral lymphocytes.

Griseofulvin

Griseofulvin is regarded as possibly carcinogenic to humans, IARC classification 2B, based on sufficient evidence of carcinogenicity in animals (see Section 3.2.2) (IARC, 1987; reviewed by De Carli and Larizza, 1988).

Hydroquinone

The IPCS (1994) concluded that there was inadequate epidemiological and experimental data to determine the carcinogenicity of hydroquinone. Recently, however, Whysner *et al* (1995) concluded that

occupational exposure to hydroquinone in photographic processing does not pose a carcinogenic risk to man.

Heavy Metals

There is inadequate evidence for carcinogenicity of lead and inorganic lead compounds in human beings (IPCS, 1995). Studies of chromosome changes in human lymphocytes from exposed individuals are conflicting. A group of 9 workers occupationally exposed to inorganic lead was studied for chromosomal defects by Verschaeve *et al* (1979b). There was a statistically significant increase in aneuploidy but according to the authors the main target for inorganic lead, in contrast to mercury compounds, is not the spindle apparatus. This was supported by the finding of an increased number of chromatid-type aberrations in the cultured lymphocytes of the workers and also by the absence of an altered chromosome-distribution pattern as compared to unexposed controls.

There is inadequate evidence of carcinogenicity in human beings for mercury and mercury compounds (IARC, 1993). Contradictory findings have been reported on the frequency of aneuploid cells in cultured lymphocytes of workers occupationally exposed to mercury. Verschaeve *et al* (1976) reported a statistically significant increase in lymphocytes with numerical aberrations from 28 persons exposed to various mercury compounds. In 1979 the same investigators (Verschaeve *et al*, 1979a) could not demonstrate chromosomal effects in 28 workers exposed to metallic mercury from a different workplace. In both studies the background frequency of aneuploid cells was high.

3.2 ANIMAL DATA

3.2.1 Effects on Germ Cells

There are many methods available to identify whether chemicals can induce aneuploidy in germ cells of experimental animals (see Section 5 and Appendix A). The literature published between 1970 and 1985 on male and female germ cell assays has been critically reviewed in the US-EPA Gene-Tox Program (Allen *et al*, 1986 and Mailhes *et al*, 1986, respectively). Only 2 out of 46 chemicals evaluated were concluded to be definitive male germ cell aneugens (42 chemicals were considered inconclusive and 2 chemicals were negative). Eight chemicals out of 16 evaluated were concluded female germ cell aneugens (5 chemicals were negative and 3 chemicals were considered inconclusive). Following these earlier reviews, the results on chemically-induced aneuploidy in male and female germ cells reported in the period 1985 - 1987 were compiled by Pacchierotti (1988). Since then several more chemicals have been tested in male germ cells, predominantly as part of the EEC programme (Adler, 1993) and in female cells (Mailhes and Marchetti, 1994). Overall, there is good evidence that chemicals are able to induce aneuploidy in germ cells of mice. The evidence for transmission of chemically-induced aneuploidy

to embryos or the F_1 progeny is much more limited to date. Although there are some methods for assessing this endpoint (cf. Appendix A) only 2 chemicals, methylmethane sulphonate (Russell, 1985) and vincristine (Danford and Parry, 1986) have been found to induce aneuploidy in the F_1 progeny.

3.2.2 Carcinogenicity Studies using Aneugens

A number of known or suspected aneugens listed below have been tested for the induction of cancer in rats and mice and for a few, there is evidence of carcinogenicity in human beings (see Section 3.1.1). It is important to note that the causative role of aneuploidy in the carcinogenic process for these chemicals has not been established.

Asbestos is carcinogenic in a number of animal species, by several routes of exposure (IARC, 1987). Asbestos induces mesotheliomas and lung carcinomas (IARC, 1987). Benomyl did not induce any carcinogenic effect in rats but increased the incidence of hepatocellular carcinomas in CD-1 mice (for review see IPCS, 1993b). Likewise, carbendazim induced a significant increase in hepatocellular carcinomas in mice strains with high spontaneous hepatic tumours (CD-1 and SPF), but not in NMRKf mice with a low incidence of spontaneous tumours, or in rats (for review see IPCS, 1993a). Cadmium and cadmium compounds are listed by IARC as having sufficient evidence of carcinogenicity in animals (IARC, 1987). Chloral hydrate is a hepatocarcinogen in male B6C3F1 mice exposed via drinking water (Daniel *et al*, 1992) and is a metabolite of the rodent carcinogens and potential human carcinogens trichloroethylene and tetrachloroethylene (IARC, 1987, Keller and Heck, 1988). Colchicine did not show any initiating activity in a 2-stage mouse skin initiation/promotion study (Roe and Salaman, 1955). Colcemid was not carcinogenic in rats when administered intravenously for 52 weeks (Schmahl and Osswald, 1970).

In animals, there are conflicting data in the literature on the carcinogenicity of benzodiazepines, like diazepam. However, a recent study of benzodiazepines (Section 3.1.1) suggests that they do not influence the risk of cancer in man (Rosenberg *et al*, 1995). Diethylstilboestrol is classified by IARC as a sufficient rodent carcinogen based on induction of tumours principally in oestrogen-responsive tissues in a number of species (IARC, 1987). Griseofulvin is classified as a rodent carcinogen inducing hepatocellular neoplasms in mice and thyroid tumours in rats but not carcinogenic in hamsters (IARC 1987; reviewed by De Carli and Larizza, 1988). There is evidence that hydroquinone induces renal adenomas in male rats (Whysner *et al*, 1995). Pyrimethamine is classified by IARC as having limited evidence of carcinogenicity in animals and non-classifiable for humans (IARC, 1987). Thiabendazole was not carcinogenic when administered in the diet to rats (Fujii *et al*, 1991).

3.2.3 *In vitro* Transformation

In vitro cell transformation models of the multistage carcinogenesis process have been used to evaluate the transforming potential of aneugens and to study the role of aneuploidy in specific stages of the neoplastic process. Commonly-used *in vitro* cell transformation assays are limited to rodent cells since human cells do not readily undergo transformation *in vitro*; the reasons for this difference are currently under investigation.

Many known or suspect aneugens including asbestos, benzene, colcemid, diethylstilboestrol, diethylstilboestrol dipropionate, 17-oestradiol, vincristine sulphate, vinblastine sulphate, benomyl, cadmium chloride, chloral hydrate, griseofulvin, pyrimethamine, diazepam, and econazole nitrate have been shown to induce transformation in primary Syrian hamster embryo (SHE) cells (Barrett *et al*, 1985; Oshimura and Barrett, 1986; Gibson *et al*, 1995). A number of aneugens have also been shown to induce transformation in a cell line derived from Syrian hamster dermal cells (Porter *et al*, 1992; Parry *et al*, 1993). The endpoint typically measured in these studies is abnormal cell growth (either morphological transformation or immortalisation) and therefore is not a direct assessment of tumorigenicity. Despite this, induction of morphological transformation and immortalisation is highly correlated with *in vivo* tumorigenicity as evidenced by studies where transformed cells are injected into animals and tumours are generated (e.g. diethylstiboestrol, Ozawa *et al*, 1989).

Importantly, non-random chromosome changes including aneuploidy have been reported to be involved in specific stages in the process of transformation of SHE cells. Perhaps the most convincing evidence of the role of chemically-induced aneuploidy in the transformation process is the observation of a non-random gain of chromosome 11 observed in 6 out of 8 asbestos-induced cell lines at early passages (Oshimura *et al*, 1986) and in 4 out of 4 diethylstilboestrol-induced cell lines (Ozawa *et al*, 1989). The authors concluded that aneuploidy is associated with an early event of carcinogenesis in some cases, as well as, a later change in others. The role of non-random aneuploidies (trisomy and monosomy) in transformation of SHE cells, particularly at the immortalisation step, an early step in the process, has been reported for a number of chemical and physical agents (Barrett and Fletcher, 1985; Suzuki *et al*, 1989, 1991; Watanabe *et al*, 1990, Afshari *et al*, 1993).

Overall, aneuploidy of specific chromosomes (trisomies and monosomies), as well as partial aneuploidy due to deletion or addition of specific chromosome regions (Endo *et al*, 1994), appears to be a critical step particularly in the immortalisation process during transformation. The genetic instability and non-random chromosome changes observed in *in vitro* cell transformation systems agree with observations made *in vivo* in animals and human beings (Sections 2.1 and 2.2).

3.3 EVALUATION

There are sufficient data demonstrating that aneugens cause adverse effects in somatic and germ cells of experimental animals. In contrast, at this time, there are insufficent data to determine with any certainty the degree to which chemically-induced aneuploidy contributes to adverse human health effects. This conclusion is based on the observation that there is no geographical variation in aneuploidy rates and is supported by the absence of epidemiological evidence (although somewhat limited) of adverse human effects from exposure to aneugens and the well established maternal age effect seen with Down's Syndrome.

4. MECHANISMS INVOLVED IN THE INDUCTION OF ANEUPLOIDY

4.1 CELLULAR AND MOLECULAR EFFECTS

There is no single molecular mechanism for the induction of aneuploidy by chemicals. Aneuploidy occurs when replicated chromosomes fail to accurately segregate between the two progeny cells. The end result is the production of cells with an abnormal number of chromosomes. Since chromosomal segregation is dependent on many different organelles and controlled by a number of metabolic pathways, aneuploidy can arise from several different mechanisms during the cell cycle (mostly during the M-phase). These include damage to essential elements for chromosome function, reduction of chromosomal pairing, induction of chromosome interchanges, effects on chromosome condensation, persistence of the nucleolus in mitosis or meiosis, increased chromosome stickiness, damage to centrioles or kinetochores, impairment of chromosome alignment, alterations in ion concentrations in mitosis (e.g. Ca^{+}), damage to the nuclear membrane and physical disruption of chromosome segregation. Such alterations can lead to reduction in chromosome pairing, induction of chromosome interchanges, impairment of chromosome alignment, physical disruption of chromosome segregation and chromosome lagging (Oshimura and Barrett, 1986).

Of primary importance in the segregation of chromosomes is the spindle apparatus. Its major functional components are microtubules, centrioles and the centromeric or kinetochore regions of the chromosomes. Other components important in spindle function include the sheath of membrane surrounding the spindle, cAMP-dependent kinases, and calcium ions (Dellarco *et al*, 1985a).

Probably the best known and studied aneugens are those that affect tubulin polymerisation and microtubular formation. Specific binding sites on tubulin have been identified (Bryan, 1972) for guanosine dinucleotides, vinca-derived alkaloids (vincristine, vinblastine), and colchicine (also colcemid and podophyllotoxin). Aneugen binding sites and subsequent effects suggest differences between lower and mammalian eukaryotes. For example, carbendazim binds much more effectively to fungal than to mammalian tubulin (Ireland *et al*, 1979; Kilmartin 1981). However, this is not true for all benzamidazoles (Kilmartin, 1981; Albertini, 1993). In contrast, colchicine has a much higher affinity for mammalian tubulin (De Brabander *et al*, 1976; Williams, 1980).

The association of chemicals with tubulin can lead to several events, most notably the inhibition of polymerisation and microtubule formation (colchicine, carbendazim). Vinblastine, as indicated above, binds to a site distinctly different from colchicine and causes the crystallisation of tubulin (Wilson and Morse, 1978). Taxol binds to tubulin but unlike colchicine it causes mitotic arrest and presumably aneuploidy (Satya-Prakash *et al*, 1986) by enhancing the polymerisation of tubulin into microtubules (Schiff *et al*, 1979).

Aneuploidy induction also arises from the failure of normal bipolar spindle formation and alteration in its regulated stabilisation or destabilisation (Hoffman *et al*, 1986). Normally, spindle fibres from opposite poles differ from one another in polarity and can interact with one another. At anaphase, there seems to be a sliding of the microtubules with respect to one another while the fibres elongate through the addition of tubulin subunits. As the interacting fibres elongate and thereby increase the distance between the poles, the microtubules attached to the kinetochores lose subunits and shorten. Chloral hydrate blocks pole-to-pole microtubule formation thus inhibiting spindle elongation and ultimately chromosome-to-pole movement. Shortened and disorientated microtubules have been reported in cells exposed to chloral hydrate . The aneugenic fungicide griseofulvin disrupts the movement of chromosomes because of its ability to block combination of microtubules with associated proteins essential for the sliding function of microtubules and thus the movement of chromosomes during cell division (reviewed by Oshimura and Barrett, 1986).

Other compounds that may cause aneuploidy by altering critical proteins in mitosis and meiosis are *p*-fluorophenylalanine (*p*-FPA) and mercury-containing chemicals. *p*-FPA, is believed to exert its action as an analog of phenylalanine effecting changes in the protein tertiary structure and function (reviewed by Oshimura and Barrett, 1986). Organic mercury compounds such as thimerosal bind to sulfydryl groups in protein and thereby interfere with microtubule assembly (reviewed by De Flora *et al*, 1994).

The centrioles are also important to the function and integrity of the spindle apparatus and as such are targets for certain aneugens. Diazepam, the active ingredient of Valium®, inhibits the separation of centrioles at prometaphase (Anderson *et al*, 1981; Hsu *et al*, 1983; Lafi *et al*, 1987). It has also been speculated that some general anaesthetics are capable of inducing aneuploidy by damaging the centrioles (reviewed by Oshimura and Barrett, 1986).

Stages in the mitotic and meiotic cell cycles which may be targets for chemical aneugens are summarised in Tables 5 and 6. It should be noted that in the mitotic cell cycle the events listed may not be discrete steps but represent continous processes.

Table 5 - Events During the Mitotic Cell Cycle Which May Be Targets for Chemical Aneugens (adapted from Dellarco *et al*, 1985b)

Stage	Events
G_1	Centriole maturation, Assembly of cytoplasmic microtubule complex (CMTC), Calmodulin synthesis
S	DNA synthesis-kinetochore duplication, Centriole (centrosome) replication, synthesis of tubulin messenger RNA
G_2	Centriole (centrosome) separation, Disassembly of CMTC, Tubulin biosynthesis
Prophase	Disassembly of CMTC completed, Chromosome coiling and condensation, Poleward centriole separation completed, Astral and central spindle assembly, Nuclear membrane breakdown
Prometaphase	Kinetochore activation and association with microtubule (MT), Chromosome movement to metaphase plate, Spindle elongation
Metaphase	Alignment of chromosomes at spindle mid-zone, Centromere separation
Anaphase A	Kinetochore-to-pole MT disassembly, Chromosome separation
Anaphase B	Chromosome separation, MT-MT interaction, Interpolar MT growth
Telophase	Kinetochore inactivation, Nuclear membrane reformation, Chromosome uncoiling and decondensation, Mitotic apparatus breakdown
Cytokinesis	Cleavage furrow initiation, Furrow position, Midbody formation, Reformation of CMTC

Other causes of aneuploidy include damage of the kinetochores (mitomycin C), increased chromosome stickiness or damage (intercalating agents, e.g. actinomycin D), improper alignment of chromosomes onto the metaphase plate (nitrous oxide), and damage to the cellular and nuclear membranes (ethanol, sodium deoxycholate). The nuclear membrane, in particular, plays a significant role in the control and regulation of spindle function since it acts as the attachment point for chromosomes and provides protection for the genetic material. In addition, Oshimura and Barrett (1986) suggest that the nuclear membrane sequesters calcium ions, which in turn regulate the transition from metaphase to anaphase.

Table 6 - Events During the Meiotic Cell Cycle Which May Be Targets for Chemical Aneugens

Stage	Oogenesis	Spermatogenesis
First division		
Prophase		
Leptotene	Genomic DNA replicated by DNA polymerase producing a tetraploid cell. Homologous sister chromatids joined at a centromere.	Primary spermatocytes formed after several mitotic divisions at puberty. First meiotic division takes 12-14 days and occurs in the testes. Spermatogenesis is an ongoing process with no "meiotic arrest".
Zygotene	Pairs of homologous sister chromatids join to form bivalents (4 chromatids)	
Early pachytene	Bivalents shorten, thicken and cross over.	
Late pachytene	Chromatids decoil or decondense	
Diplotene	Homologous pairs of chromatids begin to separate	
Metaphase I	Nuclear membrane dissolves. Bivalents line up on the meiotic spindle	
Anaphase I	Bivalents divide between 2 daughter cells	
Telophase I	Formation of secondary oocyte and first polar body, each with haploid set of chromosomes.	
Second Division		
Prophase II	(exists ?)	Secondary spermatocytes. Meiosis is completed in 24 days and results in 4 haploid spermatids
Metaphase II	(ovulation) Formation of a spindle. Meiosis is arrested until time of fertilisation	
Anaphase II	(fertilisation) Division of identical chromatids to form 2 daughter cells with a haploid genome	

There are other events in the cell cycle that are highly regulated and which if disturbed can lead to aneuploidy. The best known gene in this category is the cell cycle checkpoint gene, p53. There is considerable evidence that mutation in this gene results in chromosome instability both *in vitro* and in tumorigenesis in man. Thus Li-Fraumeni fibroblasts, derived from human beings who are heterozygous for this gene, are karyotypically unstable *in vitro* (Bischoff *et al*, 1990); mouse embryo fibroblasts, which have been manipulated to remove p53 activity, develop pronounced abnormalities of chromosome number in culture (Livingstone *et al*, 1992) and fibroblasts, derived from p53 null mice, had considerably greater genomic instability than those derived from normal or heterozygous mice (Harvey *et al*, 1993). In

man, mutation of the p53 gene has been shown to precede clonal divergence of aneuploidy cells in progression of colorectal carcinomas (Carder *et al*, 1993, 1995). Other genes, which interact with p53 and which, if mutated, could also lead to a loss of G1 checkpoint function, might also be expected to lead to genomic stability. Although there are several possible genes in this category (e.g. Meltzer, 1994) none of these have been investigated for a role in aneuploidy-induction as yet. Similarly, alteration of the G2 checkpoint occurs prior to immortalisation of cells in culture (Kaufman *et al*, 1995) and it is possible, although not yet investigated, that mutations in genes involved in this function e.g. cyclins or their associated cdk genes, could also alter genomic stability. There has also been a report of an association of 'near-diploid' aneuploidy in adenomas of the colon and specific transversions (but not all transversion or transitions) of codon 12 in k-ras-2 gene (Giaretti *et al*, 1995). However in this report aneuploidy was also seen in adenomas without mutations and the confounding factor of p53 status was not studied.

Another target which may be involved in aneuploidy is the nucleolus. It normally disappears prior to entry into metaphase. Persistence of the nucleolus, or its fractions, results in failure of synaptic chromosome pairs (bivalents) to separate leading to nondisjunction. Another example is the dependence of processes such as elongation of spindles in anaphase B on ATP which, if modified, may lead to segregation errors.

During meiosis, there are additional events not involving spindle function, including homologous chromosome pairing (synapsis) and recombination (crossing-over), during which chemical interference can induce aneuploidy (Dellarco *et al*, 1985b). Cyclophosphamide, a clastogen that also induces aneuploidy in germ cells, causes synaptic failure and fragmentation of the synaptosomal complex. Further, other meiotic aneuploidy-inducing errors include non-conjunction of homologs, nondisjunction of homologs, premature centromere division in meiosis I, failure of chromatids to separate in meiosis II, extra chromosomal replication, and chromosome loss (Hoffman *et al*, 1986).

4.2 MECHANISMS OF CHEMICALLY INDUCED POLYPLOIDY

In addition to the documented mechanisms for aneuploidy (e.g. effects on microtubule formation, centriole, kinetochore damage, etc.), other mechanisms may be responsible for polyploidy induction. Polyploidy may arise in cultured cells due to cell fusion. Inhibition of division of the cytoplasmic membrane may also give rise to polyploidy, e.g. cytochalasin B induced binucleated cells which produce polyploid metaphases (Aardema, unpublished data). Polyploidy may also arise as a consequence of endo-reduplication. In this case cells undergo two DNA-synthesis phases without cell division intervening. There is considerable evidence that compounds which block cells in G_2 (e.g. some topoisomerase inhibitors (Zucker *et al*, 1991), arsenic (Gurr *et al*, 1993), genotoxic agents such as mitomycin C (Takanari and Izutsu, 1983) and actinomycin C (Caligo and Rainaldi, 1987) can give rise to polyploidy. Some of these studies have used BrdU labelling to indicate that endoreduplicated cells arise predominantly from cells held in G_2 arrest which suggests inhibition of protein synthesis. This is

supported by the observation that the protein inhibitors actinomycin and puromycin cause G_2 arrest (Matusi *et al*, 1971; Tobey *et al,* 1966) and also induce endoreduplication (Pathak *et al*, 1975). However the situation is not clear as the protein synthesis inhibitor cycloheximide did not induce tetraploidy in Chinese Hamster Ovary (CHO) cells under conditions where arsenic effectively induced polyploidy (Gurr *et al*, 1993). Whether compounds which are able to induce gross failure of the cell division apparatus by inducing endoreduplication can also induce aneuploidy at lower doses has not been studied. However, the derivation of aneuploid cells from endoreduplicated Chinese Hamster Lung (CHL) cells induced by rotenone was demonstrated by Matsumoto and Ohta (1993). This compound is an extremely efficient inducer of endoreduplication and these authors demonstrated the formation of tripolar and tetrapolar spindles following removal of the rotenone-induced blockage and the subsequent formation of aneuploid and polyploid cells.

4.3 EVALUATION

There are fundamental differences between the meiotic and mitotic processes, e.g. the requirement for chromosome pairing, formation of chiasmata to allow recombination which is especially prolonged during the formation of oocytes. Theoretically these may give rise to qualitative differences in the response of germ and somatic cells to aneugens raising the possibility of the existence of unique germ cell aneugens.

5. METHODS FOR DETECTION OF ANEUGENS

5.1 GENERAL PRINCIPLES

There are many methods available for testing chemicals for aneugenic potential (see Appendix A). They include subcellular, submammalian, *in vitro* mammalian and *in vivo* somatic and germ cell assays and have been reviewed by Allen *et al*, 1986, Cimino *et al* (1986), Dellarco *et al* (1986), Galloway and Ivett (1986), Oshimura and Barrett (1986), Parry and Parry (1989), and Parry *et al* (1995a).

Since chromosomal segregation is dependent upon different subcellular components and controlled by a number of pathways, aneuploidy can arise by several mechanisms (Chapter 4) leading to either chromosome loss or gain. Ideally test methods should therefore be able to detect both events. Technical limitations in metaphase analysis make estimation of chromosome loss difficult therefore hyperploidy is the only accurate measurement that can be obtained. In contrast micronucleus induction methods will detect chromosome loss but not chromosome nondisjunction. Therefore, in principle, methods based on chromosome counting or micronuclei induction are useful for assessing aneugenic potential.

As induction of aneuploidy in both somatic and germ cells is of concern, and there are differences between the mitotic and meiotic processes, methods are required to assess aneugenic potential in both cell types.

5.2 EVALUATION

The advantages and disadvantages of the methods for assessing aneugenic potential of chemical substances are given in Tables 8a, 8b and 8c .

In vitro methods are useful for detecting potential aneugens and the most promising are those involving chromosome counting (directly or indirectly using fluorescent *in situ* hybridisation (FISH) techniques), polyploidy assessment, or identification of micronuclei using kinetochores/centromeres. These methods require further development and validation; they are not yet suitable for routine screening. Validation requirements are presented in Appendix A.

In vivo methods can be used to verify whether *in vitro* aneugens are capable of inducing aneuploidy in mammalian somatic cells and ultimately germ cells in the whole animal. In somatic cells micronucleus induction and chromosome counting are most relevant but are not fully validated for aneuploidy assessment.

Table 8a. - Summary of Test System Evaluation: Non-mammalian and Cell Free Systems

Assay	Advantages	Disadvantages
Plants	Easy to handle Fast Less expensive than mammalian systems	Does not reflect mammalian situation Not fully validated Used only by few laboratories
Fungi	Easy to handle and cultivate Established genetic base Fast Large database available Some assays allow detection of chromosome loss and nondisjunction	Fundamental differences in constitution of spindle apparatus between fungal and mammalian cells Limited data on the use of extrinsic mammalian metabolic activation Negative results with some potent mammalian aneugens
Drosophila	Organisms easy to cultivate Genetics well understood Tests easy to perform Metabolism similar to that of mammals	Comparative data with mammalian systems is limited New systems not validated Relevance to mammalian *in vivo* situation uncertain
Tubulin assembly	Easy to handle Mammalian target Fast	Does not reflect cellular complexity Extrinsic metabolic activation not yet used Not fully validated Compound absorption needs to be considered Data of different laboratories often not comparable

Table 8b. - Summary of test system evaluation: Mammalian *in vitro* assays.

Assay	Advantages	Disadvantages
Mitotic alterations	Provides mechanistic information	Indirect measurement Not applicable to all cell types
Micronuclei induction	Simple Speed of analysis Sample size/statistical power Potential for automation	No distinction between clastogen and aneugen possible Detects loss, not nondisjunction
Micronuclei induction + FISH	Powerful technique Ease of use Speed of analysis Sample size/statistical power	Influence by cellular phenomena Hybridisation artefacts No distinction between hyper- and polyploidy with single probe in interphase
Micronuclei induction + Cytochalasin B	Powerful technique Cell cycle control Sample size/statistical power Nondisjunction detectable in binucleated cells using FISH	No distinction of clastogen and aneugen possible Technically demanding
Chromosome counting	Direct evidence for chromosome gain and polyploidy	Technically demanding, time-consuming Low sensitivity Hypoploid cells $\rightarrow$ artefacts??

Table 8c. - Summary of test system evaluation: Mammalian *in vivo* assays.

Assay	Advantages	Disadvantages
***In vivo* somatic**		
Mitotic alterations	Provides mechanistic information	Indirect measurement Not applicable to all cell types
Micronucleus	Simple Speed of analysis Sample size/statistical power Potential for automation	No distinction of clastogen and aneugen possible Detects loss not nondisjunction
Micronucleus induction + FISH	Powerful technique Ease of use Speed of analysis Sample size/statistical power	Influence by cellular phenomena Hybridisation artefacts No distinction between hyper- and polyploidy with single probe in interphase
Chromosome counting	Direct evidence for chromosome loss/gain and polyploidy	Technically demanding, time-consuming (not polyploidy) Low sensitivity Hypoploid cells → artefacts??
***In vivo* germ**		
Analysis of secondary spermatocytes	Can analyse large numbers of cells? May be more sensitive than somatic cell assays Relevance of aneuploidy in germ cells Assess aneuploidy-induced at a sensitive stage	Technically demanding, time-consuming
Synaptonemal Complex Analysis	Investigation of mechanisms	Will only detect a few chemicals. Requires electron microscopy
Meiotic delay in males	Relatively fast pre-screen	Not yet validated
Meiotic delay in females	Relatively fast pre-screen	Requires further evaluation
Analysis of metphase II oocytes	Can expose at a sensitive stage Relevant to human aneuploidy-induction May be more sensitive than somatic assays	Technically demanding Low statistical power
Micronuclei in spermatids	Relatively fast Maybe able to assess chromosome loss	Low sensitivity? Not well developed or validated
Micronuclei in spermatids + FISH	Powerful technique Ease of use Speed of analysis Sample size/statistical power	Influence by cellular phenomena Hybridisation artefacts No distinction between hyper- and polyploidy with single probe in interphase
First Cleavage Zygotes	Simultaneous assessment of males and females Assess aneuploidy-induced at both MI and METAPHASE II	Requires mating Technically demanding

6. REGULATORY EVALUATION OF ANEUGENS

6.1 REVIEW OF COORDINATED ACTIVITIES RELATED TO ANEUPLOIDY

The initial focus of genetic toxicity testing in the early and mid 1970s concerned the detection of point mutations and structural chromosome aberrations. This was largely due to experience gained in understanding these endpoints from radiation experiments combined with the use of easy and inexpensive assays for measuring these endpoints (e.g. Ames *Salmonella A*ssay). The utility of this approach was demonstrated when it was shown that there was a good correlation between mutagens and rodent carcinogens (McCann *et al*, 1975). Concern for the potential of chemicals to induce aneuploidy grew in the late 1970s and has continued to the present. The basis for concern is the documented role of aneuploidy in adverse human health effects as reviewed in Sections 2.1 and 3.1.

The activities both by regulatory and non-regulatory groups related to aneuploidy reflects the continued interest in this area worldwide (for a historical perspective see Section 6.3 and Appendix B).

6.2 REGULATORY CONTROL OF ANEUGENS

6.2.1 Testing Guidelines

The need for an assessment of chemically-induced numerical chromosome changes is explicit or implicit in some current regulatory testing protocols and testing schemes, and has been incorporated into proposed regulatory guidelines. This assessment is either requested in the initial test battery for new chemicals or in supplementary assays for specific substances. A summary of those regulatory guidelines and testing schemes that address the assessment of numerical chromosome changes is given in Table 9. Guidelines that apply globally, like those of the Organization for Economic Co-operation and Development (OECD), for chemicals in general, and the International Conference on Harmonization (ICH) specifically for pharmaceuticals, and guidelines for individual countries are discussed in more detail below.

Table 9 - Overview Of Regulatory Guidelines and Testing Schemes For Assessing Numerical Chromosomes Changes

	Current	Future	Additional Comment
OECD	None	Report polyploidy and endoreduplication when seen. *In vivo* micronucleus test can detect whole chromosome loss.	
ICH	*In vivo* micronucleus tests have the potential to detect some aneuploidy-inducers; there may be aneuploidy-inducers that act preferentially during meiosis but there is no conclusive evidence for these chemicals	Report polyploidy and endoreduplication when seen. *In vivo* micronucleus test can detect whole chromosome loss.	
EEC	None	Harmonise with OECD and ICH	For new chemicals consider aneuploidy-induction or spindle inhibition at 100 or 1,000 t/y (500 or 5,000 t cum)
Japan	Routine assessment of polyploidy in *in vitro* cytogenetic assay	Harmonise with OECD and ICH	
Canada	*In vitro* chromosome assay should include some chromosome counts to gain information on potential aneuploidy-induction	Harmonise with OECD (and ICH ?)	
US-EPA	None	Harmonise with OECD	
US-FDA	None	Harmonise with OECD and ICH	

Organization for Economic Cooperation and Development (OECD)

The latest draft OECD guidelines for cytogenetic methods (OECD, 1996a,b) specifically state that polyploid and endoreduplicated cells should be recorded when seen. The guideline for the *in vitro* chromosome aberration test states that the assay was not designed to measure numerical aberrations, but that an increase in polyploidy may indicate that the test substance has potential to induce numerical aberrations. An increase in endoreduplicated cells may indicate that the chemical has potential to inhibit the mitotic process. The stated purpose of the *in vivo* micronucleus assay (OECD, 1996c) is also to identify chemicals that cause micronuclei as a result of lagging of chromosome fragments or whole chromosomes.

International Conference on Harmonisation (ICH)

The final draft of the International Conference on Harmonisation (ICH, 1995) document "Genotoxicity: Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals" provides guidance for the conduct and interpretation of genotoxicity tests for drugs and is intended to be applied in conjunction with existing individual country guidelines. As such, this document does not address each assay in detail, rather, only some aspects of specific tests.

In the guidance on *in vivo* tests, it is stated that the *in vivo* micronucleus test has the potential to detect some (maybe not all) aneuploidy-inducers due to the fact that micronuclei can result from whole chromosomes lagging during cell division. The potential utility of specific aneuploidy assays is mentioned, like the analysis of chromosomes in interphase nuclei using FISH. ICH concludes that negative results in *in vivo* somatic cell tests generally indicate the absence of germ cell effects and that there was no conclusive evidence for the existence of aneuploidy-inducers acting preferentially during meiosis.

Canada

In the recent guidelines from the Canadian Health Protection Branch (Canada, 1993) the role of numerical chromosome changes in somatic and heritable effects is discussed. Conventional tests to detect aneuploidy are the cytogenetic analysis of metaphase cells while the micronucleus assay can detect whole chromosome loss. This is confirmed with the use of antikinetochore or centromere-specific antibodies. In the current guidelines for conduct and reporting of results from genotoxicity assays it is recognised that existing protocols may not be optimal for measuring aneuploidy (Canada, 1991). In the *in vitro* cytogenetic assay, it is specifically stated that in order to gain information on the aneugenic potential, chromosome count distributions should be included (Canada, 1991, 1993).

Commission of the European Communities (EEC)

From a regulatory viewpoint, the EEC Guidelines on the Classification on the basis of specific effects on human health provides for the first time a definition of mutagenicity that specifically includes changes involving whole chromosomes. According to Annex VII of the same EEC Directive (EEC, 1993) an investigation of the potential for chemically-induced aneuploidy or spindle inhibition should be considered at 100 or 1,000 tonnes/annum (500 or 5,000 t cumulative) if there is potential for significant human exposure.

Japan

Aneuploidy is considered by the Japanese authorities to be an important endpoint. The standard *in vitro* cytogenetic protocol for the MHW (Ministry of Health and Welfare) and MITI (Ministry of International Trade and Industry) includes an assessment of polyploidy which is considered indicative for aneugenic potential (Ishidate, 1988a,b; J-MHW, 1990; Sofuni, 1993). There are no guidelines for the conduct of the *in vivo* chromosome aberration assay.

US Environmental Protection Agency (US-EPA)

Recently the US-EPA indicated that there are several endpoints of concern in cytogenetic tests including both structural and numerical chromosome aberrations (Dearfield *et al*, 1991). The US-EPA has indicated that substances which may induce numerical chromosomal aberrations will be evaluated on a case-by-case basis (Dearfield *et al*, 1991; Auletta *et al*, 1993).

US Food and Drug Administration (US-FDA)

There are no recommendations in the current guidelines for the regulation of food and drugs which include the assessment of numerical chromosome changes.

Draft guidelines have been published by the US-FDA (1993). The use of genetic toxicology tests for determining the ability of chemicals to cause a number of genotoxic effects including numerical chromosome changes is discussed. One of the recommended assays is the *in vivo* cytogenetic assay, including an assessment of both metaphase aberrations and micronucleus induction. In addition, non-genotoxic carcinogens are considered with the recommendation to handle such substances on a case-by-case basis. Since some aneugenic chemicals are considered "nongenotoxic" carcinogens (e.g. diethylstilboestrol), this is expected to have an impact on the testing of aneugens in general.

6.2.2 Classification Schemes

A number of international regulatory bodies have established guidelines for hazard classification of mutagens (hazard identification or qualitative risk assessment). The three main guidelines, the Classification on the Basis of a Specific Effects on Human Health in Annex VI of the EC Dangerous Substances Directive (EEC, 1993), Canada's Hazardous Products Act: Controlled Products Regulations (Canada, 1988), and US-EPA Guidelines for Mutagenicity Risk Assessment (US-EPA, 1986) are compared in Table 10, and detailed in Appendix C. Each of the procedures gives the highest level of significance to evidence obtained from epidemiological studies for the definitive association between chemical exposure and heritable mutations in man. Because of the difficulties in establishing this causal relationship, chemically-induced genetic damage in germ cells of experimental mammals is regarded as a strong indicator or (at least) equal hazard to man.

Table 10 - Comparison of the Classification Schemes for Mutagens as used by the European Community (EEC), Canada and the United States.

	EC[1]	Canada[1]	US[1]
Established human germ-cell mutagen	1	Class D, Division 2, Subdivision A 1(a)	1
Mammalian germ-cell mutagen	2a	1 (b) (i)	2
Positive in mammalian germ cells *in vivo* for chromosome aberrations	2b	1 (B) (i) or (ii)	3
Interacts with mammalian germ cell DNA or chromatin constituents	2b	1 (b) (ii)	4
Positive in somatic cell mutagenicity assays with evidence for chemical interaction with germ cells	2c	1 (b) (ii)	5, 6
Positive in *in vivo* somatic cell mutagenicity assays	3a	Class D Division 2 Subdivision B	
Positive in *in vivo* somatic cell DNA interaction assays	3b		
Operationally non-mutagen for human germ cell			7
Inadequate evidence			8

[1] For a description of the classification criteria and appropriate nomenclature see Appendix C.

The US-EPA Guidelines for Carcinogen Risk Assessment utilise somatic cell mutagenicity results only for the hazard classification of carcinogens. Evidence for chemically-induced effects in somatic cells *in vivo* is taken into account only for hazard categorisation in the Canadian and the EEC guidelines irrespective of the carcinogenicity data for hazard classification. The EEC and Canadian Guidelines give somatic cell mutagens lower hazard categories than germ cell mutagens (assuming negative results in the appropriate germ cell studies). Germ cell mutagens are classified, in the Canadian Guidelines under Division 2, Subdivision A, “Materials causing Other Toxic Effects, Very Toxic Materials”, whereas somatic cell mutagens are placed in Division 2, Subdivision B, “Materials causing Other Toxic Effects, Toxic

Material". In the EEC Guidelines, mammalian germ cell mutagens are given Category 2 and somatic mutagens Category 3.

Mutagen hazard categorisation is based primarily on studies which determine if a chemical causes, *in vivo* in either somatic or germ cells, gene mutations, structural chromosome aberrations (clastogenesis), numerical chromosome aberrations (aneuploidy), or DNA damage and repair. The criteria for categorisation are: the cellular target (germ or somatic), and the endpoint measured (gene mutations etc). The molecular mechanism by which genotoxicity is induced is currently not considered. Indeed, all mutagens (or active metabolites) are viewed as if they damage DNA directly, and accordingly without thresholds. As will be discussed subsequently (Chapter 8) it would be more appropriate to consider threshold approaches for non-DNA reactive genotoxins including aneugens in hazard and risk classification schemes.

6.3 HISTORICAL REVIEW OF REGULATORY APPROACHES TO ANEUGENS

Historically, there have been a few cases where regulatory action was taken on a chemical based solely on results from aneuploidy assays. Two relatively recent examples, benomyl and noscapine, are summarised below. A more detailed description of these and other aneugens is provided in Appendix D.

Benomyl, and its active metabolite carbendazim (methyl-2-benzimidazole carbamate) were first reported to induce aneuploidy in *Aspergillus nidulans* (Hastie, 1970) but were considered, at most, weak mutagens. The EPA risk assessment for benomyl was based on subsequent data showing rodent carcinogenicity and teratogenicity and it was concluded that the benefits of benomyl use exceeded the risks, which can be reduced by wearing protective equipment (US-EPA, 1982). The recent reviews by the International Programme on Chemical Safety on benomyl and carbendazim concluded that their toxicities were consistent with those of other spindle poisons. The mechanistic data on the selectivity of benomyl and carbendazim for fungal tubulin compared to mammalian tubulin supported the conclusion that toxic effects in man are unlikely (IPCS, 1993a,b).

The anti-tussive cough agent, noscapine is another recent example where regulatory action was taken based on induction of numerical chromosome changes. Noscapine induces polyploidy *in vitro* in mammalian cells but not *in vivo* (Ishidate, 1988a; Furukawa *et al*, 1989; Gatehouse *et al*, 1991; Mitchell *et al*, 1991; Tiveron *et al*, 1993). Due to genotoxicity concerns the UK Committee on Safety of Medicines recommended that all products containing noscapine be contraindicated in women of child-bearing potential.

6.4 EVALUATION

Regulatory concern for chemically-induced aneuploidy that began in the 1970s continues to the present.

The need for an assessment of chemically-induced numerical chromosome changes is explicit or implicit in current and/or draft regulatory guidelines either in the initial test battery or in supplementary assays for specific chemicals.

Chemicals that induce aneuploidy are classified by regulatory authorities using mutagen hazard classification schemes. These schemes do not take into account mechanisms of action or differences at the cellular level.

7. SCREENING FOR ANEUGENIC CHEMICALS

7.1 INTRODUCTION

Despite the increasing awareness of the consequences of numerical chromosome changes in human disease (Chapter 2) and the mechanisms involved in induction of numerical chromosome changes (Chapter 4) which have developed over the last 20 years, there are still a number of considerations regarding the routine testing for chemically-induced numerical chromosome changes.

Firstly, it must be determined if there is a need to routinely assess chemicals for the potential to induce numerical chromosome changes. As reviewed in Chapter 3, there are insufficient data to determine with any certainty the degree to which aneugenic chemicals contribute to adverse human health effects. Since there are sufficient data from animal studies to conclude that chemical-induced aneuploidy causes adverse effects (Section 3.2) and no reason to believe that effects in man will be dramatically different, it is concluded that it is prudent to address the aneugenic potential of chemicals in the overall safety assessment.

Secondly, if aneugenic chemicals have the potential to contribute to adverse human health effects, then the issue is how best to assess chemicals for aneuploidy-inducing activity. Currently, there are no validated short term tests that directly measure aneuploidy. There are, however, a number of efforts underway towards developing new methods to measure aneuploidy as well as development of methods that provide a "flag" or indicate the potential of a chemical to induce aneuploidy. Recently, Parry and Sors (1993) proposed a preliminary recommendation for a screening procedure that provides indicators of aneugenic potential. Their proposal consisted of the analysis of the induction of mitotic cell division aberrations in a mammalian fibroblast culture, the analysis of mitotic arrest and C-mitosis in rodent bone marrow, and the determination of meiotic delay in rodent spermatocytes. These procedures, as well as other new methods would require validation and regulatory acceptance prior to their routine use as discussed by Miller *et al* (1994).

Underlying these efforts to develop new methods for identifying aneugens is the presumption that current genotoxicity tests do not detect chemicals that have aneuploidy-inducing potential. A logical first approach is to test this assumption by determining whether aneugenic chemicals are detected in one or more of the endpoints measured in the battery of well validated, widely used and regulatory-accepted genotoxicity tests. In other words, are there pure aneugens or do chemicals that induce aneuploidy also induce mutations, chromosome aberrations, micronuclei, polyploidy, or other endpoints measured in current assays? If so, the existing assays (or modifications thereof) may be appropriate for identifying those chemicals. If not, new methods need to be developed and validated.

7.2 SCREENING STRATEGY FOR ASSESSING ANEUGENIC POTENTIAL

We have critically evaluated published and unpublished data from genotoxicity assays for known or suspected aneugenic chemicals including the set of chemicals studied in the EEC program (Table 11). The data and details of our evaluation process concerning the information relating to aneuploidy are described in Appendix E. The data relating to the commonly used standard genotoxicity tests reported in Table 11 are not referenced.

Several useful conclusions can be drawn from this database in Table 11:

- at present there are only a few chemicals that can be classified as definitive aneugens as defined by positive results *in vivo* in germ cells or somatic cells (chromosome counting or kinetochore positive micronuclei). Acrylamide, benomyl, carbendazim (the major metabolite of benomyl), chloral hydrate, colchicine/colcemid, diethylstilboestrol, griseofulvin, hydroquinone, and vinblastine/vincristine sulphate are considered definitive aneugens (shown in bold in Table 11);

- the majority of the chemicals including the definitive aneugens are negative in the Ames *Salmonella* mutation assay. This is not surprising since most known mechanisms of aneuploidy-induction do not involve direct interaction with DNA;

- the majority of the definitive aneugens that have been tested (except for hydroquinone which has conflicting results) induce polyploidy *in vitro*;

- all of the definitive aneugens sufficiently tested induce structural chromosome aberrations *in vitro* or *in vivo*;

- all of the definitive aneugens that have been sufficiently tested induce micronuclei or aneuploidy (as evidenced by chromosome counts) using bone marrow cells *in vivo*; a number of these chemicals also induce structural chromosome aberrations *in vivo*;

- in this database, there is no conclusive evidence for a "unique" germ cell aneugen, i.e. a chemical with positive results for aneuploidy-induction in germ cells but negative results for micronuclei or aneuploidy in somatic cells *in vivo*;

Table 11 - Genotoxicity of 16 Potential Aneugenic Chemicals

		Commonly Used Tests (based on standardised methods and existing literature surveys not referenced)					Other Tests for Aneuploidy (based on references given in Appendix E)			
Compound	**CAS Number**	**Ames**	**SCA *in vitro***	**Polyploidy *in vitro***	**SCA *in vivo***	**MNT *in vivo***	**Somatic *in vitro***	**Somatic *in vivo***	**Male Germ cells *in vivo***	**Oocytes *in vivo***
Acrylamide	79-06-1	-	+	ND	+	+	+	+	Inc	ND
Benomyl	17804-35-2	-	+	+(unpub)	-	+(K^+)	+	+	ND	+
Cadmium chloride	10108-64-2	+	+	Inc	+	Inc	Inc	Inc	Inc	Inc
Carbendazim	10605-21-7	-	ND	+(unpub)	-	+(K^+)	+(unpub)	+	ND	+
Chloral hydrate	302-17-0	+	ND	Inc	-	Inc	+	Inc	+	-
Colchicine	64-88-8	ND	+	+	+	+	+	+	+	+
Diazepam	439-14-5	-	-	Inc	-	-	+ (hypo)	-	Inc	-
Diethylstilboestrol	56-53-1	-	-	+	+	Inc	+	+	+	-
Econazole nitrate	24169-02-6	-	ND	-	-	Inc	-	Inc	Inc	-
Griseofulvin	126-07-08	-	+ (unpub)	+(unpub)	-	+(unpub)	+	+	ND	+
Hydroquinone	123-31-9	Inc	+	Inc	+	+	+	+	+	ND
Noscapine	128-62-1	-	-	+	ND	ND	+	-	ND	-
Pyrimethamine	58-14-0	-	+	+	ND	-	Inc	-	-	-
Thiabendazole	148-79-8	-	ND	+	ND	Inc	+	Inc	Inc	ND
Thimerosal	58-64-8	-	ND	+	-	Inc	Inc	-	-	ND
Vinblastine/Vincristine	865-21-41	+	Inc	+	+	+	+	+	+	+

SCA = structural chromosome aberration; MNT = micronucleus test; ND = not determined; + = positive in assay; - = negative in assay; Inc = inconclusive; $+K^+$ = Kinetochore positive; (unpub) = unpublished data.

- it appears that for general screening purposes, a standard genotoxicity test battery including an *in vitro* cytogenetic assay with an assessment of polyploidy/endoreduplication and/or an *in vivo* micronucleus test provides a useful initial assessment of the potential of a chemical to induce numerical chromosome changes.

A detailed description of the results obtained on polyploidy induction for the chemicals used in the EEC programme is provided in Table 12 and support the proposal to use polyploidy as an early indicator for aneuploidy.

Further support for this concept can be obtained by examination of the 779 chemicals listed by Ishidate (1988a) where both structural aberrations and polyploidy were measured in a consistent protocol. In this database, 58 out of the 779 chemicals induced increases in polyploid cells (defined as 10% or more polyploid cells) (Appendix F). It is interesting to note that induction of polyploidy is a relatively low frequency event (58/779 = 7.4%) compared to 40% of the chemicals in this same database that induced structural aberrations. In addition, Ishidate *et al* (1988) list 15 other chemicals that induce polyploidy. Of these 73 (58 + 15) polyploidy-inducing chemicals, 51 (70%) also induced structural aberrations whereas the remaining 22 (30%) did not (a number of those were reported positive in the Ames *Salmonella* Assay, other genotoxicity assays and some carcinogenicity assays, although the actual data are not provided for review).

It can be concluded that the frequency of chemicals that induce polyploidy in the absence of other genotoxic events is low. In this database a conservative estimation indicates that only around 2.3% (30% of 7.4%) of the chemicals uniquely induce polyploidy. The true frequency of chemicals that uniquely induce polyploidy may be even lower since there are undoubtedly other data for induction of chromosome aberrations for these chemicals using different protocols with different exposures, concentrations, and harvest times. In addition, it could be argued that some of the polyploidy responses were rather weak and only at high doses (e.g. hydrochlorothiazide), however, other chemicals clearly induced large increases in polyploidy at more than one concentration (e.g. garlic oil: 36 % and 47 % polyploidy at 0.015 mg/ml and 0.03 mg/ml respectively). It is recognised that there are other reported genotoxic effects for some of these chemicals (e.g. hydrochlorothiazole, has been reported to be positive in the Ames *Salmonella* Assay (US-NTP, 1989).

Similar results were obtained in a smaller, less diverse database of 30 pharmaceutical compounds where 7 of 12 (58%) of the chemicals that induced polyploidy also induced structural aberrations in human lymphocytes (Mitchell *et al*, 1995); the other 5 (42%) induced only polyploidy. As for chemicals in Ishidate's database, the biological relevance of the chemicals that only induced polyploidy is largely unknown.

Table 12 - Analysis of Aneuploidy Induction for Chemicals Used in the EEC Aneuploidy Investigation

	Cell Type										
	CHE Primary			LUC2				PBL			
	Poly	Hyper	Hypo	Poly (4n)	Hyper	Hypo	Endo	Poly	Hyper	Hypo	Endo
Cadmium Chloride	-	-	+	- 18h	-		-	-	+	+	-
Chloral Hydrate	-	-	+	+ 18h	-	+	+	+	+	+	+
Colchicine	+	+	+	+ 18h	+	+	+	+	+	+	-
Diazepam	-	+	+	+	-	+	+	-	-	+	-
Econazole	-	+	+	- 18h	+	+	-	-	-	+	-
Hydroquinone	-	+	+	- 45h	+	+	-	+	-	+	-
Pyrimethamine	-	-	+	+ 18h	+	+	+	-	-	+	-
Thiabendazole	+	-	+	+ 18h	+	-	+	+	-	+	-
Thimerosal	+	+	+	+ 45h	-	-	+	+	+	+	+
Vinblastine	ND	ND	ND	+ 18h	+	+	+	+	+	+	-
Vincristine	+	+	+	ND	ND	ND	ND	ND	ND	ND	ND

Experimental Regimen:

CHE (Chinese Hamster Embryo cells): 24 hours exposure, BrdU, analysis of 200 cells, not clear what cells analysed (M1, M2, M3), not clear how induced aneuploidy was calculated (toxicity=death ?) (Natarajan *et al*, 1993)

LUC2 (Chinese Hamster Lung Cells) : Low passage Chinese hamster pulmonary cell line, exposure was 1 cell cycle (18 hours), 2.5 cell cycles (45 hours), 3/42 hours, 12/33 recovery (Warr *et al*, 1993)

PBL (Peripheral Blood Lymphocytes) : 48 and 72 hours exposures except 5 hours exposure thiabenzole and thimerosal (Sbrana *et al*, 1993).

Overall, the results from these databases indicate that around 60-70% of polyploidy-inducing chemicals also induced structural chromosome aberrations (and in some cases gene mutations) and therefore would be detected in standard genotoxicity assays even without the assessment of polyploidy. The remaining 30-40% would have been missed, thus there is general agreement that polyploidy and endoreduplication should be reported (Galloway *et al*, 1994; OECD 1996b; Mitchell *et al*, 1995). In addition, increases in mitotic index should be recorded as they may provide useful information as to the aneugenic potential of the test chemical.

For general screening purposes, a standard genotoxicity test battery including an *in vitro* cytogenetic assay with an assessment of polyploidy/endoreduplication along with currently measured endpoints such as mitotic index, provides an efficient evaluation of a chemical's overall genotoxic potential including an indication of the potential of a chemical to induce numerical chromosome changes. Follow-up testing to define further a chemical's potential to induce aneuploidy is discussed subsequently.

7.3 PROTOCOL CONSIDERATIONS FOR ASSESSING POLYPLOIDY IN THE *IN VITRO* CYTOGENETIC ASSAY

Maximum Test Concentrations

In comparing induction of polyploidy with induction of structural chromosome aberrations, it is important to note that classic spindle poisons (colchicine, colcemid) induce structural aberrations when tested by standard methods up to the required toxicity of at least 50% *(*Arni *et al,* 1995). This is relevant since it is the standard method that would be used in screening chemicals of unknown activity. Colcemid (1,000 ug/ml) induced greater than 50% aberrant cells with an 18 hour exposure (Arni *et al*, 1995). This exposure resulted in a 25% reduction in cell number, and a 75% reduction in cloning efficiency. The data suggest that even chemicals that are considered primarily aneugenic can induce some level of clastogenicity. This has been proposed to be due to spindle/kinetochore toxicity (Tinwell and Ashby, 1991). Therefore for aneugens in general, it can be concluded that definitive aneugens would be detected by one or more endpoints in a standard cytogenetic screening assay. As is the case with colchicine, a careful examination of the concentrations at which various endpoints are included (e.g large increases in polyploidy at low doses) may indicate whether the test chemical acts primarily as an aneugen or a clastogen.

Exposure/Harvest Time

One of the most important features of the *in vitro* cytogenetic assay protocol relating to its effectiveness for detection of polyploid cells is the exposure/harvest time. In the standard protocol an asynchronous population of cells is exposed to the test chemical (treatment starts 48 hours after PHA stimulation of

human lymphocytes). The most well defined mechanism of aneuploidy induction is inhibition of the spindle. Cells progressing through G2/M during chemical exposure are probably the most sensitive population for chemically-induced spindle effects. According to current test guidelines for the *in vitro* cytogenetic assay, cells are exposed/harvested at 1.5 times the normal cell cycle, which is around 20-24 hours depending on the cell type. During this time it is expected that a proportion of cells will pass through G2/M, especially in cell lines lacking normal cell cycle checkpoints, into the next metaphase (second metaphase after treatment). This is the appropriate population of cells for measuring the effect of spindle inhibition, such as polyploidy. An exposure/harvest time of around 1.5 normal cell cycles may even take into account cell cycle delay, since without delay, a cell would normally progress from G2/M into the second metaphase in one cell cycle. This timing also is appropriate for other mechanisms of polyploidy induction, like inhibition of division of the cell membrane since this would occur during cytokinesis after the first mitosis and be observed at the following mitosis. For other mechanisms, different harvest times may be more appropriate. These theoretical considerations are supported by data published in the literature showing increases in polyploidy at harvest times around 1.5 cell cycles as discussed later.

BrdU labelling is often used to verify the appropriateness of harvest times and the population of cells to analyse in cytogenetic assays. M1 cells are considered the appropriate population for the analysis of structural aberrations (since cells with damage can be lost from the population examined at later time points) and M2 cells are the appropriate population for assessing aneuploidy since chromosome segregation errors are induced during mitosis and are evident at the second mitosis following chemical exposure. However, results from BrdU labelling studies under-represent the true proportion of cells in their second mitosis. The data presented in Appendix G at a 24 hour harvest time in human lymphocyte cultures, under conditions which correspond to 1.5 cell cycles, show that almost half of the cells have an M2 staining pattern and the other half have an M1 pattern. A proportion of cells with an M1 staining pattern are in fact in their second metaphase following chemical exposure since cells in late S/G2/M would have only incorporated BrdU during the first round of DNA synthesis before progressing into their second mitosis. Since cells in G2/M are the most sensitive population for chemically-induced spindle effects, a harvest time of 1.5 cell cycles (20-24 hours) appears to be optimal for the analysis of both chromosome aberrations and spindle effects.

The above agrees with the experience of many laboratories that observed increases in polyploidy at 1.5 cell cycles harvest time (discussed in Galloway *et al*, 1994). Of the polyploidy-inducing chemicals listed in Appendix F 56 out of 73 (77%), were tested using both 24 and 48 hours exposures. Sixteen out of the 56 (29%) chemicals, induced an increase in polyploidy at 24 hours (some also at 48 hours). In addition, 15 chemicals were tested with a shorter exposure time of 3 or 6 hours followed by 21 or 18 hours recovery (harvested at 24 hours). Seven of these 15 chemicals induced polyploidy with this regimen. This is in agreement with the unpublished data of Galloway where 23 of 72 pharmaceutical chemicals induced endoreduplication with a 3 hour exposure and 20 hour harvest time (discussed in

Galloway *et al*, 1994). Thus, it is not essential to use extremely long harvest times to observe an induction of endo-reduplication/polyploidy.

Clearly a large number of chemicals (40 out of the 56, 71%) induce polyploidy only at 48 hours. However, it is important to note that 20 out of the 40 (50%) induced an increase in aberrations at the earlier 24 hours harvest (either short or continuous exposure). Thus, in a standard cytogenetic assay where aberrations and polyploidy are assessed at sampling times of around 1.5 cell cycles, thereby including a second metaphase division (OECD, 1996b), it is expected that the majority of polyploidy inducing chemicals would be identified either by increases in aberrations or induction of polyploidy alone.

Analysis of polyploid cells

Currently, not all laboratories record polyploid and endoreduplicated cells. In addition, there are different procedures for assessing polyploidy. Some laboratories assess the frequency of polyploid cells during the recording of the mitotic index, i.e. the number of polyploid/endoreduplicated cells/1,000 interphase cells. This drastically under represents the frequency of polyploid cells since the denominator is interphase cells for which the ploidy is unknown. Some laboratories record polyploid cells while scanning for suitable metaphases for the analysis of aberrations. Again, this has the potential to alter the true frequency of polyploid cells since the scorer is focused on finding good quality metaphase spreads for aberration analysis. The recommended method for analysing the frequency of polyploid/endo-reduplicated cells is to record the number of polyploid and endoreduplicated cells separately from the 100 metaphases. Endoreduplicated cells are polyploid but they should be noted separately since their presence may provide mechanistic information.

The criteria for classifying a cell as polyploid are also important. For a screening assay, it is appropriate to record a polyploid cell as that with an obvious excess of chromosomes, without actually counting chromosomes. This makes sense especially for cell lines (e.g. CHO) with variability in chromosome number/cell. A more definitive assessment can be made by actually counting chromosomes, but this is complicated by technical artefacts and scorer error and bias.

Further Validation

At this time, the protocol for the standard *in vitro* cytogenetic assay is useful for screening chemicals that induce aneuploidy. Inherent in any screening assay is the recognition that the current *in vitro* cytogenetic protocol is not, and will not be, optimal for detecting all aneugenic-inducing chemicals. However, it is important to realise even this protocol designed for the detection of structural aberration is not optimised for all clastogens. Therefore, the appropriateness of the *in vitro* cytogenetic protocol needs to continue to be evaluated relative to our increasing understanding of chemically-induced aneuploidy. Perhaps the most important need is for the identification of more definitive *in vivo* aneugens that then can be used to assess further the *in vitro* cytogenetic assay as well as other new methods.

In vivo assays hold a key position in any screening strategy for the detection of possible aneugens and, once fully validated, should be used to overrule clear aneugenic activity observed in *in vitro* assays. This situation is identical in strategy to that previously described for the standard genotoxicology assays (Ashby, 1986; Canada, 1986, 1991). Therefore, *in vivo* assays for aneugens must be fully validated and be robust so that confidence can be ascribed to the results generated from using these assays.

7.4 FOLLOW-UP STRATEGY FOR CHEMICALS IDENTIFIED AS HAVING ANEUGENIC POTENTIAL

Since the above initial screening strategy does not include definitive measures of chromosome loss and nondisjunction, chemicals positive in one or more identifiers for aneuploidy require follow-up testing which should focus on more definitive assessments of aneuploidy. An assessment of structure-activity of the test chemical relative to other known aneuploidy-inducers may provide predictions for their aneugenic potential. Potential mechanisms of induction of polyploidy or endoreduplication should also be considered since polyploidy may arise by various mechanisms (e.g. cell fusion, inhibition of cell membrane synthesis or G_2 arrest) (see Chapter 4).

Some assays for assessing chromosome loss and nondisjunction are being developed and show promise. For instance the *in vitro* micronucleus assay is being proposed as an alternative/replacement for the standard chromosome aberration test (Albertini et al, 1995; Kirsch-Volders and Albertini, 1997; Li et al, 1993; Matsuoka *et al*, 1993; Migliore *et al*, 1987; Miller, 1996; Miller *et al*, 1996) due to its ability to detect both structural aberration and chromosome loss. The induction of chromosome loss is detected by the use of kinetochore or centromeric probes. These probes can also be used in a follow-up *in vivo* micronucleus assay. An even more definitive assessment of aneugenic-inducing potential may be made using primary cells, for example, the *in vitro* human lymphocyte binucleated micronucleus assay, where both the consequences of chromosome loss in the form of micronuclei, and nondisjunction in the two daughter nuclei can be measured using fluorescence *in situ* hybridisation probes.

Several advantages of the micronucleus test *in vitro* can be put forward:

- Potential of not only detecting clastogens, but additionally aneuploidy inducing chemicals
- Simplicity of the method
- Fast and inexpensive assay
- Higher statistical power ($\rightarrow$ more cells evaluated)
- Potential for automation using image analysis
- Using the method of binucleated cells mechanistic information (in case of positive results) can be easily obtained (e.g. by FISH)

For chemicals positive in the more definitive aneuploidy assays including a somatic cell *in vivo* assay, further consideration should include potential germ cell aneuploidy and/or carcinogenicity effects. To date there is no conclusive evidence for the existence of unique germ cell aneugens.

7.5 EVALUATION

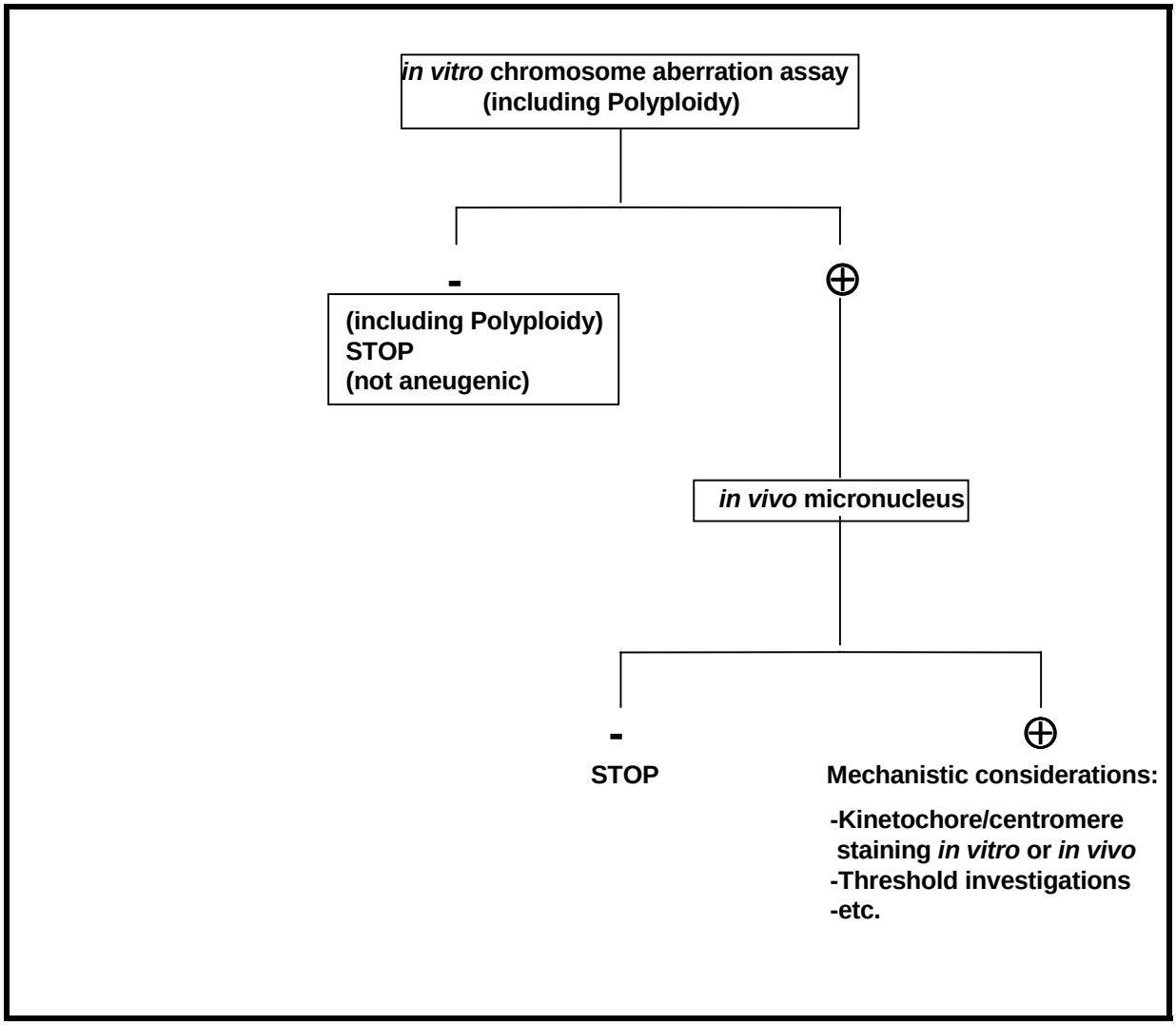
It is clear from the published literature that, at present, there is no single aneuploidy specific assay that is sufficiently well validated to be routinely used for the detection of aneugens and therefore, data generated from any of these assays must be treated with great caution until such validation has been successfully completed. It has been implied by many authors that a class of aneugen specific chemicals exists i.e. chemicals capable of disturbing the mitotic apparatus leading to aneuploidy, yet which are negative in the standard genotoxicity assays for gene mutation, chromosomal damage or DNA repair. There is, however, from all the published work on known or suspect aneugens, no clear evidence to support this suggestion. No pure aneugens or pure clastogens have been identified although there is clear evidence for chemicals being primarily aneugens or clastogens. It may be that a low level of clastogenicity is the inevitable consequence of the effects of aneugenic compounds on the spindle apparatus and that clastogens will produce some fragments that happen to contain a centromere.

A proposal for a screening approach for detection of potential aneugenic chemicals is outlined in Figure 2. A standard *in vitro* cytogenetic assay including an assessment of polyploidy provides a useful initial assessment of the potential of a chemical to induce numerical chromosome changes. In the case of positive findings in the standard *in vitro* cytogenetic assay it is prudent to investigate whether the chemical has the ability to express activity *in vivo.* For that purpose it is recommended an *in vivo* micronucleus assay is performed.

If the test compound leads to the induction of micronuclei *in vivo* complementary studies to distinguish micronuclei formed from chromosome loss from those originating from chromosome breakage (e.g. kinetochore staining *or* FISH with centromeric probes either *in vitro* or *in vivo*), i.e. important information on the genotoxic properties are to be gathered.

As a future perspective one could envisage including the micronucleus test *in vitro* in a testing strategy as an alternative/replacement for the CA *in vitro* as proposed recently (Miller *et al*, 1996; Kirsch-Volders and Albertini, 1997).

Figure 2 - Screening approach for detection of potential aneugenic chemicals



8. RISK CHARACTERISATION OF ANEUGENS

8.1 EVIDENCE INDICATING THRESHOLDS FOR THE GENOTOXIC EFFECTS OF ANEUGENS

Certain types of environmentally-induced genetic damage, such as aneuploidy-induction through the inhibition of spindle function, do not involve the direct interaction of the suspect agent or an active metabolite with DNA (Chapter 4). The interaction of an aneugen with its target receptor (such as tubulin) often exhibits a characteristic dose-response pattern which may include a threshold level (Dellarco *et al,* 1985a). The damage is impacted by pharmacokinetic factors, since the binding to the target receptor is usually reversible and repeated exposure (particularly at low doses) may not be additive. These properties are different than those of other genotoxic substances - mutagens and clastogens - which interact with DNA covalently and for which the occurence of thresholds is not currently accepted.

For chemicals which exert their effects by binding to a macromolecular receptor, the magnitude of the response (such as the inhibition of microtubular function) is related to the affinity of the chemical to its receptor, the reversibility of the binding, and the degree of the occupancy of the macromolecular binding sites by the compound. In a dose-response relationship, the maximal biological response is reached when all receptor sites are occupied. The threshold is obtained at the dose level above which a critical number of receptor sites must be occupied for the biological event to commence.

There is a generally held belief that aneugens have thresholds. Recently a clear indication for threshold effects has been obtained for tubulin binding compounds (Elhajouji *et al*, 1995). In general, thresholds have been established for polyploidy; for aneuploidy there is an indication that most substances show a no-observed-effect-level (NOEL) (cf. Table 12).

The studies cited in Table 13 support the conclusion that thresholds exist for spindle poisons. It is anticipated that by using the available test methods for aneuploidy, threshold levels for aneugens acting by different mechanisms will be established. Mammalian cells *in vitro* provide the best potential (Parry *et al*, 1995b) although these techniques have been primarily applied with the purpose of developing new assay methods.

Table 13 - Threshold or NOEL Levels determined in Various Cell Systems for Benomyl, Carbendazim, Colcemid, Colchicine and Vincristine Sulphate.

Compound	Cell System	Dose Range (μg/ml)	Genotoxic Endpoint Measured	Threshold or NOEL (μg/ml)	Reference
Benomyl	Human x mouse hybrid cells (R3-5)	1.5 - 15.0	Aneuploidy	1.5	Athwal and Sandhu, 1985
	V79/AP4 Chinese Hamster cells	2.5 - 10.0	Polyploidy	2.5	Rainaldi *et al*, 1987
	Chinese Hamster Ovary cells	1.0 - 10.0	Kinetochore- + micronuclei (aneuploidy)	1.0	Eastmond and Tucker, 1989
	Human lymphocytes	0.025 - 4.0	Aneuploidy	0.1	Georgieva *et al*, 1990
	Human-Chinese hamster hybrid cell (EUBI)	2.0 - 8.0	Aneuploidy/ polyploidy	2.0	Zelesco *et al*, 1990
Carbendazim	Human x mouse hybrid cells (R3-5)	0.015 - 15.0	Aneuploidy/ polyploidy	approx 1.0 (aneuploidy) approx 8.0 (polyploidy)	Parry *et al*, 1993
	Human lympocytes	0.03 - 2.0	Centromere + micronuclei	0.5	Elhajouji *et al,* 1995
Colcemid	Chinese Hamster Ovary cells	0.01 -0.07	Aneuploidy	0.01	Cox and Puck, 1969
	Human x mouse hybrid cells (R3-5)	0.005 - 0.10	Polyploidy	0.01	Athwal and Sandhu, 1985
	VP79/AP4 Chinese Hamster cells	0.0125-0.0375	Polyploidy	0.0125	Rainaldi *et al*, 1987
	Human fibroblasts (JHU-1)	0.003 - 0.02	Aneuploidy	0.01	Tsutsui *et al*, 1990
Colchicine	Human diploid fibroblasts	0.001 - 0.01	CREST- +micronuclei (aneuploidy)	0.001	Bonatti *et al,* 1992
	Chinese Hamster Luc2 cells	0.001 - 1.0	Kinetochore-labelled micronuclei (aneuploidy)	0.001	Lynch and Parry, 1993
	Human lymphocytes	0.001-0.04	Centromere +micronuclei	0.015	Elhajouji *et al*, 1995
Vincristine sulphate	Syrian Hamster Embryo cells	0.0003 - 0.03	Heteroploidy	0.001	Tsutsui *et al*, 1986
	Human lymphocytes	0.02 - 0.05	Nuclei with >3 hybridisation regions	0.04	Eastmond and Pinkel, 1990
	Human fibroblasts	0.001 - 0.01	Aneuploidy	0.003	Tsutsui *et al*, 1990

8.2 EXTRAPOLATION FROM ANIMAL DATA TO HUMAN RISK

Extrapolation of animal mutagenicity, including aneugenicity, data to man needs to consider differences in metabolism between rodents and man. There is little information available on the metabolism of specific aneugens in different species. Theoretically, however, one factor could contribute to species differences which is specific to the induction of aneuploidy. Recent studies of centromere organisation point to important structural differences among eukaryotes (yeast, rat, mouse and man) (Willard, 1992; Tomkiel and Earnshaw, 1993; Jeppesen *et al,* 1993; Doheny *et al*, 1993; De Stoppelaar *et al*, 1995) and these could contribute to differences in chromatid disjunction. It is possible, if not probable, that the influence of aneugens interfering with centromere function may show clear differences in rodents and human beings. Tsutsui *et al* (1990) have shown already that human fibroblasts are less sensitive than those of the Syrian hamster to the induction of aneuploidy by 4 chemicals: colcemid, vincristine, diethylstilboestrol, and 17 β-oestradiol.

In considering the extrapolation of germ cell effects from rodents to human beings other factors need to be considered. For example the timing of ovulation in relation to mating differs in rodents and man. Also there may be differences in selection against aneuploid gametes or embryos in different species. Some evidence for this exists from the frequencies of aneuploidy in embryogenesis in rodents and man (c.f. Section 2.2). To date, there is no information on the relevance of the data obtained in experimental animals for human beings and this is an area requiring further investigation.

8.3 PROPOSAL FOR A UNIFIED SCHEME FOR MUTAGEN HAZARD IDENTIFICATION

As pointed out in Section 6, three major hazard classification schemes (detailed in Appendix C) address the concern for mutagen induction of heritable damage in the human population. One major drawback, common to all three schemes, is that the mechanism by which the mutation has occurred is not taken into hazard classification. Yet unlike direct DNA damaging agents, thresholds can be established for mutagens exerting their action through non-DNA binding targets. Aneugens which bind tubulin are an example.

One argument raised by proponents of the current schemes is that hazard identification deals with qualitative (hazard) and not quantitative (risk) assessment. One noticeable deviation from this concept is the criteria for classifying carcinogenic substances by the EEC in which mechanistic and quantitative data is used. The EEC scheme consists of three categories of carcinogens. Category 1 compounds are “substances known to be carcinogenic to man”. Category 2 compounds are “substances which should be regarded as if they are carcinogenic to man”. Category 3 compounds are “substances which cause concern to man owing to possible carcinogenic effects but in respect of which the available information is

not adequate for making a satisfactory assessment". In the EEC Guidelines, when it is not clear if a chemical should receive Category 2 or Category 3, a number of quantitative and mechanistic considerations are given to determine if Category 3 is appropriate including:

- lack of genotoxicity in short-term tests *in vivo* and *in vitro,* and/or the existence of a secondary mechanism of action with the application of a practical threshold above a certain dose level;

- existence of a species-specific mechanism of tumour formation (e.g. by specific metabolic pathway) irrelevant for man.

Thus dose, threshold, mechanism (genotoxic versus non-genotoxic), relevance to man etc. which pertain more to risk than hazard are being used by the EEC for carcinogen classification.

Bearing in mind that threshold effects have been demonstrated for some aneugens similar considerations should be given to mutagen classification by the regulatory authorities.

The complicating factor is that with mutagens the cellular target (germ versus somatic) is the determinant factor in category classification. The current EEC Guidelines can be used as a basis for a modified mutagen hazard classification system. Such a scheme must reflect the following levels of concern:

- substances causing heritable mutations in human beings (data from epidemiology studies are of greater importance than data generated from mammalian germ cell mutagens assays and which in turn are more important than data generated for mammalian somatic cell mutagens assays);

- substances or reactive metabolites which bind to DNA (for which there is no acceptable threshold) are of greater concern than mutagens exerting their action through binding to other critical macromolecules (tubulin, enzymes, etc) for which a threshold can be established;

- the relevance of a specific mechanism of mutation induction in man (e.g. specific metabolic activation in the test system) that may be irrelevant to man.

8.4 EVALUATION

Spindle inhibitors show threshold effects in the conduct of tests for aneuploidy. Similar dose response curves may be expected for other classes of aneugens acting on other non-DNA targets. Such test responses would normally result in the classification of the substance as a mutagen.

Particular consideration should be given to substances which bind to critical macromolecules other than DNA for which a threshold can be established. Such threshold concepts should lead to a modified harmonised mutagen hazard classification scheme.

9. CONCLUSIONS

In this report the following topics were addressed:

- the significance of aneuploidy for human health,
- the mechanisms of aneuploidy induction,
- an evaluation of the methods used for detecting aneugens,
- recommendations for the testing of aneugens, and
- recommendations for the risk characterisation of chemicals with aneugenic potential.

On the basis of this evaluation the following conclusions are reached.

Aneuploidy plays a major role in adverse human health effects including cancer and congenital abnormalities. There are insufficient data to determine with any certainty if aneugenic chemicals contribute to these disorders. However, animal studies provide clear evidence for the role of chemically-induced aneuploidy in disease. Since there is no reason to assume that the effects in man will be different, aneugenic potential should be addressed in the overall safety assessment of chemicals.

To ensure that hazard identification for aneugens is appropriately addressed the following recommendations are made:

- the inclusion of an early indicator of aneuploidy such as polyploidy and endoreduplication, or mitotic index in the standard *in vitro* cytogenetic assay after further validation;
- the acceptance by the regulatory authorities of the *in vitro* micronucleus test in mammalian cells as an alternative method for the standard *in vitro* cytogenetic tests;
- the use of centromere or chromosome specific probes in both *in vitro* and *in vivo* assays where required to differentiate clastogenic from aneugenic mechanisms; these probes will provide a more definitive measure of aneuploidy and mechanism of induction;
- the use of appropriate germ cell aneuploidy tests as follow-up tests for positive findings in mammalian somatic cells *in vivo*; at this time there is no evidence for the definitive existence of unique germ cell aneugens.
- the modification of mutagen classification schemes to take into account consideration of non-DNA reaction mechanisms.

Appropriate (quantitative) risk assessment of aneugens would require:

- incorporation of mechanistic information and identification of critical targets (DNA and non-DNA);
- establishing a dose-response relationship and definition of no effect levels (thresholds).

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APPENDIX A - METHODS FOR THE DETECTION OF ANEUGENS

There are many methods available for the testing for the aneugenic potential of test substances, including subcellular, submammalian, *in vitro* mammalian and *in vivo* somatic and germ cell assays. Methods for aneuploidy have been reviewed by Allen *et al* (1986), Cimino *et al* (1986), Dellarco *et al* (1985), Galloway and Ivett (1986), Oshimura and Barrett (1986) and Parry and Parry (1989). Specific recommendations on protocol design for the most developed and validated tests were made by Parry *et al* (1995b). Some assays assess single mechanisms only (e.g. the tubulin assay) and as such are more appropriate for mechanistic studies than for screening tests. Other assays should be considered as "indicator" tests where one of the stages of mitotic or meiotic divisions or events possibly correlated with aneuploidy-induction (e.g. cell division aberrations or mitotic arrest or meiotic delay) are studied. Definitive tests for aneuploidy require the counting of whole chromosomes or centromeres. While tests in cell-free systems and *in vitro* give an indication of the potential of the chemical to induce aneuploidy, the *in vivo* studies allow an estimation of the relevance of this potential for human health.

There is no single molecular mechanism for the induction of aneuploidy by chemicals. Since chromosomal segregation is dependent upon many different organelles and controlled by a number of pathways, aneuploidy can arise from several different mechanisms. These include the damage of essential elements for chromosome function, reduction of chromosome pairing, induction of chromosome interchanges, effects on chromosome condensation, persistence of the nucleolus in mitosis or meiosis, increased chromosome stickiness, damage to centrioles or kinetochores, impairment of chromosome alignment, alterations of ion concentrations during mitosis, damage to the nuclear membrane, physical disruption of chromosome segregation and effects on microtubules (Oshimura and Barrett, 1986). In addition mutation of cell cycle control genes such as the G_1S and G_2M checkpoint gene, p53, can lead to aneuploidy. Details of these mechanisms and chemicals affecting their function are described in Chapter 4.

Since aneuploidy may arise from various mechanisms, which give rise to either chromosome loss or chromosome gain, test methods should be able to detect both events. However, technical limitations often make the estimation of chromosome loss very difficult. In methods using chromosome counts as the endpoint, hypotonic treatment and spreading of metaphases without breakage of the cell membrane is difficult to achieve. Even with scoring of interphase cells by FISH, insufficient penetration of the probe and spatial overlap of the two signals must be considered. Consequently the indication of hyperploidy, and not hypoploidy, is a more definitive method for assessing changes in chromosome number. Whereas the drawback of sensitivity of detecting chromosome loss applies to the chromosome counting methods, those methods based on micronuclei induction will detect chromosome loss but not chromosome nondisjuntion. There is no evidence to date that any chemical will induce specifically

chromosome loss or nondisjuntion. Therefore, in principle, methods based on chromosome counting or micronucleus induction should both be useful for screening for aneugenic potential.

As induction of aneuploidy in both somatic cells and germ cells is of concern, and there are differences between the mitotic and meiotic process, methods are required to assess aneugenic potential in both cell types. Although most of the targets are identical, homologous chromosome pairing and crossing over are restricted to meiosis. In germ cell assays consideration must be paid to differences in gametogenesis in males and females and methods are available to screen for aneugenic potential at various developmental stages in both sexes.

The methods available for detecting aneugens are described in detail below.

A.1 NON-MAMMALIAN METHODS

Various plants, fungi, and the fruit fly *Drosophila melanogaster* have been used by geneticists for many years. Chromosomes of *Vicia* or *Allium* species are suitable for analysis. Several fungal strains are genetically well understood and therefore used in aneuploidy testing. *Drosophila melanogaster* is easy to cultivate and has a short generation time. Culturing of plants, fungi and *Drosophila* needs less sophisticated equipment than that required for mammalian cells. For these reasons various investigators have proposed the use of these organisms for the assessment of aneuploidy-induction of chemicals. The methods used are described briefly below but due to their limited relevance to mammals they are not discussed in detail.

A.1.1 Plants

Tests with plants are less expensive and time consuming than mammalian cell test systems. Aneuploidy-induction in plants has been investigated cytogenetically in mitotic and meiotic cells and morphologically in assays, where the colour of leaf spots or stripes is monitored (Vig, 1975, 1978; Sandhu *et al*, 1988; Redei and Sandu, 1988). The data base for chemicals inducing aneuploidy in plants is limited. For the present, plant systems cannot be considered to be validated sufficiently and it is difficult to extrapolate from plant data to human hazard.

A.1.2 Fungi

Four species *Aspergillus nidulans*, *Neurospora crassa*, *Sordaria brevicollis* and *Saccharomyces cerevisiae* have been primarily used to study the induction of aneuploidy by chemicals. Since the chromosomes of these species are too small for routine visual examination, induced aneuploidy is detected by genetic methods. Fungi may be used to investigate the induction of aneuploidy during both

vegetative mitotic cell division and meiotic reduction division (Griffiths *et al*, 1986; Käfer *et al,* 1986; Resnick *et al*, 1986; Albertini and Zimmermann, 1991). The conditions which lead to meiosis and spore formation in fungi may not be representative of those which lead to gamete formation in higher organisms (Parry and Parry, 1987).

Many of the compounds tested in fungi have not been evaluated in mammalian cell assays. For those few chemicals for which comparative data exists, the results have not always agreed with those in mammalian assays (Albertini, 1993). Potent mammalian aneugens such as colchicine and vinblastine were negative in yeast and *Aspergillus*. Differences in affinity of chemicals to mammalian or fungal tubulin, the persistence of the nuclear envelope throughout cell division in fungi but not in mammalian cells and differences in the attachment of centromeres to the spindle in fungal and mammalian cells are possible explanations for these differences (Parry and Parry, 1987; Albertini, 1993). Due to this, findings in fungi cannot be easily extrapolated to mammals.

A.1.3 *Drosophila*

The fruit fly *Drosophila melanogaster* has many advantages for use in environmental screening procedures. It is easy to cultivate, has a short generation time (10-14 days) and its genetic markers are very well known. Indirect mutagens are activated efficiently by an enzyme system which is comparable to that of mammals (Valencia *et al*, 1984).

Drosophila is primarily used to detect induced aneuploidy in germ cells. The usefulness of the somatic cell assays is very limited as nondisjunction of the large autosomes leads to large and lethal disturbances of the genetic balance of the organisms (Würgler, 1992). Zimmering *et al* (1986) reviewed the literature on data concerning chemically induced aneuploidy in *Drosophila* published before 1985. A general comparison of the effects induced by aneugens in *Drosophila* and in mammalian systems has not been performed, thus the relevance for mammals of data obtained with *Drosophila* is not yet known.

A.2 CELL FREE SYSTEMS (TUBULIN ASSEMBLY ASSAYS)

Aneuploidy can also be assessed using the major subcellular component of the cell division process by investigating effects on tubulin assembly. Microtubules are a highly dynamic part of the cytoskeleton and found in all eukaryotic cells. Tubulin and microtubule associated proteins are the main constituents of the spindle (Dustin, 1984). Mammalian brain tubulin has been studied in detail and much is known about its behaviour *in vitro.* The tubulin assembly assay allows the identification of compounds which induce aneuploidy by interaction with tubulin proteins and may allow the differentiation of aneugens acting on spindle components and those acting via other mechanisms. Tubulin assembly is determined

photometrically (Gaskin, 1982; Albertini *et al*, 1985; Hartley-Asp *et al*, 1985; Wallin *et al*, 1988). Aneugens have been shown to either slow down or accelerate tubulin assembly.

Data on compounds interfering with tubulin assembly have been published (Albertini *et al*, 1985; Hartley-Asp *et al*, 1985; Sharp and Parry, 1985). Many groups of compounds have been tested (Görschel-Stewart *et al*, 1985; Albertini *et al*, 1988; Brunner *et al*, 1991). Data on some compounds tested in different laboratories are not consistent. For example 10 chemicals have been tested within the CEC Aneuploidy Project on tubulin. Five of the compounds were found to interfere with tubulin assembly in one laboratory (Brunner *et al*, 1991) whereas Wallin and Hartley-Asp (1993) found 9 chemicals inhibited tubulin assembly and only one compound did not interfere with assembly of microtubules, but produced morphologically detectable (electron microscopy) aberrant forms. Differences in microtubule preparation methods as well as in the species used may play a role in the observed differences between laboratories. Clearly, this assay needs further validation, if it is to be used as a screening method.

A.3 *IN VITRO* MAMMALIAN CELL METHODS

Since aneuploidy can arise from different events, various test systems and techniques have been developed using primary, transformed, or hybrid cells. Primary cells have the advantage of a stable diploid karyotype. The major systems are subsequently described.

A.3.1 Mitotic Alterations

Many parameters related to spindle and chromosomes during mitotic division can be evaluated by microscopic observation of alterations in cell morphology during division. The technique involves careful fixation to avoid spindle shrinkage. The cells are stained with brilliant blue and safranine which stain the spindle and the chromosomes respectively (Parry *et al*, 1982; Warr *et al*, 1993). The parameters studied include: multipolar spindle, chromosome dislocation from the spindle, lagging chromosomes, chromosome clusters, scattered chromosomes, etc.. Consequently, as any significant effect on the spindle can be detected additional knowledge on the mechanism of action of the test substances is obtained. Of the 10 known or suspected aneugens tested in the EEC 4th Environmental Research and Development Programme all except cadmium chloride yielded positive results in LUC2 cells while all were positive in DON, an immortal cell line.

The presence of C-metaphase morphology of mitosis and measurement of the mitotic index has been used to evaluate the ability of chemicals to affect the spindle. In C-mitosis the spindle is inactivated. When the inactivation is complete (full C-mitosis) the chromosomes are contracted and scattered over the cell and tetraploid restitution nuclei are formed after chromosome division. Intermediate disturbance occurs when the spindle is not completely inactivated (partial C-mitosis) but is unable to accomplish

normal chromosome distribution. The result is the formation of multipolar anaphases (Sbrana *et al*, 1993).

A.3.2 Induction of Micronuclei

The use of *in vitro* micronucleus assays to evaluate chemical aneugens has been proposed (Oshimura and Barrett, 1986; Parry and Parry 1987; Ford *et al* 1988; Miller *et al* 1994) despite the fact that this assay detects chromosome loss and nondisjunction events. The tests are based on the scoring of additional small nuclei in dividing cells. Micronuclei arise from chromosome fragments and/or whole chromosomes lagging during anaphase movement. The *in vitro* micronucleus test has been used with a number of cell types including cell lines (e.g. Bonatti *et al*, 1983) and human lymphocytes (e.g. Heddle, 1976) because of the simplicity and the speed of the assay. The cytokinesis-block technique described by Fenech and Morley (1985) allows the analysis of micronuclei in cells that have undergone one cell division cycle following chemical treatment since they appear as binucleated cells. This provides a measure of cytotoxicity and/or mitotic delay and increases the sensitivity of the assay by limiting scoring to cells which have divided in culture.

To differentiate the micronuclei arising from lagging fragments from those formed by whole chromosomes in *in vitro* assays, the following techniques have been used:

Morphological Assessments

Morphological assessments of micronuclei include measurements of the size (Fergusson *et al*, 1993; Van Hummulen *et al,* 1995b) and DNA content (Thomson and Perry, 1988). Since micronuclei were shown to undergo DNA synthesis, cell cycle effects should be taken into account and DNA content should preferably be assessed in G_1 cells (Van Hummelen *et al*, 1995b). However, these methods are less reliable for cells that have a large variation in chromosome size such as human cells.

Immunolabeling of Kinetochores or Centromeres

Whether micronuclei result from chromosome breakage or segregation, errors cannot be defined without investigating the content of the micronucleus. Different techniques can be used to establish directly the presence of centromeres. This includes C-banding (Banduhn and Obe, 1985) which stains the centromeric regions dark, especially in the mouse, but this method is not very reliable. Antikinetochore antibodies (CREST) (Moroi *et al*, 1980) to label chromosomal kinetochore proteins (Vig and Swearngin, 1986) were widely used for the detection of chemical aneugens (Degrassi and Tanzarella, 1988; Eastmond and Tucker, 1989a,b; Nüsse *et al*, 1987, 1988) but their specificity is limited due to the intercross reactivity of antibodies and variation between donors. The presence of centromeric DNA in

micronuclei of cells exposed to various spindle poisons can also be evaluated by *in situ* hybridisation with centromere specific DNA probes (Eastmond and Pinkel, 1990; Van Hummulen, 1995a). Since some chemicals might interfere with kinetochore proteins, and thus may hinder binding to CREST antibodies, FISH with centromeric probes is the technique of choice. This method has been refined by using dual probes for centromeric and pericentromeric DNA to distinguish centromeric structural damage from whole chromosome loss.

A.3.3 Chromosome Counting

Chromosome counting provides the most direct measure of an increase in number of chromosomes. In order to obtain well-spread metaphases for chromosome analysis, cells are treated with hypotonic solution, fixed, dropped on cold microscopic slides and air-dried. With this procedure, the cells usually burst and consequently the changes in the chromosome number in a metaphase may be an artefact arising from gain or loss of chromosomes by the drastic preparation procedure (Natarajan, 1993). To overcome this problem, cells are grown on a cover glass which are then treated and fixed *in situ*. When carefully carried out, the cytoplasm is retained and all the chromosomes remain within the cell boundary and numbers can be easily counted (Dulout and Natarajan, 1987). Many parameters such as mitotic index, cell cycle progression (obtained by addition of BrdU in the medium and differential staining) and numerical chromosome aberrations, can be evaluated in this assay.

Using this technique Natarajan *et al* (1993) have studied the 10 chemicals used in the EEC programme, which are known or suspected aneugens in Chinese Hamster Embryonic Fibroblasts (CHEF) without metabolic activation. Of these substances only pyrimethamine was not detected, possibly due to a requirement for metabolic activation of this substance.

Due to the ease of conduct of this assay, it appears promising for routine testing following validation.

Hybrid Cell Lines

Sandhu *et al* (1988) described an *in vitro* assay for aneuploidy-induction in R3-5, the mouse-human hybrid cell line that contains the human chromosome 2. The original cell line is Hypoxanthine Guanine Phospho Ribosyl Transferase (HGPRT)-deficient; the human chromosome has been genetically manipulated such that it carries the *Escherichia coli* Ecogt gene, thus making R3-5 cells sensitive to 6-thioguanine. Therefore, loss of the human chromosome can be selected by plating cells in media containing 6-thioguanine. Very few aneugens have been tested in the model (George *et al*, 1993). However it must be noted that this assessment is not specific for aneuploidy since mutations can also be induced by direct DNA-damaging agents. In addition, inherent instability of hybrid cell lines and the development of chromosome specific probes as described below decreases the utility of these methods.

Fluorescence in situ hybridisation (FISH) with Chromosome Specific Probes

In the past few years, DNA sequences (probes) which hybridise to blocks of repetitive DNA on specific chromosomes have been developed. Probes for all human chromosomes and many rodent chromosomes are currently available. The probes may be for either the whole chromosome or for the pericentromeric region. *In situ* hybridisation with pericentric probes results in the staining of a compact chromosomal region which can be easily detected on metaphase chromosomes or within interphase nuclei. A significant increase in the incidence of cells with extra chromosomes, including polyploidy, as identified by the specific probes can be assessed in interphase nuclei (Raimondi *et al*, 1989; Eastmond and Pinkel, 1990; Vagnarelli *et al*, 1990).

The determination of aneuploidy is performed by simply counting the number of FISH labelled regions representing a particular chromosome of interest within the cell (Eastmond and Pinkel, 1990) without karyotyping. Scoring is rapid; 1,000 cells can be examined in approximately 90 minutes (Eastmond and Pinkel, 1990). Potential drawbacks with this method include insufficient penetration of the probe and spatial overlap of signals leading to overestimation of hypoploidy. The latter may be overcome by the use of confocal microscopy. It is important to note that premature separation of centromeres can lead to an overestimation of hyperploidy. Also, a break in the middle of the hybridisation region may be incorrectly scored as a hyperdiploid cell. This suggests that this assay might be prone to false positives when clastogens are tested. The simultaneous use of centromeric and telomeric probes can be used to distinguish aneugenic from clastogenic events (Eastman *et al*, 1993). Additional studies including a battery of aneugens as well as clastogens should be used in the future to fully validate this new method. It should be kept in mind that hybridisation is simple and the high contrast of the staining makes the partial or complete automation of this assay feasible.

A.4 *IN VIVO* SOMATIC CELL METHODS

The *in vitro* methods descibed above will only detect the intrinsic potential of chemicals to induce aneuploidy and it is necessary to follow-up the results of the *in vitro* assays with tests in whole animals. Cimino *et al* (1986) compiled and reviewed the available assays for evaluating aneuploidy in mammalian somatic cells *in vivo*. They concluded that very few studies had been specifically designed for that purpose. Most of the available data arose from ancillary numerical observations on clastogenicity studies *in vivo* and was insufficient to allow judgement on the accuracy of the assessment for aneuploidy-induction. Despite scientific advances, there continues to be no single sufficiently validated *in vivo* somatic cell assay for routine use. A number of different endpoints can be used in *in vivo* assays, including mitotic alterations, micronucleus induction and chromosome counting.

A.4.1 Mitotic Alterations

Mitotic alterations including C-mitoses, increases in mitotic metaphases, cell cycle delay, clumping, contraction of chromosomes, chromatid separation, anaphase and telophase bridges, chromosome lagging, multipolar spindles and diplochromosomes have been used to measure the induction of aneuploidy *in vivo* following treatment with chemicals. Mitotic effects were first described by Levan (1938) following treatment of *Allium cepa* with colchicine and various authors have suggested that these effects are possibly indicative of aneuploidy in other systems (Hsu *et al*, 1983; Liang and Satya-Prakash, 1985).

Miller and Adler (1992) as part of the EEC sponsored programme (Section 7.2) tested a series of 10 known or suspect spindle poisons in the bone marrow of mice using three endpoints; changes of mitotic index, induction of chromatid contraction and spreading and decreases of anaphase frequencies. Five out of the ten chemicals tested were positive, the other five showed no induction of C-mitotic effects under the conditions of test. It is not possible to evaluate the predictivity of this assay as it is not known which of the 10 chemicals tested are *in vivo* clastogens or aneugens. It should be noted that there is the potential to induce similar types of mitotic effects due to toxic effects.

It should also be noted that failure to record C-mitotic effects with the currently used procedures does not preclude the possibility of the chemical being an aneugen. In fact Adler *et al* (1993) reported that acrylamide did not induce mitotic arrest in bone marrow but meiotic delay and aneuploidy was observed in spermatocytes. To develop this assay further, investigation of the choice of treatment, the sampling times, the definition of artefacts and the standardisation of the observations of chromosome spreading and contraction will be required.

A.4.2 Micronucleus Induction

The mouse bone marrow micronucleus assay is widely accepted as a well validated and recommended method of assessing the clastogenic activity of chemicals *in vivo* (Mavournin *et al*, 1990). As micronuclei can result from whole lagging chromosomes as well as from the acentric chromosomal fragments produced by clastogens it was suggested by Cimino *et al* (1986) that the assay should be developed and validated for the *in vivo* detection of aneugens. The conventional assay (Heddle, 1973; Schmid, 1973; 1975) cannot differentiate between the two events. Adler *et al* (1991) as part of the EEC sponsored programme, investigated the ability to detect aneugens using the standard mouse bone marrow micronucleus test with May-Grunwald/Giemsa or Wright staining. The results showed that the technique detected 3 out of the 10 known or suspected aneugens that were tested. It is not known if all these 10 known or suspected aneugens actually expressed such activity *in vivo*. The negative results that were obtained with econazole and chloral hydrate were unexpected in light of the fact that these two materials

had previously been shown to induce C-mitotic effects in mouse bone marrow cells *in vivo* (Miller and Adler, 1989. However, it should be noted that the relationship between C-mitotic effects and aneuploidy is not well defined and that Russo and Levis (1992b) have reported chloral hydrate as positive in the mouse bone marrow micronucleus test using a longer harvest time. These results therefore indicate that the sensitivity of the standard mouse bone marrow micronucleus test for the detection of *in vivo* aneugens is unknown. In addition, it should be noted that the micronucleus test will detect chromosome loss but not nondisjunction and therefore may miss a proportion of aneugenic events.

Several methods have been developed to allow discrimination between the micronuclei produced by the chromosome breaking activity of clastogens and those arising from spindle malfunctions caused by aneugens. These include measuring DNA content (Heddle and Carrano, 1977; Vanderkerken *et al*, 1989; Van Hummulen *et al*, 1993), measuring the diameter (Yamamoto and Kikuchi, 1980) or area (Vanderkerken *el al*, 1989; Vanparys *et al*, 1990) of the micronuclei, the presence of C-band positive material in the micronuclei (Vanderkerken *et al*, 1989), micronucleus morphology (Yamamoto and Kikuchi, 1980; Tinwell and Ashby, 1991) and *in situ* hybridisation (Hogstedt and Karlsson, 1985). All the above methods have been reported as successful to varying degrees in discriminating between the micronuclei produced by clastogenic events and aneugenic events. Although the methods may be automated it should be noted that the assays are either technically difficult to perform or currently lack the validation that would be required prior to use as a routine assay. Furthermore these methods do not definitively confirm the presence of centromeres in the induced micronuclei.

In recent years, several publications have described more definitive methods for assessing micronuclei containing whole chromosomes. *In vitro* immunofluorescence methods for differentiating micronuclei containing kinetochores have been described (Degrassi and Tanzarella, 1988; Hennig *et al*, 1988; Thomson and Perry, 1988; Eastmond and Tucker, 1989a,b; Hennig, 1990). The method involves the use of an antikinetochore antibody originally discovered in the serum of CREST syndrome patients (Fritzler *et al*, 1980; Moroi *et al*, 1980). The antibody is used to detect the presence of kinetochores in the micronuclei which in turn indicates the presence of whole chromosome(s) or centric fragment(s).

A number of studies have been conducted using the CREST antikinetochore antibody to differentiate between micronuclei produced by aneugens and clastogens *in vivo* (Gudi *et al*, 1990; Miller and Adler, 1990; Miller *et al*, 1991). In these studies the effects of various known or suspected aneugens and clastogens were investigated in the mouse micronucleus assay. Analysis was conducted on the total erythrocyte population due to the inability of the technique to allow differentiation between the erythrocyte types. (In the standard mouse bone marrow micronucleus assay the micronuclei are measured in the polychromatic erythrocytes). Miller and Adler (1990) highlighted problems with unspecific staining, insufficient penetration of the antibodies into the micronuclei and the fact that the counterstaining with Hoechst 33258 did not allow differentiation between polychromatic and normochromatic erythrocytes. Miller *et al* (1991) also confirmed that counterstaining with Hoechst 33258, DAPI or propidium iodide did

not allow discrimination between the polychromatic and normochromatic erythrocytes. In contrast, acridine orange staining does allow this discrimination. Gudi *et al* (1990) who also used whole bone marrow smears reported that the presence of nucleated cells interfered with the accurate evaluation of the micronuclei due to the background fluorescence observed.

In attempts to overcome these problems a number of modified assays have been developed (Gudi *et al* 1990, 1992; Krishna *et al*, 1992). However, since mutagens can interfere with the synthesis of chemical proteins, false negative results for kinetochore staining have been reported. In addition, heterogeneity between CREST sera leads to variability in results and difficulty in interpretation. To overcome this, methods with newly developed direct centromere probes have been developed. Miller *et al* (1991) recently reported a fluorescent *in situ* hybridisation (FISH) method of detecting micronuclei containing centromeres using a DNA probe for mouse gamma satellite DNA. The results published by these authors are promising. Of course even with this technology, chromosome breakage at the centromere can not be excluded.

Further improvement of the analysis of centromere containing micronuclei include the use of flow cytometric and image analyses.

A.4.3 Chromosome Counting

The investigation of aneuploidy by evaluating hyperdiploidy in somatic cells by chromosome counting has been used for many years and provides direct evidence of chemically-induced aneuploidy. The method requires expert analysis and is time consuming. Unlike *in vitro* assays, techniques are not available *in vivo* to allow preservation of the cytoplasm of the target cells for accurate quantification of the number of chromosomes per cell. The analysis of hypoploidy, which can be the result of artefactual chromosome loss due to the cell processing and slide preparation techniques used, should therefore not be used to determine chemically induced aneuploidy *in vivo*.

Henderson and Regan (1986) and Zijno *et al* (1989) have reported the simultaneous assessment of cell cycle delay, hyperdiploidy and sister chromatid exchange in mouse bone marrow metaphases differentially stained after incorporation of BrdU. A number of parameters remain to be defined in this assay, for example the selection of appropriate sampling times. The effect of pretreatment of the animals with the spindle poisons, such as colchicine, to facilitate collection of the metaphases for analysis should not be problematic as long as a method is used to assess second division cells for hyperploidy.

In a review of the EEC Aneuploidy project, Adler (1993) reported that 4 of the 10 chemicals tested in two laboratories using hyperdiploidy in mouse bone marrow cells *in vivo* as an endpoint were positive, whereas 1 of the 10 chemicals tested as negative in both laboratories. It is of concern that 5 of the 10

chemicals tested yielded discordant results between the testing laboratories. These differences are difficult to interpret due to the significant differences in dose levels, sampling times and sample sizes that were used. Further validation of this assay type using strictly defined protocols is needed. In addition, the data demonstrate that the use of a single dose level and a single sampling time are not sufficient to obtain a reliable result (Adler, 1993).

The development of rodent chromosome specific probes should make the assessment of aneuploidy easier and less time consuming since interphase cells can be used.

A.5 *IN VIVO* GERM CELL METHODS

The induction of aneuploidy in germ cells is of concern as it may give rise to individuals with birth defects. There are fundamental differences between the meiotic process and the mitotic process e.g. requirement for chromosome pairing, formation of chiasmata to allow recombination, prolonged duration of meiosis in oocytes. Theoretically these may give rise to qualitative differences in response of germ cells and somatic cells to aneugens and the possibility of unique germ cell aneugens. The EEC programme on aneuploidy provided some support for this hypothesis (Adler, 1993) and there are a number of chemicals which have been reported to induce positive responses in germ cells but not somatic cells. However, interpretation of these results is confounded by inconsistencies between results in different laboratories and the use of deficient protocols. Pacchierotti and Mailhes (1991) point out that increased sensitivity of the METAPHASE II oocyte assay compared to assessing aneuploidy in bone marrow could occur due to the optimisation of exposure to the sensitive period in the former, while the latter involves exposure of asynchronous cells and possible dilution of damage by cells not exposed in their most sensitive period. The relative sensitivity of somatic and germ cells is an area requiring further work in order to assess the role of germ cell assays in a strategy for detection of aneuploidy.

Another area requiring investigation is the assessment of differences in response between males and females (Mailhes and Marchetti, 1994). It is recognized that female germ cells contribute more to the load of aneuploidy in human beings than male cells (Hassold, 1986) but it is not yet known whether one sex is more sensitive than the other to the induction of chemical aneugens in test animal species. Further information on this point is required to determine whether aneuploidy assays using a single sex would be sufficiently predictive of germ cell effects in both sexes.

As described above for the aneuploidy test systems in somatic cells, there are a variety of endpoints available for assessment. The major endpoint is chromosome counting but other approaches, as detailed below, are also used. Critical factors in the conduct of aneuploidy assays in germ cells are an in-depth understanding of the gametogenesis processes and skill at recognising meiotic stages and

analysing meiotic chromosomes in assays which use chromosome counts or morphology as the endpoints.

A.5.1 Male Germ Cells

Analysis of Secondary Spermatocytes

Secondary spermatocytes, which are in the second mitotic division, are the cell type of choice for aneuploidy studies in the male germ line. They can be used to assess the induction of aneuploidy in the mitotic spermatogonial divisions as well as the first meiotic division, although in practice the treatment protocol is usually chosen to allow assessment of post-mitotic divisions. The method for obtaining good quality chromosome spreads is well-established (Evans *et al*, 1964; Liang and Pacchierotti, 1988, Tease *et al*, 1993) and the method is the most widely used for assessment of germ cell aneugens. Assessment is made of the number of dyads (chromosomes) present and this is made easier and more accurate by the use of C-banding to identify centromeres. As previously recommended, only hyperploidy should be assessed due to the possibility of technical artifacts inducing hypoploidy.

The assay has been used to detect aneuploidy-induced by a number of chemicals, for example, cyclophosphamide (Pacchierotti *et al*, 1983), chloral hydrate (Russo *et al*, 1984), adriamycin, cytosine arabinoside (Liang *et al*, 1986), ethanol (Hunt, 1987), nitrotriacetic acid (Costa *et al*, 1988), colchicine, econazole, hydroquinone, vinblastine, diazepam, cadmium (Miller and Adler, 1992) and X-irradiation (Russo *et al*, 1983). A detailed evaluation of the data generated with the method is contained in Allen *et al*, (1986). Although the method is fairly widely used, inconsistent results are found between laboratories (Adler, 1993, Leopardi *et al*, 1993) and a number of variables which are likely to influence the performance of this assay require further investigation. These are detailed below.

Choice of Species and Strain

It is not yet known what contribution the choice of species and strain makes to the reported differences between laboratories. Differences in the quality of meiotic preparations obtained in different strains may also affect the performance of the assay (Liang *et al*, 1985).

Time of Treatment and Harvest

Treatment time must be chosen to ensure that cells are exposed at the most sensitive stage. Many reports in the literature use a single harvest time and the results must be treated as preliminary, particularly in the case of negative responses. The optimal time may be affected by chemically-induced cell progression delay (as well as variability due to absorption and metabolism of the test substance) and,

as these cannot be predicted in advance, multiple harvest times should be used. Differences in sensitivity in induction of aneuploidy by chloral hydrate at different stages of spermatogenesis have been reported with the mitotic gonadal stages being most sensitive (Russo *et al*, 1984). In contrast, metaphase I, diplotene and late pachytene stages were much more sensitive to aneuploidy-induction by X-rays than earlier meiotic and spermatogonial stages (Russo *et al*, 1983). Quantitative differences in response at different harvest times have also been reported for p-fluorophenylalanine (Brook and Chandley, 1986) and colchicine, chloral hydrate and vinblastine (Miller and Adler, 1992).

Dose Selection

Rarely is the rationale for dose selection given in publications. This leads to a lack of confidence in negative results. Little is known of dose responses to aneugens but it should be appreciated that compounds may be effective over a very narrow dose range. Therefore little confidence can be attached to negative results obtained using only a single dose. In addition, doses close to the lethal dose have occasionally been used to demonstrate a positive response (Hunt, 1987; Costa *et al*, 1988) and the use of such high doses is questionable.

Inclusion of Colchicine

Colchicine is used by some workers to increase the number and quality of metaphase II cells (Liang *et al*, 1985; Liang and Pacchierotti, 1988) but is not favoured by others who object to the possible interference in the use of an aneugen and its confounding effect on estimation of meiotic delay (Adler, 1993).

Assessment of Micronuclei in Spermatids

Methods for the detection of micronuclei in spermatids have been described (Tates *et al*, 1983; Lahdetie, 1988) and these have been used to study the induction of clastogenicity and nondisjunction by environmental mutagens. The method was shown to be insensitive to induction of nondisjunction in a mouse strain with a high nondisjunction frequency (Tates and de Boer, 1984) but it might be useful for detecting chromosome lagging. Vinblastine was negative in this assay despite evidence that the chemical reached the target tissue (Tates, 1992) and also induced hyperploidy in secondary spermatocytes (Miller and Adler, 1992). This may indicate a low sensitivity of this endpoint although the interpretation is confounded by a report from another laboratory of a negative response in secondary spermatocytes (Liang *et al*, 1986). In contrast an increased sensitivity of this endpoint is indicated by the results of Russo and Levis (1992a), who reported the induction of micronuclei in spermatids by chloral hydrate and EDTA, whereas EDTA did not induce hyperploidy in mouse secondary spermatocytes (Zordan *et al*, 1990). The methods have been refined by incorporating an antikinetochore antibody or by the use of FISH to distinguish between clastogens and aneugens (Collins *et al*, 1992, Kallio and Lahdetie, 1993). Further work is required to critically evaluate the sensitivity of this endpoint.

Other approaches

Assessment of Post-Meiotic Stages

The induction of numerical chromosome changes in germ cells can be assessed by the incidence of progeny with numerical chromosome abnormalities. Aneuploidy-induced in metaphase II can only be detected by studying post-meiotic stages. Although the F_1 progeny can be assessed at birth this is an expensive option and is insensitive due to the selection against aneuploid embryos. The preference is therefore to use either one-cell zygotes, pre-implantation or early post-implantation embryos. The use of one-cell zygotes allows the chromosome complement of both the father and the mother to be assessed separately as the parental sets remain separated and are morphologically different. This method has been used by a number of workers (Albanese, 1987a; Mailhes *et al*, 1990; Marchetti *et al*, 1992).

Analysis of Synaptonemal Complexes

Analysis of the synaptonemal complex (SC) by electron microscopy (Allen *et al*, 1988a,b) provides a possible approach to the detection of chemicals which act by disrupting homologous chromosome pairing at pachytene. SC preparations can be made from both male and female germ cells. Analysis of these preparations is carried out by electron microscopy and therefore requires expertise and specialised equipment.

Meiotic Delay

The numbers of spermatogonial mitoses, metaphase I and metaphase II cells can be scored to assess the induction of meiotic delay (Miller and Adler, 1992). This endpoint has the advantage of being considerably faster than chromosome counting and has been suggested as a screening assay for germ cell aneugens after standardisation and validation of the protocol (Miller and Adler, 1992; Adler, 1993). Further work needs to be performed to determine whether this endpoint is accurate at differentiating cytotoxic chemicals from aneugens.

Frequency of Chiasmata or Univalents

Chiasmata frequency or the incidence of univalents in metaphase I (indicative of lack of homologue pairing) have been assessed as endpoints for detection of aneugens (Polani and Jagiello, 1976; Liang *et al*, 1986). The lack of correlation between the incidence of univalents and aneuploidy at metaphase II from a variety of studies suggests this endpoint may not be an accurate measure of aneugenic potential (Polani and Jagiello, 1976; Allen *et al*, 1986; Liang *et al*, 1986; Tease and Cattanach, 1986; Liang and Pacchierotti, 1988).

Fluorescent in situ hybridisation (FISH)

The use of nonradioactive *in situ* hybridisation methods with chromosome specific probes for analysis of loss or gain of whole chromosomes in spermatids provides another potential approach. This has been applied to the study of nondisjunction in human semen samples (Coonen *et al*, 1991; Holmes and Martin, 1993). Variability in aneuploidy frequencies between different laboratories has been reported and there are some technical problems associated with probe penetrance and hybridisation efficiency to be overcome but the approach appears promising. It has not yet been applied to experimental animals. Such methods may be an improvement on the assessment of heterochromatin patterns (Tates, 1979) or Y-bodies in spermatids, which were prone to artefactual results.

A.5.2 Female Germ Cells

The analysis of chromosome damage during oogenesis is technically more demanding and the numbers of cells available for analysis are fewer than in the male. The first meiotic division of oogenesis occurs during gestation, where the cells are arrested at the diplotene stage and remain at this stage until just prior to ovulation. At this time, cells continue the meiotic division and proceed to metaphase II. They remain at this stage until the egg is fertilised, at which time, meiosis is completed. Aneuploidy may be assessed in either metaphase I, metaphase II or early embryonic cells. All of these test systems are at an early stage of validation; their technical nature and the small number of laboratories experienced in the techniques has limited the extensive investigation of the critical parameters involved.

Analysis of Metaphase II Oocytes

The analysis of metaphase II can detect aneuploidy induced in the mitotic divisions which occur pre-natally or in metaphase I. However the treatment period is usually chosen to enable assessment of aneuploidy-induced in metaphase I only. As such, this is most relevant to the human situation where marker studies indicate most aneuploidy in human beings arises from nondisjunction at metaphase I. Superovulation is usually used in order to obtain sufficient cells for analysis. The animals are treated with pregnant mare's serum and human chorionic gonadotrophin (HCG) and metaphase II cells are collected approximately 16 hours later. The ovulated oocytes can be collected from the oviduct and chromosome spreads prepared. As described for the assessment of secondary spermatocytes, the endpoint is the number of dyads present. Hyperploidy must be distinguished from hypoploidy to avoid artifacts due to chromosome loss during chromosome preparation.

The method has been fairly widely used to detect aneuploidy induced by a number of substances, for example, methylmercury (Mailhes, 1983), colchicine (Mailhes and Yuan, 1987; Mailhes *et al*, 1990), vinblastine sulphate (Russo and Pacchierotti, 1988; Mailhes *et al*, 1993a), benomyl (Mailhes and Aardema, 1992), cadmium chloride (Watanabe *et al*, 1977, 1979), cyclophosphamide (Becker and

Schoneich, 1982), norethindrone acetate (Rohrborn and Hansmann, 1974), trenimon (Becker and Schoneich, 1982) and griseofulvin (Tiveron *et al*, 1992; Mailhes *et al*, 1993b). Negative responses have been reported for pyrimethamine, diethylstilboestrol diphosphate and chloral hydrate (Mailhes *et al*, 1988, 1993a), 6-mercaptopurine, phenylalanine and fluorophenylalanine (Brook and Chandley, 1985), 4-chloromethylbiphenyl (Brook, 1982) and cyclophosphamide (Yuan and Mailhes, 1987). A number of variables have been identified which could affect the performance of this assay. These are detailed below.

Choice of Species and Strain

Mice and Chinese and Syrian hamsters are the species which have been most frequently used. There may be differences between species in sensitivity to aneugens. Sugawara and Mikamo (1980) were able to analyse metaphase II oocytes in Chinese hamsters after a dose of 3mg/kg colchicine, but this dose completely blocks progression from metaphase I to metaphase II in mice (Mailhes and Yuan, 1987). The possibility of strain and species differences has led to difficulties in interpretation where differing results have been found by different investigators, for example, differential response to cyclophosphamide (Becker and Schoneich, 1982; Yuan and Mailhes, 1987).

Method of Obtaining Ovulated Oocytes

There does not appear to be any increase in background frequency of aneuploidy induced by superovulation (Golbus, 1981) and the use of this technique does not impose any interpretation problems. In contrast, the use of an *in vitro* ovulation step induces aneuploidy (Golbus, 1981).

Time Course

Experiments have been conducted to determine the optimal time for administration of the test chemical. The optimal timing may depend on the mode of action of the test chemical. For example, colchicine impairs microtubule formation and consequently must be present during microtubule assembly for effect. Mailhes and Yuan (1987) reported the maximum induction of hyperploidy by colchicine was found when it was administered at the same time as the human chorionic gonadotrophin (HCG), corresponding to 8 hours before metaphase I. Lower frequencies were found when treatment was performed 2-4 hours before or after HCG treatment. Treatment at this time is also effective in inducing hyperploidy by vinblastine (Russo and Pacchierotti, 1988). Pacchieriotti *et al* (1989) showed that griseofulvin induced hyperploidy when administered at 6 hours prior to metaphase I but not at 8 hours. The difference between this result and that reported for colchicine is not clear as the target for griseosulvin also appears to be microtubule associated proteins. In another study griseofulvin induced hyperploidy when administered at the same time as HCG (Mailhes *et al*, 1993a). At this stage it is only possible to state that different dosing regimes may be required to detect activity of other aneugens to allow for differences

in targets during the meiotic process and for cell cycle delay and absorption and distribution of the test substance. Further work is required to establish whether a single optimal treatment time (probably simultaneous with the HCG injection) would be sufficiently sensitive for detecting female germ cell aneugens. A possible way of keeping the size of the study manageable, while still ensuring that different stages are exposed, is to use split doses, as employed by Mailhes *et al* (1993a). This approach requires further evaluation.

Dose Selection

Rarely is the rationale for dose selection given in publications, resulting in a lack of confidence in negative results. The choice of dose may be limited by having sufficient metaphase II cells available for analysis. In their critical review of the test method Mailhes *et al* (1986) recommend that the top dose induces "significant toxicity" and state "it would be most appropriate to base the doses on toxicity to the oocytes themselves, but this is both time consuming and expensive". As discussed for aneuploidy induction during male gametogenesis, a number of widely spaced doses are recommended to detect compounds effective over a narrow dose range.

Study Design

Sample size is limited by technical feasibility. Improvements in the assay have led to more consistent spontaneous aneuploidy levels in different laboratories and this should allow the assessment of the sample sizes required for obtaining adequate statistical power. It should be noted that large numbers of animals are required to obtain a reasonable number of analysable cells. Mailhes *et al* (1988) obtained 2-8 (average 4-5) analysable oocytes per animal and reported that it is possible to process 25 animals in a 3 hour period.

Analysis of Metaphase I Oocytes

Metaphase I oocytes can only be obtained by induction of ovulation by exogenous gonadotrophic hormones or *in vitro* culture techniques (Tease *et al*, 1993). It is difficult to obtain sufficient cells for analysis without use of a mitotic inhibitor. Aneuploid metaphase I oocytes are the result of nondisjunction or chromosome lagging during the mitotic divisions which occur prenatally. Therefore an assay based on this endpoint would require treatment of pregnant animals and assessment of the offspring. Such an assay would be complex, very expensive and has a role as a research tool only. Based on the present state of our knowledge it does not seem likely to give any information on aneugenic potential over that obtainable from analysis of metaphase II oocytes.

Other approaches

Meiotic Arrest

Metaphase I oocytes are not normally ovulated and consequently the frequency of ovulated metaphase I oocytes can be used to detect whether meiotic arrest or delay has occurred. Increased frequencies have been detected following exposure to colchicine (Mailhes and Yuan, 1987), griseofulvin (Pacchierotti *et al*, 1989; Tiveron *et al*, 1992; Mailhes *et al*, 1993b), benomyl (Mailhes and Aardema, 1992) and vinblastine sulphate (Russo and Pacchierotti, 1988). Although the database is small, there is no evidence of chemicals which induce meiotic delay and do not also induce aneuploidy in metaphase II oocytes. The numbers of polyploid oocytes or metaphase II oocytes associated with a polar body can also be measured. It has been suggested that polyploidy may result from gross damage whereas lower doses of an aneugen may have more subtle effects resulting in hyperploidy (Mailhes and Aardema, 1992).

Assessment of Post-Meiotic Stages

As described above for males, post-meiotic stages can also be used to assess induction of aneuploidy in the female. For this the females are mated following superovulation and chemical treatment. Colchicine is used to arrest the cells in metaphase. One-cell embryos have been used to demonstrate the induction of aneuploidy by ethanol (Kaufmann, 1983), X-irradiation (Tease, 1982), griseofulvin (Marchetti *et al*, 1992) and vinblastine in female mice (Albanese, 1987b). Preimplantation embryos have been used to show the induction of aneuploidy in female mice treated in the preovulatory phase with triazoquinone (Hansmann and Rohrborn, 1973) and cadmium chloride (Watanabe and Endo, 1982). The disadvantage of the assessment of post-meiotic stages, particularly post-one-cell embryos, is the possibility of selection against aneuploid cells. However for some chemicals this approach may be more sensitive than the assessment of metaphase oocytes. Generoso *et al* (1989) showed treatment with nocodazole of metaphase II oocytes (i.e. treatment at around the time of sperm entry) was more effective in inducing embryonic death (possibly induced by chromosome malsegregation) than treatment prior to ovulation. Similarly Watanabe and Endo (1982) found higher frequencies of aneuploidy in preimplantation embryos following treatment with cadmium than they had previously found in metaphase II oocytes (Watanabe *et al*, 1977).

A.5.3 Methods for assessing aneuploidy in embryos or F_1 progeny

Autosomal aneuploidy is lethal in mice, with deaths occurring in embryonic development for monosomies and during mid-gestation for trisomies. Therefore tests are either based on the detection of sex chromosome aneuploidies using genetic markers on the X chromosome or by using stocks that have a high frequency of nondisjunction (e.g. Robertsonian translocation bearing animals) and detecting complementation that gives rise to chromosomally balanced survivors (reviewed by Russell, 1986). Very large sample sizes are required to obtain a conclusive result, the methods are extremely costly and

require the use of special stocks of mice. One advantage they have over cytogenetic methods is their ability to detect chromosome loss. Very few agents have been assessed in these assays. Most work has been performed with radiation, although an experiment on methylmethane sulphonate showed induction of nondisjunction in the sex chromosome aneuploidy test (Russell, 1986). Danford and Parry (1986) used the sex chromosome method to study induction of aneuploidy by vincristine in mice.

A.6 VALIDATION REQUIREMENTS FOR ANEUPLOIDY TEST METHODS

There are two levels of validation required for assays detecting aneuploidy. The first level is the validation of the individual tests to be validated and the second the requirement to validate the strategy for detecting aneugens as discussed in Section 7. Before accepting a new assay as validated for regulatory purposes a number of points must be addressed.

Accurate Detection of the Endpoint of Interest

Short-term tests for aneuploidy are designed to test whether compounds can induce changes in somatic cells (which may contribute to carcinogenicity) or in germ cells (which may contribute to birth defects and reproductive loss). Since there are no definitive human germ-cell aneugens at this time, and only a few human carcinogens have been proposed to involve induction of aneuploidy, chemicals with established aneuploidy-inducing effects in rodent germ cells or somatic cells must be used in the validation of new assays. Based on the data reviewed the following chemicals are concluded to be definitive aneugens in germ and in somatic cells *in vivo*: benomyl, carbendazim, colchicine, griseofulvin, hydroquinone, vinblastine sulphate. This conclusion is based on the fact that consistent, positive results have been reported for these chemicals. Some of these chemicals, like hydroquinone, may induce structural chromosome aberrations in addition to induction of aneuploidy. These chemicals require special consideration. The appropriate data set to use in validating *in vivo* somatic cell aneuploidy assays are the definitive germ-cell aneugens. Likewise, *in vitro* aneuploidy assays must be validated against known *in vivo* aneugens.

Determination of the Appropriate Protocol

It is essential to know what factors affect the sensitivity of the test system so that evaluators of the test results can determine whether appropriate protocols have been used. For example, factors such as harvest times, effects of toxicity and treatment duration may be applicable using *in vitro* studies and choice of species, treatment duration, toxicity limits and strain may be applicable using *in vivo* studies. Clearly though, a screening assay is not going to be optimal for every chemical. The decision to use an assay or not has to be based on whether it appears to be adequate with the knowledge at the time.

Reproducibility between Laboratories and within Laboratories

Comparative trials should be conducted with several laboratories using an acceptable set of test substances. These should be chosen on the basis of established effects as discussed above, and considering different mechanisms. Non-aneugens together with known aneugens that cover a range of toxicity should be used. Coded samples eliminate bias in scoring.

Criteria for a Positive and Negative Response

Sufficient data should be available to define the background range and to determine biologically meaningful responses. The appropriate statistics and the power of the assay need to be considered. The consistency of background levels of aneuploidy in the test needs to be evaluated so that consideration can be given to evaluating responses against concurrent controls or pooled or historical controls. For example, the use of pooled controls has been used in the spermatocyte assay to improve the statistical power of the assay (Miller and Adler, 1992).

Other Considerations

Data required under official regulations, must be produced according to good laboratory practice and may be produced at contract laboratories. On the basis of these criteria, no short-term tests for direct measurement of aneuploidy can currently be considered to be validated. None of the test systems have been adequately evaluated in terms of sensitivity, specificity or accuracy in detecting established aneugens. Although it is not possible to generalise across all methods, most of them have not been fully investigated for protocol factors affecting sensitivity. The EC aneuploidy project has provided the most comprehensive study to date on interlaboratory reproducibiltiy. However, the results indicate significant differences between laboratories. This is now being studied as part of a second project. This approach may lead to the validation of some of the test methods.

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APPENDIX B - HISTORICAL PERSPECTIVE OF COORDINATED ACTIVITIES RELATED TO ANEUPLOIDY

DATE	AGENCY/SPONSOR	FUNCTION	PURPOSE	RESULTS/CONCLUSIONS
1978	National Institute of Environmental Health (NIEHS)	Workshop: Systems to Detect Induction of Aneuploidy by Environmental Mutagens (US-EPA, 1979)	To assess systems to detect aneuploidy	Need for short-term tests National Toxicology Program (NTP) initiated studies at NIEHS in yeast and *Drosophila*
1983	US-EPA Reproductive Effects Assessment Group (REAG)	Workshop:"Approaches for Assessing the Risk of Aneuploidy-induction by Chemicals" (Dellarco *et al*, 1985)	To assess mechanisms, progress of test development, and to compile a chemical data base	Need for studies into mechanisms of aneuploidy Aneuploidy Data Review Committee established 1984
1984	US-EPA Federal Register	Regulatory Guidelines	Provide guidelines for analysis of mutagenicity data	Guidelines have not been set for assays to analyse aneuploidy. Lack of well validated test methods. Limited understanding of mechanisms.
1984	US-EPA Aneuploidy Data Review Committee Formed (directed by REAG and Health Effects Research Labs within Office of Res. and Dev. US-EPA)	Review of data from test systems (Dellarco *et al,* 1986a)	To review specific test methods database; provide guidance for test development and validation	New assays needed Standardisation of existing assays needed
1985	US-EPA/NIEHS	Symposium: "Aneuploidy: Etiology and Mechanisms" (Dellarco *et al*, 1986b)	Review of test systems	New assays needed Standardisation of existing assays needed
1986	US-EPA Federal Register	Regulatory Guidelines	Provide guidelines for analysis of mutagenicity data	Several endpoints of concern: Point mutations Structural chromosome damage Numerical chromosome changes (Aneuploidy) Assays need further development/validation.
1986-1990	EEC - STEP Programme	Coordinated research programme in Europe (Parry and Natarajan, 1993)	To evaluate assay systems for detecting aneugens, provide recommendations to EC authorities	Recommendation for tiered screen for division abnormalities

DATE	AGENCY/SPONSOR	FUNCTION	PURPOSE	RESULTS/CONCLUSIONS
1988	US-EPA	Publication (Dellarco, V.L and Jacobson-Kram, Aneuploidy Part B: Induction and Test Systems, 1988)	Provide updated opinion on numerical chromosome changes	"The Agency acknowledges aneuploidy as an important consideration in assessing human risk and will use information on numerical chromosome abnormalities to the extent possible"
1991	US-EPA	Proposed Office of Pesticides Programme (OPP) Guidelines	Provide guidelines for genotoxicity testing	Results from initial batteries may require confirmation which may include an assessment of numerical chromosome alterations
1991	EEC	1991 Directives	Provide directives for classification of dangerous substance	Included numerical chromosome changes in definition of mutation
1991-1995	EEC - STEP Aneuploidy Programme	Investigate methods, mechanisms, provide guidance to EC	Follow-up programme	Estimated initial recommendation to EC 1996
1992	NATO, US Dept. Energy, Commission of European Community	Workshop: "Chromosome Segregation and Aneuploidy"	To review mechanisms of aneuploidy-induction and test methods	
1993	EEC	New Guidelines	Provide guidelines for genotoxicity testing	Include a clause for considering the potential of a chemical to induce numerical changes when exposure is significant
1993	Canadian Health and Welfare	New Guidelines	Provide guidelines for genotoxicity testing	Requests analysis of numerical chromosome changes *in vitro*
1994 1996	OECD	Proposed New Guidelines (Revised 1996)	Provide guidelines for genotoxicity testing worldwide	Requests specific scoring of polyploid cells *in vitro* which was previously not specified. A detailed review paper is being written to establish whether a separate oecd guideline should be formulated for numerical chromosome changes.
1986-1990	EEC - STEP Programme	Coordinated Research Programme (Parry and Natarajan, 1993)	To evaluate assay systems for detecting aneugens and provide recommendations to the EC authorities	Recommendation of a tiered screen for division abnormalities

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APPENDIX C - HAZARD CLASSIFICATION SCHEMES APPLICABLE TO ANEUGENS

C.1 INTRODUCTION

A number of international regulatory bodies have established guidelines for classifying mutagens (hazard identification or qualitative risk assessment). These include the EEC Classification on the Basis of Specific Effects on Human Health (EEC, 1993), Canada's Hazardous Products Act: Controlled Products Regulations (Canada 1988), and US-EPA Guidelines for Mutagenicity Risk Assessment Guidelines (US-EPA, 1986). The focus of all three schemes is the potential of chemical mutagens to induce heritable mutations in man. The most convincing evidence is that obtained from well conducted epidemiological studies demonstrating a strong association between chemical exposure and heritable damage in man.
To date, despite the fact that a number of genetically transmitted disorders are being recognised in the human population, no substance has been classified as a human germ-cell mutagen. This is because:

- any specific mutation is a rare event,
- only a small fraction of the estimated thousands of human genes, and conditions, are useful markers,
- human genetic variability,
- small numbers of offspring per individual,
- long generation times,
- only disorders caused by dominant mutations, some sex-linked recessive mutations and certain chromosomal aberrations can be detected in the first generation after their occurrence, and
- conditions caused by autosomal recessive mutations or by polygenetic traits may go unrecognised for many generations.

Because of the current difficulties in establishing a causative relationship between mutagen exposure and heritable effects in man, chemically-induced heritable genetic damage in appropriately conducted animal studies should be regarded as if they are (at least) equally hazardous to man.

C.2 EEC CLASSIFICATION OF MUTAGENIC SUBSTANCES

The EEC has established an hierarchical three-category system for the classification of mutagens (EEC, 1993).

Category 1

Substances known to be mutagenic to man. There is sufficient evidence to establish a causal association between human exposure to a substance and heritable genetic damage.

To place a substance in Category 1, positive evidence from human mutation epidemiology studies will be needed. Because it is extremely difficult to obtain reliable information from studies on the incidence of mutations in human populations or on possible increases in their frequencies, there are to date no compounds in this category.

Category 2

Substances which should be regarded as if they are mutagenic to man. There is sufficient evidence to provide a strong presumption that human exposure to the substance may result in the development of heritable genetic damage, generally on the basis of: appropriate animal studies or other relevant information.

Compounds in this category are positive in assays showing either mutagenic effects or other cellular interactions relevant to mutagenicity in germ cells of mammals, or mutagenic effects in somatic cells of mammals with clear evidence that the chemical or a relevant metabolite reaches the germ cells.

Methods described to place substances in Category 2 include:

2a: *in vivo* germ-cell mutagenicity assays, specific locus, heritable translocation and dominant lethal tests;
2b: *in vivo* assays showing relevant interaction with germ cells (usually DNA). These include assays for chromosome abnormalities as detected by cytogenetic analysis including aneuploidy, sister chromatid exchanges, unscheduled DNA synthesis, germ cell DNA adduct formation or other assays for DNA damage. Positive results in these assays would normally be supported by positive *in vivo* somatic cell mutagenicity assays in mammals or man (Category 3).
2c: *in vitro* assays (stated in the Guidelines as *in vitro*, but actually means *in vivo*; see category 3)) showing mutagenic effects in somatic cell mutagenicity assays in combination with toxicokinetic methods or other techniques that demonstrate the chemical or relevant metabolite reaches the germ cells.

Category 3

Substances which cause concern for man owing to possible mutagenic effects. There is evidence from appropriate mutagenicity studies, but this is insufficient to place the substance in Category 2.

Category 3 compounds are positive in assays showing mutagenic effects or other cellular interactions relevant to mutagenicity in somatic cells in mammals *in vivo*.
Methods described to place compounds in this category include:

3a: *in vivo* somatic-cell mutagenicity assays (bone marrow micronucleus or metaphase analysis, metaphase analysis of peripheral lymphocytes, mouse spot test).
3b: *in vivo* somatic-cell DNA interaction assays (SCE, UDS, DNA binding or damage in somatic cells).

Compounds in Category 1 or Category 2 are assigned symbol T (toxic) and risk phrase R46: “May cause heritable damage"; Category 3 compounds receive symbol Xn (harmful) and risk phrase R40: “Possible risk of irreversible effects".

With reference to aneuploidy, the Guidelines state that alterations leading to mutations "may involve a single gene, a block of genes, or a whole chromosome" and that "effects on whole chromosomes may involve structural or numerical changes".

C.3 CANADA

Canada's Hazardous Products Act (Canada, 1988) places mutagens under Class D-Poisonous and Infectious Material, Division 2: Material Causing other Toxic Effects. Germ-cell mutagens are in Subdivision A: Very Toxic Material whilst somatic mutagens are in Subdivision B: Toxic Materials. In contrast a chemical placed in Division 1 is a “material causing immediate and serious toxic effects”.

Subdivision A: Very Toxic Material

1. A pure substance or test mixture fall into Subdivision A of Division 2 of Class D-Poisonous and Infectious Material if:
1.1 there is epidemiological evidence that shows a causal connection between exposure of persons to the substance or mixture and heritable genetic effects; or
1.2 there is evidence of mutagenicity in mammalian germ cells *in vitro* as shown by:

- positive results in a study that measures mutations transmitted to offspring, or
- positive results in an *in vivo* study showing chemical interaction with genetic materials of mammalian germ cells and positive results in an *in vivo* study assessing either gene mutation or chromosomal aberration in somatic cells.

2. The experimental evidence referred to in paragraph 1.1 shall be obtained
2.1 in accordance with test methods described in the "Introduction to the OECD Guidelines on Genetic Toxicity Testing and Guidance on the Selection and Application of Assays", dated March 1, 1987 published in the Third Addendum to the OECD *Guidelines on Genetic Toxicology for Testing of Chemicals*; and

2.2 using testing strategies described in the *Guidelines on the Use of Mutagenicity Tests in the Toxicological Evaluation of Chemicals*, dated 1985, published under the authority of the Minister of National Health and Welfare and the Minister of the Environment.

Subdivision B: Toxic Material

A pure substance or tested mixture falls into Subdivision B of Division 2 of Class D - Poisonous and Infectious Material if evidence of mutagenicity in mammalian somatic cells *in vivo* is obtained in a test to assess either gene mutations or chromosomal aberration carried out

- in accordance with test methods described in the “Introduction to OECD Guidelines on Genetic Toxicology Testing and Guidance on the Selection and Application of the Assays”.
- using testing strategies described in the *Guidelines on the Use of Mutagenicity tests in the Toxicolgical Evaluation of Chemicals,* dated 1986, published under the authority of the Canadian Minister of National Health and the Minister of the Environment.

C.4 US-EPA GUIDELINES FOR MUTAGENICITY RISK ASSESSMENT

The central theme of the Guidelines for Mutagenicity Risk Assessment (US-EPA, 1986) "is to provide judgement concerning the weight of evidence that an agent is a potential human mutagen capable of inducing transmitted genetic changes, and if so to provide a judgment on how great an impact this agent is likely to have on public health". Although the EPA emphasises its concern with risk associated with both germ-cell and somatic-cell mutations, the "guidelines are only concerned with genetic damage as it relates to germ-cell mutations". Somatic cell effects are in the Guidelines for Carcinogen Risk Assessment.

In evaluating chemically-induced mutagenic hazard (qualitative risk assessment), the primary mutagenic endpoints of concern are point mutations, structural chromosome aberrations, and numerical chromosome aberrations. Numerical aberrations are defined as gains or losses of whole chromosomes (e.g.trisomy, monosomy) or sets of chromosomes (haploidy, polyploidy). Many mutations can directly induce alterations in DNA. Although EPA recognises that some mutagens may induce mutations through non-DNA binding mechanisms (e.g. induced lesions in non-DNA targets such as tubulin) the guidelines give no practical distinction between hazard associated with DNA damaging mutagens and those which exert their action by other mechanisms. Since chemicals causing numerical chromosome aberrations (aneuploidy, polyploidy) may not be detected if evaluated in tests only for DNA damage, gene mutations, chromosomal breakage or rearrangement, the regulations mention the need to consider tests for such compounds in the total assessment of mutagenic hazards.
The EPA describes the evidence for germ cell mutations in terms of:

- **Direct Evidence** from mutational germ-cell studies in mammals (specific locus, heritable translocation locus tests, for example),
- **Sufficient Evidence** which demonstrates the interaction of an agent with mammalian germ-cell DNA or other chromatin constituents, or that the chemical induces such endpoints as UDS, SCEs, or chromosomal aberrations in germ cells, or
- **Suggestive Evidence** which describes adverse gonadal effects such as sperm abnormalities following acute, subchronic or chronic toxicity testing, or findings of adverse reproductive effects (e.g. decreased fertility) consistent with the chemical's interaction with the germ cells.

In this respect, the hazard determination may include non-mutagenicity endpoints such as reproduction, metabolism, or knowledge of mechanism of action.

The EPA established the following eight categories (in decreasing order of strength of evidence) for consideration regarding the potential of an agent to induce heritable damage in man:

1. Positive results derived from human germ-cell mutagenicity studies, when available, will constitute the highest level for human mutagenicity.
2. Valid positive results from studies on heritable mutational events (of any kind) in mammalian germ cells.
3. Valid positive results from mammalian germ-cell chromosome aberration studies that do not include an intergeneration test.
4. Sufficient evidence of a chemical's interaction with mammalian germ cells, together with valid positive mutagenicity test results from any two assay systems, at least one of which is mammalian (*in vivo* or *in vitro*). The positive results may both be for gene mutations or both for chromosome aberrations; if one is for gene mutations and the other for chromosome mutations, both must be from mammalian systems.
5. Suggestive evidence for a chemical's interaction with mammalian germ cells, together with valid positive mutagenicity evidence from two assay systems as described under 4. above, when combined with sufficient evidence for a chemical's interaction with mammalian germ cells.
6. Positive mutagenicity test results of less strength than defined under 4. combined with suggestive evidence for a chemical's interaction with mammalian germ cells.
7. Although definitive proof of non-mutagenicity is not possible, a chemical could be classified operationally as a non-mutagen for human germ cells, if it gives valid negative test results for all endpoints of concern.
8. Inadequate evidence bearing on either mutagenicity or chemical interaction with mammalian germ cells.

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APPENDIX D - REGULATORY EVALUATION OF KNOWN ANEUGENS

Benomyl (CAS No 17804-35-2)

One example that illustrates the impact of aneuploidy data on the development of a chemical occurred in the late 1970's when the US-EPA reviewed the pesticide benomyl (see Voytek, 1985; Dellarco and Jacobson-Kram, 1988).

Benomyl and its metabolite carbendazim (methyl-2-benzimidazole carbamate, CAS No 10605-21-7) had been reported to induce aneuploidy in *Aspergillus nidulans* (Hastie, 1970) but were considered at most weak mutagens due to predominantly negative results in assays for mutations and structural chromosome aberrations. As there was no precedence for conducting a risk assessment on aneuploidy, additional data from cancer, teratology and mutation assays were requested by the US-EPA. Since then it has been reported that benomyl induces carcinogenic and teratogenic effects as well as aneuploidy in *in vitro* and *in vivo* in somatic and in germ cells (Mailhes and Aardema 1992; IPCS, 1993a; Van Hummelen *et al*, 1995). The risk assessment for benomyl was based on the carcinogenic and teratogenic effects and it was concluded that the benefits of benomyl use exceeded the risks in use, which can be reduced by wearing protective equipment (US-EPA, 1982).

As part of the decision process, the US-EPA requested suggestions from the "Benomyl Scientific Advisory Panel" (SAP) methods for estimating human risk from chemicals which cause spindle effects (US-EPA, 1982). The SAP indicated that a multigeneration reproductive test, dominant lethal assay, and *in vivo* cytogenetic assay in sperm and bone marrow were the best methods available for determining human risk from exposure to spindle poisons. The US-EPA concluded that these methods were not sensitive enough and that new methods were needed. The US-EPA also stated that inhibition of tubulin activity can affect many cellular processes, therefore the appropriate toxicological endpoints of concern need to be defined before a risk assessment can be conducted.

Taking into consideration the kinetics of the affinity of spindle inhibitors for tubulin and the subsequent inhibition of tubulin polymerisation, the SAP came to the conclusion that a threshold existed for chemicals with spindle effects such as benomyl, colchicine, and the vinca alkaloids. The evidence for the existence of thresholds for aneuploidy-inducing chemicals is discussed in Section 7 of this report. In contrast, the US-EPA concluded that it could not be unequivocally demonstrated that a threshold for spindle effects existed based on the available data, again in part because they felt the relevant toxicities were unknown (US-EPA, 1982). Thus, the spindle effects were not used in the decision process. In contrast, in the recent benomyl review by IPCS, it was concluded that the toxicities observed with benomyl (IPCS, 1993a) and carbendazim (IPCS, 1993b) were consistent with what has been seen with other spindle

poisons, and are due to the binding of the inhibitor to tubulin. The mechanistic data on the selectivity of benomyl and carbendazim for fungal tubulin compared to mammalian tubulin supported the conclusion that toxic effects in man are unlikely (IPCS, 1993a,b).

Colchicine (CAS No 64-86-8) and ***Colcemid*** (CAS No 477-30-5)

Colchicine and the synthetic version colcemid, are model compounds for studies of aneuploidy induction *in vitro* and *in vivo*. Colchicine is used for the treatment of gout. According to the Physician's Desk Reference (1993), colchicine has been reported to adversely affect spermatogenesis in animals and azoospermia in man has been reported. Colchicine is listed as having the ability to cause foetal harm in pregnant women and it is contraindicated in pregnant patients due to teratogenic effects. Relative to carcinogenicity, the Physician's Desk Reference states that "Since colchicine is an established mutagen, its ability to act as a carcinogen must be suspected...".

Griseofulvin (CAS No 126-07-8)

Griseofulvin has been shown to induce numerical chromosome changes *in vitro* and *in vivo*. Griseofulvin is fungistatic and used in the treatment of fungal infections in keratin, particularly skin, hair, and nails. According to the Physician's Desk Reference (1996), griseofulvin is listed as having colchicine-like effects. Suppression of spermatogenesis has been reported in rats but this has not been confirmed in man. Due to the teratogenicity of griseofulvin, warnings indicate that the substance should not be prescribed to pregnant patients or individuals contemplating pregnancy. The established rodent carcinogenicity of griseofulvin is not taken into account in the contraindications or precautions for use of this drug.

Noscapine (CAS No 912-60-7)

A more recent example where regulatory action was taken based on induction of numerical chromosome changes was on the anti-tussive agent, noscapine. Noscapine had been shown to induce polyploidy *in vitro* in mammalian cells but not *in vivo* (Ishidate, 1988; Furukawa *et al*, 1989; Gatehouse *et al*, 1991; Mitchell *et al*, 1991; Tiveron *et al*, 1993). Despite the fact that only *in vitro* effects were induced, the UK Committee on Safety of Medicines recommended that all products containing noscapine be contraindicated in women of child-bearing potential (due to genotoxicity concerns) and that all products become prescription only (SCRIP, 1991). It has since been reported that noscapine induces transformation of Syrian hamster dermal cells indicating carcinogenic potential (Porter *et al*, 1992).

Vinblastine sulphate (CAS No 143-67-9) and ***Vincristine sulphate*** (CAS No 57-22-7)

Vinblastine sulphate and vincristine sulphate are chemotherapeutic agents. Their chemotherapeutic effects are attributed to their ability to inhibit the mitotic spindle thereby causing arrest of dividing cells. Aspermia has been reported in men administered vinblastine sulphate (Physician's Desk Reference, 1993). Women are advised to avoid becoming pregnant while using vinblastine and vincristine sulphate (Physician's Desk Reference, 1993).

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APPENDIX E - EVALUATION OF ANEUGENIC CHEMICALS DERIVED IN PART FROM THE CEC PROGRAMME

E.1 INTRODUCTION TO DATA COMPILATION

An in-depth literature search on aneuploidy (including key words such as micronuclei, aneuploidy/hyper-/hypoploidy, polyploidy, FISH, kinetochore staining, endoreduplication, C-mitosis, cell cycle delay, chromosome counting, mitotic abnormalities, etc.) was performed. The following databases were searched:

DATASTAR: Toxline; Medline.

Data on the following test substances were evaluated:

Acrylamide
Benomyl
Cadmium Cl
Carbendazim
Chloral hydrate
Colchicine
Diazepam
Diethylstilboestrol
Econazole/Econazole nitrate
Griseofulvin
Hydroquinone
Noscapine
Pyrimethamine
Thiabendazole
Thimerosal
Vinblastine sulphate/Vincristine

The data were compiled into an EXCEL spreadsheet recording the conclusions made by the authors of the paper as well the evaluation made by the Working Group.
The data set is subdivided according to:

1. In vitro mammalian systems

A) Human lymphocytes *in vitro* (*in vitro* H. LY.)

ANEU H.P. FIBRO.	Aneuploidy human primary fibroblasts
POLY LYMPHO.	Polyploidy human lymphocytes
POLYPL. FIBR.	Polyploidy human primary fibroblasts

B) *in vitro* other cells

The following endpoints were evaluated for mammalian systems *in vitro*:

chromosome counting,
micronuclei, (including: size, CREST etc),
FISH/interphase,
mitotic delay,
C-mitosis,
nondisjunction,
spindle disturbances.

The following criteria were used for assessment of *in vitro* data:

Negative:	Data presented reasonably, No effects observed.
Positive:	Data presented reasonably, Dose-dependent effect.
Inconclusive:	Highest dose used not sufficiently justified, No signs of toxicity observed, Sampling time(s) not adequate, No dose-response, Effects not sufficiently obvious (based on statistical and biological significance and considering the variability in the negative controls), Excessive variation of effects, No data shown.

The criteria for the overall Task Force conclusion were:

Negative:	If at least one of the TF judgments is 'negative' and there is no 'positive'.
Positive:	If half or more of the TF judgments are 'positive'.
Inconclusive:	If the majority of the TF judgments are 'inconclusive'.

2. Somatic cells in vivo

The following endpoints were evaluated for somatic cells *in vivo*:

chromosome counting (hyper-/hypoploidy; polyploidy),
mitotic abnormalities,
micronuclei,
FISH/DNA content.

The following criteria were used for assessment of *in vivo* somatic data:

Negative:	Number of animals and evaluated cells acceptable,

Highest dose used considered to be adequate,
Signs of toxicity observed,
Sampling times acceptable,
Data presented reasonably,
No effects observed.

Positive: Number of animals and evaluated cells acceptable,
Effects sufficiently obvious (based on statistical **and** biological significance and considering the variability in the negative controls),
Dose-dependent effect,
Data presented reasonably.

Inconclusive: Number of animals and evaluated cells too low,
Highest dose used not sufficiently justified,
No signs of toxicity observed,
Sampling time(s) not adequate,
No dose-response,
Effects not sufficiently obvious (based on statistical **and** biological significance and considering the variability in the negative controls),
Excessive variation of effects,
No data shown.

The criteria for the overall Task Force conclusion were:

Negative: If at least one of the TF judgments is 'negative' and there is no 'positive'.

Positive: If half or more of the TF judgments are 'positive'.

Inconclusive: If the majority of the TF judgments are 'inconclusive' and there is no 'negative' for micronucleus test.

3. Germ cells (in vivo)

The following endpoints were evaluated for germ cells:

Male germ;
Synaptonemal complex damage,
mitotic delay,
spermatid micronuclei,
secondary spermatocytes chromosome counts.

Female germ;
oocyte chromosome counts.

The following criteria were used for assessment of *in vivo* germ line data:

Negative: No reported increase in hyperploid or polyploid cells or micronculei.

Positive: Significant increase in hyperploid or polyploid cells or micronuclei.

Inconclusive: Both positive and negative results reported.

The criteria for the overall Task Force conclusion were:

Negative: If at least one of the TF judgments is 'negative' and there is no 'positive'.

Positive: If half or more of the TF judgments are 'positive'.

Inconclusive: If the majority of the TF judgments are 'inconclusive' and there is no 'negative'.

E.2 PRESENTATION OF THE DATA

Date is presented in EXCEL-Data Sheets and Graphs.

E.2.1 The EXCEL-Data Sheets

The data is presented in the following manner.

Chemical		Chloral hydrate			Colchicine		
Interpretation ⇒		Author		TF	Author		TF
Test System ⇓	Nr.						
***in vitro* H. LY.**							
vitro c-mitosis	10 P	1.4		P	P 1.4		P
vitro MN	19 P	1.5a		I iec	P 1.5a		I iec
vitro MN+CREST	28 P	1.11		P	P 1.11		P
3H-FISH-C9	30 P	1.9		I a			
vitro chrom. count	33 P	1.4		I spp	P 4		P
.....							

.....

Overall Assessment		
in vitro	**positive**	**positive**
***in vivo* (somatic)**	**inconcl.**	**positive**
***in vivo* germ cells male**	**positive**	**positive**
***in vivo* (oocytes)**	**negative**	**positive**

An abbreviated citation code (1.x; 2.x etc.) corresponding to each publication was recorded (cf. Nr).

Abbreviations used in EXCEL data Sheet are:

Test system

MN	Micronuclei
CREST	Kinetochore (CREST antiserum)
FISH	Fluorescence *in situ* hybridisation
H.LY.	Human Lymphocytes
FIBR.	Fibroblasts
POLY.	Polyploidy

Number

Nr.	Sequential number - Refers to position on x-axis of graphs for the compound

Result interpretation

Author	Authors conclusion
TF	Task Force Evaluation
P	Positive
N	Negative
I	Inconclusive
number(s)	References
ndr	no dose/concentration response
ntt	not tested to sufficient toxicity
spp	single point positive
spn	single point negative
nds	no data shown
tfa	too few animals per group
tfc	too few cells
ecn	evaluation criteria not adequate
sna	statistics not adequate
iec	incomplete evaluation criteria
sss	small sample size
a	abstract
itd	insufficient doses
itc	inadequate time
ied	insufficient experimental detail
u	unvalidated test system

References Nr. after Author conclusion

The number refers to the attached reference lists.

1.x	*in vitro* H.LY. & Fibroblasts
2.x	*in vitro* other cells
3.x	*in vivo* somatic
4.x	*in vivo* male germ
5.x	*in vivo* female germ (oocyte)

Chemical		Acrylamide		Benomyl		Cadmium Cl		Carbendazim	
Interpretation ⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
***in vitro* H. LY.**									
vitro mitotic delay	1								
vitro interphase	5			P 1.1	P				
vitro interphase	6			P 1.1	P				
vitro interphase	7			P 1.2	I ssp				
vitro C-mitosis	8					I 1.4	N		
vitro MN	15					P 1.5a	I iec		
vitro MN	16					N 1.5b	I iec		
vitro MN size	17								
vitro MN+C	18								
vitro MN+CREST	19								
vitro MN+CREST	20								
3H-FISH-C9	22			P 1.9	I a				
3H-FISH-CY	23			P 1.9	I a				
vitro chrom. count	24					P 1.4	I ssp		
vitro chrom. count	25								
ANEU H.P. FIBRO.									
vitro mitotic arrest	2								
vitro spindle dam.	3								
vitro diff. staining	4					N 1.18	N		
vitro MN+CREST	21					P 1.16	P		
vitro chrom. count	26								
vitro chrom. count	27								
POLY LYMPHO.									
vitro chrom. count	9								
vitro chrom. count	10								
vitro chrom. count*	11					N 1.4	N		
vitro chrom. count end*	12					N 1.4	N		
POLYPL. FIBR.									
vitro chrom. count	13								
vitro chrom. count	14								
***in vitro* other cells**									
vitro spindle disturbance	1								
vitro spindle disturbance	2								
vitro metaph arrest	3	P 2.4	P			N 2.40	N		
vitro metaph arrest	4								
vitro metaph arrest	5								
vitro metaph arrest	6								
V79 not specif.	7	P 2.20	abstract						
SHE not specif.	8								
BHK not specif.	9								

Chemical		Acrylamide		Benomyl		Cadmium Cl		Carbendazim	
Interpretation ⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
Don not specif.	10								
vitro polyploidy	11					N 2.41	N		
vitro polyploidy	11					N 2.40	N		
vitro micronuclei	13			P 2.26	P	N 2.45	N		
vitro micronuclei	14			P 2.36	abstract	P 2.1	I spp		
vitro micronuclei	15			P 2.43	P	N 2.42	N		
vitro micronuclei	16								
vitro micronuclei	17								
vitro mark chrom.	18			P 2.3	P				
vitro mark chrom.	19			P 2.38	P				
vitro nondisjunct.	20			P 2.13	P				
vitro nondisjunct.	21								
vitro chrom count	22					N 2.40	N		
vitro chrom count	23			P 2.2	P	P 2.41	P		
vitro chrom count	24								
vitro chrom count	25								
vitro chrom count	26								
vitro chrom count	27								
***in vivo* somatic**									
AGT (Av. Gen. Time)	1			P 3.19	I nds	N 3.2	I spn		
AGT (Av. Gen. Time)	2								
AGT (Av. Gen. Time)	3								
AGT (Av. Gen. Time)	4								
Mitotic abnormal.	5	P 3.6	P						
Mitotic index	6								
C-mitosis	7					N 3.1	N		
C-mitosis	8								
MN (simple)	9	P 3.27	P	P 3.21	P	P 3.14	I tfc		
MN (simple)	10			P 3.19	I nds	N 3.12	N	P 3. 20	P
MN (simple)	11			P 3.20	P	N 3.2	I spn		
MN (simple)	12					N 3.18	N		
MN (simple)	13					P 3.15	I ecu		
MN (simple)	14					P 3.16	P		
MN (simple)	15					N 3.26	I tfa		
Polyploidy	16					N 3.2	N nds		
Polyploidy	17								
Polyploidy	18								
Aneupl. or polyploidy	19	P 3.7	P						

Chemical		Acrylamide		Benomyl		Cadmium Cl		Carbendazim	
Interpretation ⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
MN+CREST	20								
MN+Centrom. DNA	21								
Hyperploidy	22								
Hyperploidy	23					N 3.2	I spn		
Hyperploidy	24								
Hyperploidy	25								
***in vivo* Male Germ**									
SC damage	1								
Mitotic delay	2	P 4.9	I u, ied			P 4.1	P u		
Spermatid MN	3								
Spermatid MN	4								
Spermatid MN	5								
Second. sperm.	6	P 4.9	I ied			P 4.1	I sna		
Second. sperm.	7	N 4.11	I itc			N 4.2	I idt		
Second. sperm.	8								
Second. sperm.	9								
***in vivo* (oocyte)**	11			P 5.1	P	N 5.2	N	P 5.6	P
	12					I 5.4	I		
	13					P 5.5	P		
	14								
	15								
	16								
	17								
Overall Assessment									
in vitro			**positive**		**positive**		**inconclu.**		**positive**
***in vivo* (somatic)**			**positive**		**positive**		**inconclu.**		**positive**
***in vivo* (male germ cells)**			**inconclu.**		**ND**		**inconclu.**		**ND**
***in vivo* (oocytes)**			**ND**		**positive**		**inconclu.**		**positive**

Chemical		Chloral hydrate		Colchicine		Diazepam		Diethylstilboestrol	
Interpretation ⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
***in vitro* H. LY.**									
vitro mitotic delay	1							P 1.13	P
vitro interphase	5	P 1.2	P						
vitro interphase	6								
vitro interphase	7								
vitro C-mitosis	8	P 1.4	P	P 1.4	P	P1.4	P	P 1.12	I spp, sna
vitro MN	15	P 1.5a	I iec	P 1.5a	I iec	N 1.5a	I iec		
vitro MN	16	P 1.5b	I iec	P 1.5b	I iec			I 1.6	I iec
vitro MN size	17	N 1.22	N	P 1.22	P	P 1.22	I sss		
vitro MN+C	18							P 1.12	P
vitro MN+CREST	19	P 1.11	P	P 1.10	P			P 1.10	P ssp
vitro MN+CREST	20			P 1.11	P				
3H-FISH-C9	22	P 1.9	I a					P 1.9	I a
3H-FISH-CY	23	P 1.9	I a					P 1.9	I a
vitro chrom. count	24	P 1.4	I spp	P 4	P	N 1.4	N		
vitro chrom. count	25								
ANEU H.P. FIBRO.									
vitro mitotic arrest	2					P 1.20	I iec		
vitro spindle dam.	3								
vitro diff. staining	4							P 1.19	P
vitro MN+CREST	21	P 1.16	P ssp	P 16	P	P 1.16	P ssp		
vitro chrom. count	26							P 1.17	I ndr
vitro chrom. count	27							P 1.23	I a
POLY LYMPHO.									
vitro chrom. count	9							P 1.13	I spp
vitro chrom. count	10							P 1.12	I sna
vitro chrom. count*	11	N 1.4	I spp	P 4	P	N 1.4	N		
vitro chrom. count end*	12	N 1.4	I ndr	N 4	N	N 1.4	N		
POLYPL. FIBR.									
vitro chrom. count	13							P 1.17	I ndr
vitro chrom. count	14							P 1.23	I a
***in vitro* other cells**									
vitro spindle disturbance	1			P 2.16	abstract	P 2.29	P	P 2.16	abstract
vitro spindle disturbance	2			P 2.24	P			P 2.33	P
vitro metaph arr.	3	P 2.40	P	P 2.7	P	P 2.8	P	P 2.18	P
vitro metaph arrest	4			P 2.31	I no data	P 2.9	P	P 2.11	P
vitro metaph arrest	5			P 2.10	P	P 2.40	P	P 2.10	P
vitro metaph arrest	6			P 2.40	P			P 2.39	P
V79 not specif.	7								
SHE not specif.	8							P 2.27	abstract
BHK not specif.	9							P 2.28	I no data

Chemical		Chloral hydrate		Colchicine		Diazepam		Diethylstilboestrol	
Interpretation⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
Don not specif.	10			P 2.30	abstract	P 2.30	abstract		
vitro polyploidy	11	N 2.41	N	P 2.41	P	N 2.41	N		
vitro polyploidy	12	P 2.40	P	P 2.40	P	N 2.40	P		
vitro micronuclei	13	P 2.5	P	P 2.5	P	N 2.42	N	P 2.26	P
vitro micronuclei	14	P 2.1	P	P 2.1	P	P 2.45	P		
vitro micronuclei	15	P 2.42	P	P 2.26	P				
vitro micronuclei	16			P 2.42	P				
vitro micronuclei	17								
vitro mark chrom.	18			P 2.3	P				
vitro mark chrom.	19								
vitro nondisjunct.	20			P 2.13	P				
vitro nondisjunct.	21			P 2.12	abstract			P 2.12	abstract
vitro chrom count	22	P 2.40	P			P 2.40	I hypo	P 2.34	P
vitro chrom count	23	P 2.41	I spp	P 2.2	P			P 2.17	abstract
vitro chrom count	24			P 2.14	P	P 2.25	I hypo	P 2.15	P
vitro chrom count	25			P 2.40	P	P 2.41	I hypo	P 2.35	P
vitro chrom count	26			P 2.41	P			P 2.41	P
vitro chrom count	27							P 2.44	P
***in vivo* somatic**									
AGT (Av. Gen. Time)	1	P 3.2	I	P 3.3	P	N 3.2	N	P 3.9	P
AGT (Av. Gen. Time)	2			P 3.8	P			P 3.21	P
AGT (Av. Gen. Time)	3								
AGT (Av. Gen. Time)	4								
Mitotic abnormal.	5			P 3.6	P				
Mitotic index	6								
C-mitosis	7	P 3.1	I	P 3.1	P	N 3.1	N		
C-mitosis	8								
MN (simple)	9	P 3.26	P					N 3.20	N
MN (simple)	10	N 3.2	N	P 3.3	P	N 3.12	N	N 3.5	N nds, a
MN (simple)	11	N 3.18	N	N 3.18	N	N 3.2	I spn	N 3.22	N
MN (simple)	12	N 3.12	N	P 3.12	P			P 3.23	I tfa, tfc
MN (simple)	13	P 3.17	I spp					N 3.24	N
MN (simple)	14	P 3.19	I ndr					N 3.25	I tfa
MN (simple)	15								
Polyploidy	16	N 3.2/10	N nds	P 3.3	P	N 3.2	N nds	N 3.5	N nds
Polyploidy	17			P 3.8	P	N 3.10	N nds		
Polyploidy	18			P 3.10	P				
Aneupl. or polyploidy	19								

Chemical		Chloral hydrate		Colchicine		Diazepam		Diethylstilboestrol	
Interpretation⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
MN+CREST	20	P 3.26	P	P 3.13	P				
MN+CREST	21			P 3.26	P				
MN+Centrom. DNA	22			P 3.13	P				
Hyperploidy	23	P 3.2	I spp	P 3.3	I spp	N 3.2	N	N 3.9	N
Hyperploidy	24								
Hyperploidy	25								
Hyperploidy	26								
***in vivo* Male Germ**									
SC damage	1			P 4.10	P u				
Mitotic delay	2	P 4.1	P u	P 4.1	P u	P 4.1	I u, sna		
Spermatid MN	3	P 4.5	P						
Spermatid MN	4	N 4.6	I itc						
Spermatid MN	5	P 4.13	P						
Second. sperm.	6	P 4.1	P	P 4.1	P	P 4.1	I sna	P 4.4	P
Second. sperm.	7	N 4.2	N	P 4.2	P	N 4.2	N	P 4.12	I ied
Second. sperm.	8	P 4.3	P						
Second. sperm.	9	P 4.4	P						
***in vivo* (oocyte)**	11	N 5.2	N	P 5.8	P	N 5.14	N	N 5.2	N
	12	N 5.7	N	I tfc 5.9	I			N 5.7	N
	13			P 5.10	P				
	14			P 5.11	P				
	15			P 5.2	P				
	16			P 5.12	P				
	17			P 5.13	P				
Overall Assessment									
in vitro			**positive**		**positive**		**positive**		**positive**
***in vivo* (somatic)**			**inconclu.**		**positive**		**negative**		**positive**
***in vivo* (male germ cells)**			**positive**		**positive**		**inconclu.**		**positive**
***in vivo* (oocytes)**			**negative**		**positive**		**negative**		**negative**

Chemical		Econazole Nitrate		Griseofulvin		Hydroquinone		Noscapine	
Interpretation ⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
***in vitro* H. LY.**									
vitro mitotic delay	1								
vitro interphase	5			P 1.1	P				
vitro interphase	6			P 1.1	P				
vitro interphase	7								
vitro C-mitosis	8	N 1.4	N			P 1.4	P		
vitro MN	15	P 1.5a	I iec			I 1.5a	I iec		
vitro MN	16	N 1.5b	I iec			P 1.5b	I iec		
vitro MN size	17					P 1.22	I sss		
vitro MN+C	18								
vitro MN+CREST	19					P 1.7	P spp		
vitro MN+CREST	20					P 1.8	P		
3H-FISH-C9	22			P 1.9	I a				
3H-FISH-CY	23			P 1.9	I a				
vitro chrom. count	24	N 1.4	N			N 1.4	N		
vitro chrom. count	25								
ANEU H.P. FIBRO.									
vitro mitotic arrest	2								
vitro spindle dam.	3							P 1.21	P
vitro diff. staining	4								
vitro MN+CREST	21	N 1.16	N			N 1.16	N		
vitro chrom. count	26								
vitro chrom. count	27								
POLY LYMPHO.									
vitro chrom. count	9								
vitro chrom. count	10							P 1.21	P
vitro chrom. count*	11	N 1.4	N			P 1.4	P		
vitro chrom. count end*	12	N 1.4	N			N 1.4	N		
POLYPL. FIBR.									
vitro chrom. count	13								
vitro chrom. count	14								
***in vitro* other cells**									
vitro spindle disturbance	1								
vitro spindle disturbance	2								
vitro metaph arr.	3	N 2.40	N	P 2.37	I no data	P 2.21	abstract		
vitro metaph arrest	4					I 2.40	I		
vitro metaph arrest	5								
vitro metaph arrest	6								
V79 not specif.	7								
SHE not specif.	8								
BHK not specif.	9								

Chemical		Econazole Nitrate		Griseofulvin		Hydroquinone		Noscapine	
Interpretation ⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
Don not specif.	10								
vitro polyploidy	11	N 2.41	N			N 2.41	N		
vitro polyploidy	12	N 2.40	N			N 2.40	N		
vitro micronuclei	13	N 2.45	N			N 2.45	N		
vitro micronuclei	14	N 2.1	N			P 2.1	P		
vitro micronuclei	15	N 2.42	N			N 2.42	N		
vitro micronuclei	16								
vitro micronuclei	17								
vitro mark chrom.	18								
vitro mark chrom.	19								
vitro nondisjunct.	20								
vitro nondisjunct.	21								
vitro chrom count	22	P 2.40	I ndr			I 2.40	I ndr		
vitro chrom count	23	P 2.41	P			P 2.41	P		
vitro chrom count	24								
vitro chrom count	25								
vitro chrom count	26								
vitro chrom count	27								
***in vivo* somatic**									
AGT (Av. Gen. Time)	1	N 3.2	I spn			P 3.3	P		
AGT (Av. Gen. Time)	2								
AGT (Av. Gen. Time)	3								
AGT (Av. Gen. Time)	4								
Mitotic abnormal.	5								
Mitotic index	6	P 3.9	P nds			N 3.9	N nds		
C-mitosis	7	P 3.1	P			P 3.1	P		
C-mitosis	8	P 3.9	P			P 3.9	P		
MN (simple)	9								
MN (simple)	10	N 3.12	N	N 3.18	N	P 3.3	P	N 3.16	N nds, a
MN (simple)	11	N 3.2	I spn			P 3.14	P nds, a		
MN (simple)	12	P 3.26	I tfa			P 3.12	P		
MN (simple)	13	P 3.26	P			P 3.26	P		
MN (simple)	14								
MN (simple)	15								
Polyploidy	16	N 3.2	N nds			N 3.3	N		
Polyploidy	17	N 3.10	N nds			N 3.10	N		
Polyploidy	18								
Aneupl. or polyploidy	19								

Chemical		Econazole Nitrate		Griseofulvin		Hydroquinone		Noscapine	
Interpretation ⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
MN+CREST	20	P 3.26	P			P 3.13	P		
MN+CREST	21					P 3.26	P		
MN+Centrom. DNA	22					P 3.13	P		
Hyperploidy	23	N 3.2	I spn			P 3.3	I spp		
Hyperploidy	24								
Hyperploidy	25								
Hyperploidy	26								
***in vivo* Male Germ**									
SC damage	1								
Mitotic delay	2	P 4.1	P u			P 4.1	I spp		
Spermatid MN	3								
Spermatid 7MN	4								
Spermatid MN	5								
Second. sperm.	6	P 4.1	P			P 4.1	P		
Second. sperm.	7	N 4.2	N			P 4.2	P		
Second. sperm.	8								
Second. sperm.	9								
***in vivo* (oocyte)**	11	N unpub	N	P 5.15	P			N 5.19	N
	12			P 5.16	P				
	13								
	14								
	15								
	16								
	17								
Overall Assessment									
in vitro			**negative**		**positive**		**positive**		**positive**
***in vivo* (somatic)**			**inconclu.**		**positive**		**positive**		**negative**
***in vivo* (male germ cells)**			**inconclu.**		**ND**		**positive**		**ND**
***in vivo* (oocytes)**			**negative**		**positive**		**ND**		**negative**

Chemical		Pyrimethamine		Thiabendazole		Thimerosol		Vinblastine sulphate (*= Vincristine)	
Interpretation ⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
***in vitro* H. LY.**									
vitro mitotic delay	1								
vitro interphase	5								
vitro interphase	6								
vitro interphase	7								
vitro C-mitosis	8	N 1.4	N	P 1.4	P	P 1.4	I spp	P 1.4	I spp
vitro MN	15	N 1.5a	I iec	N 1.5a	I iec	I 1.5a	I iec	P 1.5a	I iec
vitro MN	16	N 1.5b	I iec	N 1.5b	I iec	N 1.5b	I iec	P 1.6*	I iec
vitro MN size	17								
vitro MN+C	18								
vitro MN+CREST	19							P 1.10*	P
vitro MN+CREST	20								
3H-FISH-C9	22								
3H-FISH-CY	23								
vitro chrom. count	24	N 1.4	N	N 1.4	N	P 1.4	I spp	P 1.4	I spp
vitro chrom. count	25								
ANEU H.P. FIBRO.									
vitro mitotic arrest	2								
vitro spindle dam.	3								
vitro diff. staining	4								
vitro MN+CREST	21	N 1.16	N	P 1.16	I ecn	N 1.16	N	P 1.16	P
vitro chrom. count	26							P 1.17*	I spp
vitro chrom. count	27							P 1.23*	I a
POLY LYMPHO.									
vitro chrom. count	9								
vitro chrom. count	10								
vitro chrom. count*	11	N 1.4	N	P 1.4	P	P 1.4	P spp	P 1.4	P
vitro chrom. count end*	12	N 1.4	N	N 1.4	N	P 1.4	P spp	N 1.4	N
POLYPL. FIBR.									
vitro chrom. count	13							P 1.17*	I spp
vitro chrom. count	14							P 1.23*	I a
***in vitro* other cells**									
vitro spindle disturbance	1								
vitro spindle disturbance	2								
vitro metaph arr.	3	P 2.40	P	P 2.40	P	P 2.40	P	P 2.22	I no data
vitro metaph arrest	4							P 2.31	I no data
vitro metaph arrest	5							P 2.40	P
vitro metaph arrest	6								
V79 not specif.	7								
SHE not specif.	8								
BHK not specif.	9								

Chemical		Pyrimethamine		Thiabendazole		Thimerosol		Vinblastine sulphate (*= Vincristine)	
Interpretation ⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
Don not specif.	10							P 2.30	abstract
vitro polyploidy	11	N 2.41	N	P 2.41	P	P 2.41	P	P 2.41	P
vitro polyploidy	12	P 2.40	P	P 2.40	P	P 2.40	P	P 2.40	P
vitro micronuclei	13	N 2.42	N	P 2.42	P	N 2.42	N	P 2.36	abstract
vitro micronuclei	14	N 2.45	N	P 2.45	P	P 2.1	I spp	P 2.1	P
vitro micronuclei	15					N 2.45	N	P 2.42	P
vitro micronuclei	16							P 2.43	P
vitro micronuclei	17							P 2.45	P
vitro mark chrom.	18								
vitro mark chrom.	19								
vitro nondisjunct.	20								
vitro nondisjunct.	21								
vitro chrom count	22	P 2.40	I	P 2.40	P	N 2.40	N	P 2.40	P
vitro chrom count	23	N 2.41	I spp	P 2.41	P	P 2.41	P	P 2.41	P
vitro chrom count	24								
vitro chrom count	25								
vitro chrom count	26								
vitro chrom count	27								
***in vivo* somatic**									
AGT (Av. Gen. Time)	1	N 3.2	I spn	P 3.2	I spp	N 3.2	I spn	P 3.4	P
AGT (Av. Gen. Time)	2							P 3.8	P
AGT (Av. Gen. Time)	3							P 3.9	P
AGT (Av. Gen. Time)	4							P 3.11	P
Mitotic abnormal.	5								
Mitotic index	6								
C-mitosis	7	N 3.1	N	N 3.1	N	N 3.1	N	(P 3.1 *)	P
C-mitosis	8								
MN (simple)	9			N 3.16	N nds, a				
MN (simple)	10	N 3.12	N	N 3.12	N	N 3.12	N	P 3.12	P
MN (simple)	11	P/I 3.2	I	P/I 3.2	I spp	N 3.2	I spn	P 3.18	P
MN (simple)	12	N 3.26	I tfa	P 3.26	P	P 3.26	P	P 3.26	P
MN (simple)	13								
MN (simple)	14								
MN (simple)	15								
Polyploidy	16	N 3.2	N nds	N 3.2	N nds	N 3.2	N nds	P 3.4	P
Polyploidy	17			N 3.5	N nds			P 3.8	P
Polyploidy	18								
Aneupl. or polyploidy	19								

Chemical		Pyrimethamine		Thiabendazole		Thimerosol		Vinblastine sulphate (*= Vincristine)	
Interpretation ⇒		Author	TF	Author	TF	Author	TF	Author	TF
Test System ⇓	Nr.								
MN+CREST	20	P 3.26	I tfa	P 3.26	P	P 3.26	P	P 3.13	P
MN+CREST	21							P 3.26	P
MN+Centrom. DNA	22							P 3.13	P
Hyperploidy	23	N 3.2	I spn	N 3.2	I spn	N 3.2	I spn	P 3.4	P
Hyperploidy	24							P 3.8	P
Hyperploidy	25							P 3.9	P
Hyperploidy	26							P 3.11	P
***in vivo* Male Germ**									
SC damage	1							P 4.10	P u
Mitotic delay	2	N 4.1	N u	N 4.1	N	N 4.1	N u	P 4.1	P u
Spermatid MN	3							P 4.8	P
Spermatid MN	4								
Spermatid MN	5								
Second. sperm.	6	N 4.1	N	N 4.1	I ecn	N 4.1	N	P 4.1	P
Second. sperm.	7	N 4.2	I idt	P 4.2	P	N 4.2	I idt	N 4.7	I itc
Second. sperm.	8								
Second. sperm.	9								
***in vivo* (oocyte)**	11	P 5.20						P 5.21	P
	12	N 5.7	N					P 5.7	P
	13								
	14								
	15								
	16								
	17								
Overall Assessment									
in vitro			**inconclu.**		**positive**		**inconclu.**		**positive**
***in vivo* (somatic)**			**negative**		**inconclu.**		**Inconclu.**		**positive**
***in vivo* (male germ cells)**			**negative**		**inconclu.**		**negative**		**positive**
***in vivo* (oocytes)**			**negative**		**ND**		**ND**		**positive**

E.2.2 Graphical Presentation

A data set consisting of a discrete number of bioassays/endpoints and qualitative values (Working Group conclusion for each assay/endpoint) can be easily represented in a bar graph. This was accomplished for each chemical in the Aneuploidy Data Base by dividing into four categories of assays:

in vitro human lymphocytes/fibroblasts
in vitro, other cells
in vivo, somatic cells
in vivo, germ cells

In the bar-graph the x-axis unit values correspond to the various test systems/endpoints. The sequence (Nr.) of bioassays/endpoints plotted on the x-axis is listed in the corresponding Excel-Data Sheet row. The assays/endpoints are grouped according to strength of evidence for induction of chromosome gain/loss in the corresponding test system.

The y-axis values correspond to the response function for each individual test system/endpoint, defined as follows:

+1.0	Author and TF conclusion positive response
+0.5	Author conclusion positive/TF conclusion inconclusive or negative
-1.0	Author and TF conclusion negative response
-0.5	Author conclusion negative/TF conclusion inconclusive or positive

Thus a discrete array of tests/endpoints and responses defined a readily interpretable profile for each chemical concerning aneuploidy-induction.

An example is given below

Appendix E - Figure 1A: Activity profile for benomyl → *in vitro* assays

Appendix E - Figure 1B: Activity profile for benomyl → *in vivo* assays

Appendix E - Figure 2A: Activity profile for cadmium chloride → *in vitro* assays

Appendix E - Figure 2B: Activity profile for Cadmium chloride → *in vivo* assays

Appendix E - Figure 3A: Activity profile for chloral hydrate → *in vitro* assays

Appendix E - Figure 3B: Activity profile for chloral hydrate → *in vivo* assays

Appendix E - Figure 4A **Activity profile for colchicine → *in vitro* assays**

Appendix E - Figure 4B: Activity profile for colchicine → *in vivo* assays

Appendix E - Figure 5A: Activity profile for diazepam → *in vitro* assays

Appendix E- Figure 5B: Activity profile for diazepam → *in vivo* assays

Appendix E - Figure 6A: Activity profile for diethylstilboestrol (DES) → *in vitro* assays

Appendix E - Figure 6B: Activity profile for diethylstilboestrol (DES) → *in vivo* assays

Appendix E - Figure 7A: Activity profile for econazole nitrate → *in vitro* assays

Appendix E - Figure 7B: Activity profile for econazole nitrate → *in vivo* assays

Appendix E - Figure 8A: Activity profile for griseofulvin → *in vitro* assays

Appendix E - Figure 8B: Activity profile for griseofulvin → *in vivo* assays

Appendix E - Figure 9A: Activity profile for hydroquinone → *in vitro* assays

Appendix E - Figure 9B: Activity profile for hydroquinone → *in vivo* assays

Appendix E - Figure 10A: Activity profile for pyrimethamine → *in vitro* assays

Appendix E - Figure 10B: Activity profile for pyrimethamine → *in vivo* assays

Appendix E - Figure 11A: Activity profile for thiabendazole → *in vitro* assays

Appendix E - Figure 11B: Activity profile for thiabendazole → *in vivo* assays

Appendix E - Figure 12A: Activity profile for thimerosal → *in vitro* assays

Appendix E - Figure 12B: Activity profile for thimerosal → *in vivo* assays

Appendix E - Figure 13A: Activity profile for vinblastine → *in vitro* assays

Appendix E - Figure 13B: Activity profile for vinblastine → *in vivo* assays

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APPENDIX F - REVIEW OF POLYPLOIDY INDUCING CHEMICALS

Table HA: Review of chemicals that induce polyploidy *in vitro* [10% or more polyploid or aberrant cells considered +; Ishidate, 1988; unless designated (1) Ishidate *et al, 1988*]

Chemical Name	Polyploidy	Exp./Rec.	Aberration	Exp/Rec.	Cell Type	Other
A649 (1)	+	52/0	+	52/0	Human lymphocytes	Ames -
acridine	-	24/0	+	24/0	CHL	
	+	48/0	+	48/0		
	-	3/21 -S9	-	3/21 -S9		
	-	3/21 +S9	+	3/21 +S9		
allylisothiocyanate (1)	+	24/24	+	24/24	B241	Ames +
9-aminoacridine HCl	+	24/0	+	24/0	CHL	
	+	48/0	+	48/0		
2-amino-9H-pyrido[2,3-B]indole acetate	+	24/0	-	24/0	CHL	Ames +
	+	48/0	-	48/0		Carc +
	-	3/21 -S9	+	3/21 -S9		
	-	3/21+S9	+	3/21+S9		
2-amino-3-methyl-9H-pyrido[2,3-P]indole acetate	-	24/0	-	24/0	CHL	Ames +
	+	48/0	-	48/0		Carc +
	-	3/21 -S9	+	3/21 -S9		
	-	3/21 +S9	+	3/21 +S9		
asbestos chrysotile (1)	+	48/0	-	48/0	SH fibro	
			+	72/0	hum lymph	
asbestos crocidolite (1)	+	48/0	-	48/0	SH fibro	
			+	72/0	hum lymph	
bendroflumethiazide	-	24/0	-	24/0	CHL	Ames -
	+	48/0	+	48/0		
benzaldehyde	+	6/18 -S9	+	6/18 -S9	CHL	Ames -
	+	6/18 +S9	-	6/18 +S9		
1,3-bis(2-chloroethyl)-1-nitrosourea	-	24/0	+	24/0	CHL	Ames+
	+	48/0	+	48/0		MN +
bleomycin HCl	-	24/0	+	24/0	CHL	MN +
	+	48/0	+	48/0		
N-butyl-n-nitrosourethane	-	24/0	+	24/0	CHL	Ames+
	+	48/0	+	48/0		Carc +
calcium citrate	+	24/0	-	24/0	CHL	Ames -
	+		-	48/0		
caprolactam		6/18 +S9	-	6/18 +S9	CHL	Ames -
	+	6/18 -S9	+	6/18 -S9		MN -
	+					Carc -
cellulase	-	24/0	+	24/0	CHL	Ames -
	+	48/0	+	48/0		
chlorine dioxide powder (3.5% in Ca silicate)	-	24/0	-	24/0		Ames -
	+	48/0	-	48/0		
cinnamic aldehyde	-	24/0	+	24/0	CHL	Ames +
	+	48/0	+	48/0	B241	MN -
	+ (1)	24/24	+ (1)	24/24	B241	
citronella oil	-	24/0	-	24/0	CHL	
	+	48/0	-	48/0		
creatinine	-	24/0	+	24/0	CHL	
	+	48/0	+	48/0		
1,2-dibromoethane (1)	+	27/0	+	27/0	V79-CL-15	
N, N dibutyl-N-nitrosourea	-	24/0	+-	24/0	CHL	Ames +
	+	48/0		48/0		

Chemical Name	Polyploidy	Exp./Rec.	Aberration	Exp/Rec.	Cell Type	Other
1,3-dichloropropene	- - - +	24/0 48/0 6/18 -S9 6/18 +S9 * *mouse S9	+ - + +	24/0 48/0 6/18 -S9 6/18 +S9	CHL	Ames +
dichlorvos (1)	+	27/0	+	27/0	V79-CL-15	Ames+ Carc ?
diethylene glycol	- - - +	24/0 48/0 6/18 -S9 6/18 +S9	- + - +	24/0 48/0 6/18 -S9 6/18 +S9	CHL	Ames -
DES -	+ + (1) + (1)	24/0 48/0 36/0 32/0	- - + (1) - (10	24/0 48/0 36/0 32/0	CHL CHL CH1-L human lymph	Ames - MN + Carc +
4,4 dimethoxydiphenyl amine	- - + -	24/0 48/0 6/18 -S9 6/18 +S9	+ + - +	24/0 48/0 6/18 -S9 6/18 +S9	CHL	Ames -
2,4-dinitrochlorobenzene	+ + - -	24/0 48/0 6/18 -S9 6/18 +S9	+ - + -	24/0 48/0 6/18 -S9 6/18 +S9	CHL	
2,4 dinitrophenyl thio-cyanate (1)	+	27/0	-	27/0	V79-CL-15	
estradiol (1)	+	32/0	-	32/0	human lymph	
ethenzamide	- + - -	24/0 48/0 3/21 -S9 3/21 +S9	- + - +	24/0 48/0 3/21 -S9 3/21 +S9	CHL	Ames -
ethionamide	+ -	24/0 48/0	- +	24/0 48/0	CHL	Ames - Carc -
ethyl acetate	- +	24/0 48/0	- +	240 48/0	CHL	Ames - MN -
ethylcinnamate	+ -	24/0 48/0	- -	24/0 48/0	CHL	Ames -
n-ethyl-n'-nitro-n-nitroso-guanidine	- +	24/0 48/0	+ +	24/0 48/0	CHL	Ames + Carc +
n-ethyl-n-nitrosurea	- +	24/0 48/0	+ +	24/0 48/0	CHL	Ames+ MN + Carc +
ethyl vanillin	- +	24/0 48/0	- -	24/0 48/0	CHL	Ames -
fiberglass code100 (1)	+	48/0	-	48/0	SH fibro	
formaldehyde	+ -	24/0 48/0	+ +	24/0 48/0	CHL	Ames + Carc -
gardenia red A	- +	24/0 48/0	+-	24/0 48/0	CHL	
garlic oil	- +	24/0 48/0	- -	24/0 48/0	CHL	
griseofulvin (1)	+ +	60/0 60/0	+ +	60/0 60/0	human lymph human EUE	Ames - MN - Carc +

Chemical Name	Polyploidy	Exp./Rec.	Aberration	Exp/Rec.	Cell Type	Other
n-hexane	-	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		
	-	3/21 -S9	+	3/21-S9		
	-	3/21+S9	-	3/21+S9		
hydrochlorothiazide	-	24/0	-	48/0	CHL	Ames -
	+	48/0	-			
6-mercaptopurine	-	24/0	-	24/0	CHL	Ames +
	-	48/0	+	48/0	CHL	MN +
	+ (1)	52/0	+ (1)	52/0	human lymph	Carc -
metformin HCl	-	24/0	+	24/0	CHL	Ames -
	+	48/0	+	48/0		
methylchlothiazide	-	24/0	-	24/0	CHL	Ames -
	+	48/0	+	48/0		
methyl-2-benzimidazole carbamate (1)	+	32/0	-	32/0	human lymph	
4,4'-methylenebis(2-chlor aniline)	-	24/0	-	24/0	CHL	
	+	48/0	-	48/0		
	-	6/18 -S9	-	6/18 -S9		
	-	6/18 +S9	-	6/18 +S9		
methyl-n-methylanthranilate	-	24/0	-	24/0	CHL	
	+	48/0	-	48/0		
naphthyl n-nitroso-n-methyl carbamate	-	24/0	+	24/0	CHL	Ames +
	+	48/0	+	48/0		MN - Carc -
nicotinamide	-	24/0	+	24/0	CHL	Ames -
	+	48/0	+	48/0		
nitrobenzene	-	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		
nitrogen mustard (1)	+	52/0	+	52/0	human lymph	Ames+ Carc +
5-nitro-1-naphthonitrile (1)	+	27/0	-	27/0	V79-CL-15	
1-nitropyrene	-	24/0	-	24/0	CHL	Ames +
	+	48/0	-	48/0		
m-nitrotoluene	-	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		
o-nitrotoluene	-	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		
p-nitrotoluene	-	24/0	-	24/0	CHL	Ames + (1)
	+	48/0	-	48/0		
noscapine HCl	+	24/0	-	24/0	CHL	Ames -
	+	48/0	+	48/0		
nylidrin HCl	-	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		
perillaldehyde	-	24/0	+	24/0	CHL	Ames -
	+	48/0	+	48/0		MN -
phenobarbital sodium	-	24/0	-	24/0	CHL	Ames +
	+	48/0	-	48/0		Carc ?
propazine	-	24/0	-	24/0	CHL	
	+	48/0	-	48/0		
riboflavin	+	24/0	+	24/0	CHL	Ames -
	+	48/0	+	48/0		MN -
salicylamide	-	24/0	-	24/0	CHL	Ames -
	+	48/0	+	48/0		MN -
sandalwood oil	-	24/0	-	24/0	CHL	
	+	48/0	-	48/0		

Chemical Name	Polyploidy	Exp./Rec.	Aberration	Exp/Rec.	Cell Type	Other
12-0-	-	24/0	-	24/0	CHL	Ames -
tetradeconoylphorbol-	-	48/0	-	48/0	CHL	Carc -
13-acetate	+ (1)	72/0	+ (1)	72/0	human lymph	
thiabendazole	-	24/0	-	24/0	CHL	Ames +
	+	48/0	-	48/0		
o-toluidine	+	6/18 -S9	-	6/18 -S9	CHL	Ames +
	-	6/18+S9	+	6/18+S9	CHL	(1) Carc + (1)
tribromethane	-	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		MN -
	-	3/21 -S9	-	3/21 -S9		Carc -
	-	3/21 +S9	+	3/21 +s9		
try-p1 acetate	-	24/0	+	24/0	CHL	Ames +
	-	48/0	+	48/0		Carc +
	+	3/21 -S9	+	3/21 -S9		
	-	3/21 +S9	-	3/21 +S9		
vamidothion (1)	+	27/0	+	27/0	V79-CL-15	Ames +

Table HB: Chemicals that induce polyploidy but not chromosome aberrations *in vitro* [clear positive (+) results only, from Ishidate, 1988]

Chemical Name	Polyploidy	Exp./Recov	Aberration	Exp./Recov	Cell type	Other
calcium citrate	+	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		
chlorine dioxide powder	-	24/0	-	24/0		Ames -
(3.5% in Ca silicate)	+	48/0	-	48/0		
citronella acid	-	24/0	-	24/0	CHL	
	+	48/0	-	48/0		
dinitrophenyl thiocyanate	+	27/0	- (1)	27/0	V79-CL-15	
estradiol	+	32/0	-(1)	32/0	human lymph	
ethylcinnamate	+	24/0	-	24/0	CHL	Ames -
	-	48/0	-	48/0		
ethyl vanillin	-	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		
garlic oil	-	24/0	-	24/0	CHL	
	+	48/0	-	48/0		
hydrochlorothiazide	-	24/0	-	48/0	CHL	Ames -
	+	48/0	-			
methyl-2-benzimidazole carbamate	+	32/0	- (1)	32/0	human lymph	
4,4'-methylenebis(2-chlor aniline)	-	24/0	-	24/0	CHL	
	+	48/0	-	48/0		
	-	6/18 -S9	-	6/18 -S9		
	-	6/18 +S9	-	6/18 +S9		
methyl-n-methylanthranilate	-	24/0	-	24/0	CHL	
	+	48/0	-	48/0		
nitrobenzene	-	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		
1-nitropyrene	-	24/0	-	24/0	CHL	Ames +
	+	48/0	-	48/0		
m-nitrotoluene	-	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		
o-nitrotoluene	-	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		
p-nitrotoluene	-	24/0	-	24/0	CHL	Ames +
	+	48/0	-	48/0		
nylidrin HCl	-	24/0	-	24/0	CHL	Ames -
	+	48/0	-	48/0		
phenobarbital sodium	-	24/0	-	24/0	CHL	Ames+ (Carc?)
	+	48/0	-	48/0		
propazine	-	24/0	-	24/0	CHL	
	+	48/0	-	48/0		
sandalwood oil	-	24/0	-	24/0	CHL	
	+	48/0	-	48/0		
thiabendazole	-	24/0	-	24/0	CHL	Ames +
	+	48/0	-	48/0		

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APPENDIX G - HUMAN LYMPHOCYTE CELL CYCLE DATA

The UK Industrial Genotoxicology Group conducted a collaborative trial to measure variability in cell cycle times in human lymphocyte cytogenetic studies (Henderson *et al*, in press). In this trial several different parameters were investigated for their effect on cell cycle time such as donor variation, inter-experimental variation and culture conditions. The data generated from this study are useful in determining appropriate harvest times for determination of aneuploidy and polyploidy and the relevant data are summarised below.

As a part of the trial each laboratory used their own standard cytogenetics culture conditions and incorporated bromodeoxyuridine at 48 hours and harvested the cultures at 72 hours. The individual laboratory times for cultures in the absence of metabolic activation were : 13.4, 14.5, 14.3, 13.5, 13.1, 15.1, 14.6, 14.3, 13.6 and 16.1 hours. Similar cell cycle times were found in each laboratory for cultures treated in the presence of S9.

Data has been extracted from this compilation in order to determine the proportion of second metaphases which would be present in cultures harvested at a time corresponding to 1.5 cell cycles (as used in regulatory cytogenetic studies). The data are based on experiments where the cells were cultured for 48 h after initiation and bromodeoxyuridine was added for 24 hour before harvest. Only those cultures where the cell cycle time was approximately 16 h (+ 2 h) have been selected from the complete data set as this would correspond to a harvest time of approximately 24 h.

The incidence of metaphase 1 (M1) and metaphase 2 (M2) cells found under these conditions is given below. At a harvest time equivalent to approximately 1.5 cell cycles there are almost equal numbers of M1 and M2 cells, indicating this is probably the optimal time point for both chromosome aberrations and polyploidy (although compound induced cell cycle delay is not assessed here of course). The average generation time (AGT) is calculated from the distribution of cells in M1, M2 and M3.

	M1 (%)	M2 (%)	M3 (%)	Mean AGT (h)
Lab 1	46	54		15.7
Lab 2	48	52		15.8
Lab 3	46	54		15.7
Lab 4	43	49	8	14.6
Lab 5	45	51	4	15.2
Lab 6	56	39	6	16.1
Lab 7	57	43		16.8
Lab 8	36	64		14.7

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APPENDIX H - GLOSSARY

Allele: One of two or more alternative forms of a gene occupying the same locus on a particular chromosome.

Aneuploidy: Condition of cells or individuals which have one or a few whole chromosomes more or less than the typical number of the line or species in question.

Chromatid One of the two visibly distinct longitudinal subunits of the replicated chromosome.

Chromosome: In prokaryotes the circular DNA molecule containing the entire genetic material of the cell. In eukaryotes one of the threadlike structures in the nucleus carrying the genetic information.

Centromere: The region(s) of each chromosome where the spindle fibres attach during mitosis or meiosis.

Dyad: The two chromatids connected at the centromere which make up one chromosome in the first meiotic division.

Gene Copy Number: Number of the same gene in one cell.

Gene Dosage: The number of alleles in a particular genotype.

Gene Product: Molecules (proteins, enzymes) produced by the cell ribosomes based on the information of a gene.

Genome Mutation: Alteration in the number of chromosomes.

Genotoxic: Toxic for the genome. The term includes induction of gene, chromosome and genome mutations as well as effects which are detected with indicator tests such as unscheduled DNA repair, sister chromatid exchange, DNA adduct formation etc.

Hemizygous: Situation when genes are present only once in a genotype and not in the form of pairs of alleles.

Heterozygous:	Diploid or polyploid cells or individuals having different alleles at one or more genes in homologous chromosome segments.
Heterokaryon:	A fungal cell, spore or mycelium possessing genetically different nuclei in a common cytoplasm.
Homozygotes:	Diploid or polyploid cells or individuals having identical alleles at all genes in homologous chromosome segments.
Meiosis:	The process of cell division in germ cells.
Mitosis:	The process of cell division in somatic cells.
Monogenic Disorders:	Genetic imbalance resulting from chromosome loss.
Monosomy:	Loss of a single chromosome during mitosis or meiosis; usually resulting from chromosome nondisjunction or chromosome lagging during cell division.
Nondisjunction:	Failure of sister chromatids in mitosis, or paired chromosomes in meiosis to migrate to opposite poles during cell division.
Nucleus Organiser Region:	The chromosome region which is active in nucleolus formation and which contains DNA complementary to ribosomal RNA.
Oncogenes:	Genes which are responsible (or partially responsible) for the process of malign transformation. In most cases mutated proto-oncogenes.
Proto-oncogenes:	Genes involved in the control of cell growth and differentiation.
Recessive Mutation:	Mutation that leads to a gene that fails to express phenotypically its presence in the heterozygous genotype.
Tumour Suppressor Gene:	Genes which prevent by their products the uncontrolled growth of the cell. In most cases they are recessive.
Trisomy:	Addition of a single chromosome during mitosis or meiosis generally as a result of nondisjunction.

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