The Muntjac Spindle Staining Assay:

An investigation of the ability of this assay

to detect potential aneuploidy inducing agents.

A thesis submitted for the degree

of Master of Philosophy by

Sally Ann Pascoe

Department of Biology and Biochemistry

Brunel University

June 1988

ABSTRACT

Department of Biology and Biochemistry
Brunel University, Uxbridge.

Sally Ann Pascoe
1988

The Muntjac Spindle Staining Assay: An investigation of the ability of this assay to detect potential aneuploidy inducing agents.

An assay using muntjac fibroblasts in conjunction with a differential spindle and chromosome stain was developed and evaluated for its ability to detect aneuploidy inducing agents. The effects of the spindle poison colcemid and the human carcinogen diethylstilboestrol were compared with those obtained after treatment of the cells with a number of compounds each believed to impair mitotic spindle function via a different mechanism. Vinblastine and nocodazole were both shown to have colcemid like effects suggesting a common mode of action and potential aneugenic activity. p-Fluorophenylalanine affected the spindle morphology resulting in an accumulation of atypical metaphases but without significant chomosome loss. Hydroquinone induced metaphase arrest and significant spindle damage but at doses very close to the toxic limits for this compound. Lastly, acenaphthene appeared to have no significant effect on mitosis at doses up to toxic levels.

The ability of the muntjac fibroblasts to express aneuploid elements in recovery cell populations following treatment with colcemid and p-fluorophenylalanine was also examined. The results were inconclusive although an induction of endoreduplication was indicated. Although both the spindle staining assay and recovery protocol require further refinement and validation, it was concluded that the assay was able to detect aneugenic agents especially those which act via the spindle.

CONTENTS

* alternative spelling, diethylstilbestrol

PAGE

1. INTRODUCTION

1.1 Aneuploidy

Aneuploidy is a genetic imbalance. This condition was identified and defined by Täckholm in 1922, whereby a cell or organism possessing fewer or more chromosomes than an exact multiple of the normal haploid number, was called an aneuploid (Bond and Chandley, 1983). This definition has now been broadened to include segmental or partial aneuploidy which refers to a condition in which fragments of chromosomes, rather than whole chromosomes are responsible for the aneuploid state (Hook, 1985). When there is one more than the diploid number of chromosomes (or chromosome segments) the condition is known as a trisomy and when there is one less chromosome (or chromosome segment) than the diploid number it is a monosomy. The two principal errors at cell division, which can be either spontaneous or induced, leading to aneuploidy are non disjunction and chromosome loss. Nondisjunction results from the failure of a homologous pair of chromosomes to separate normally so that both enter one daughter cell leaving none for the other. This results in one cell being trisomic for that chromosome and one cell monosomic. Simple chromosome loss will result in one normal cell and one cell monosomic for that chromosome. Consequently trisomy is the hallmark of prior non disjunction whereby monosomy is not (Bond and Chandley, 1983) (Fig. 1.1).

Aneuploid mammalian cells are characterised by differences in their biological behaviour compared to normal cells. When such

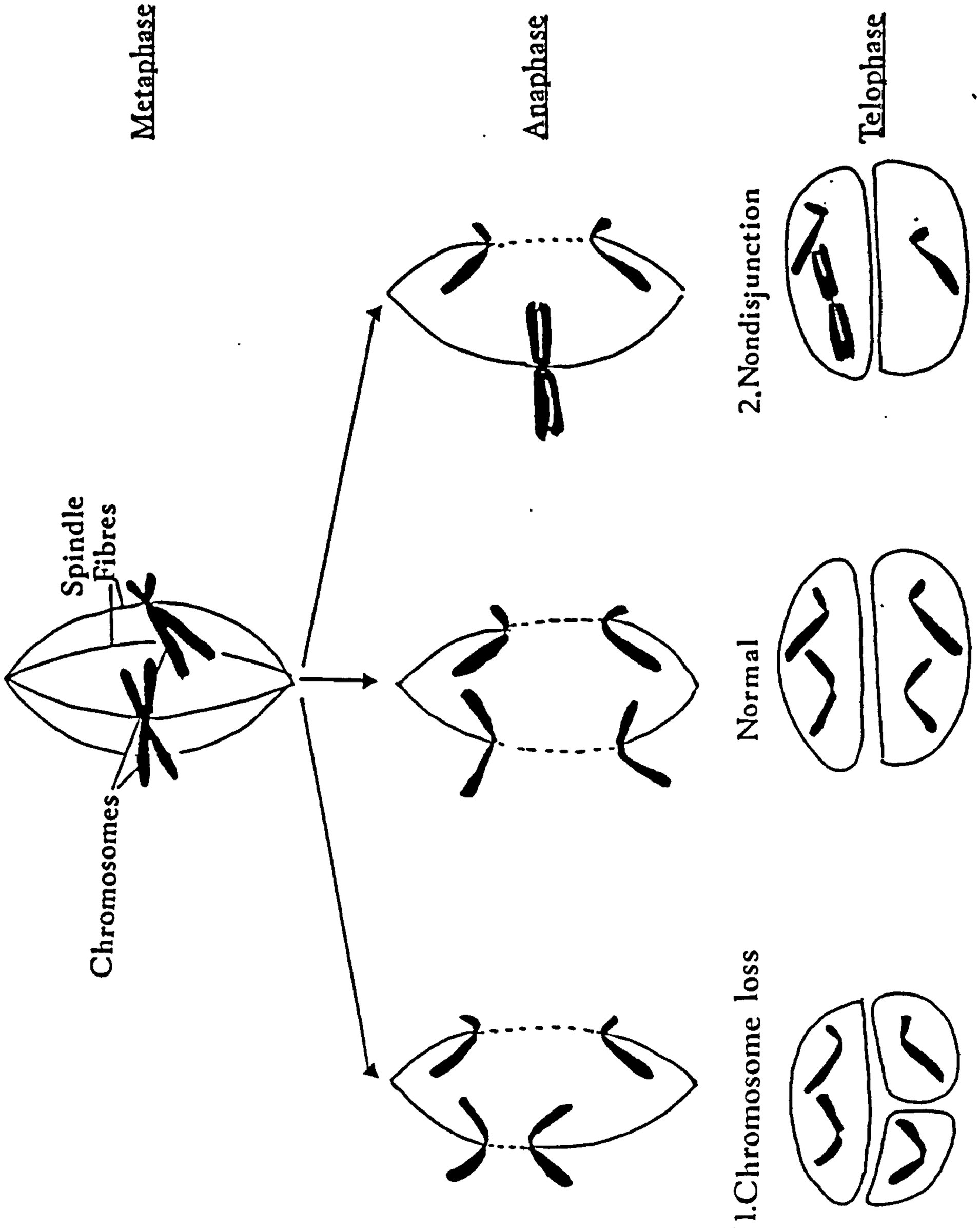


Fig.1.1 The two principal errors of cell division leading to aneuploidy.

changes occur in the meiotic cells of a species such as man, they may lead to spontaneous abortion and to a range of congenital abnormalities. It is known that approximately 15% of all clinically recognised pregnancies abort spontaneously and of the 50-60% showing karyotypic abnormalities approximately 70% are aneuploid (Bond and Chandley, 1983). This means that the vast majority of aneuploidy conceptions are expelled prenatally, however of those which survive to term, trisomy of chromosomes 13, 18 and 21 and sex chromosome aneuploids predominate. Trisomy 13, 18 and 21 are associated with the syndromes of Patau, Edwards and Down respectively. Each of these conditions involves small chromosomes which contain large areas of heterochromatin which is presumed to be genetically inert. This may explain why these conditions are more viable than other trisomies and monosomies of autosomal chromosomes.

The more common sex chromosome aneuploids which can survive to term are the 450X monosomy and 47XXX trisomy conditions in females and the 47XXY and 47XYY trisomic conditions in males. The monosomic condition in females is associated with Turner's syndrome and the 47XXY condition in males is associated with Klinefelter's syndrome (for review see Bond and Chandley, 1983).

In addition to the physical and/or mental disabilities suffered by all aneuploid individuals, to a greater or lesser extent, most will be infertile (Bond and Chandley, 1983) and some will have an increased chance of malignant tumour development (Evans, 1985).

Aneuploidy in somatic cells has strong associations with the development of malignant tumour cells, as illustrated by the genetic traits prone to cancer in aneuploid individuals. Although it has still not been established whether aneuploidy precedes the development of malignant tumour cells, chromosome aberrations have been found in some premalignant conditions.

Conti et al (1986) demonstrated the presence of aneuploid cells in chemically induced early skin papillomas. Aneuploid clones eventually displaced the diploid stem line at later stages. Squamous cell carcinomas, similarly induced, were also highly aneuploid. This suggests that the genomic imbalance produced by aneuploidy may be related to the malignant conversion of the benign skin papillomas.

Non random numerical chromosome changes are often seen in the blastic stage of some human leukaemias as well as in the acute and chronic phases. These often involve a trisomy of chromosome 8. Solid tumours are difficult to karyotype and therefore less evidence exists for the presence of aneuploidy within them. However trisomy of chromosome 7 and monosomy of chromosome 9 have been found as the sole karyotypic changes in some cases of bladder carcinoma. Non random numerical chromosome changes have also been reported in rodent tumours and trisomies of particular chromosomes in virally and chemically induced sarcomas, carcinomas and leukaemias in rat and Chinese hamster (Oshimura and Barrett, 1986).

The hypothesis that aneuploidy may play a role in cell transformation has been strengthened by the experimental work of Tsutsui et al (1983) with the synthetic oestrogen diethylstilboestrol (DES) and Syrian hamster embryo cells in culture. DES was shown to cause mitotic arrest resulting in the production of hyper and hypodiploidy without an increase in chromosome aberrations at doses which also induced morphological and neoplastic transformation. From these results it was postulated that aneuploidy had a role in the cell transformation process and possible carcinogenesis with nondisjunction as a key mechanism.

The mechanisms by which aneuploidy may induce the detrimental effects within germ and somatic cells may involve a change in gene dosage, balance or expression. A change in gene dosage may result in an increase or decrease of a gene product or changes in the rate of expression of a gene. The possible consequences of which may be reduced reproductive capability and congenital abnormalities. A change in gene balance could lead to a genetic trait prone to cancer and the phenotypic expression of a recessive mutation may be responsible for cell transformation (Oshimura and Barrett, 1986). Therefore the important feature of both somatic and germ cell aneuploidy is not the abnormal total number of chromosomes but the identity of the missing or extra chromosome and the genes that it may carry.

The maintenance of a stable karyotype is dependent upon the exact replication, disjunction and segregation of the chromosomes at division. To this end special division related components exist in eukaryotic cells which have to operate in a strictly controlled fashion. The structure and function of these components will now be discussed.

1.2 The division related components

These can be divided into two main areas:

1) The mitotic apparatus, including microtubules and associated proteins, membranes and centrioles.
2) The chromosomes and kinetochores.

1.2.1. The mitotic apparatus

Microtubules form the bulk of the mitotic apparatus. They also form the centrioles, cilia and flagella and are involved in the maintenance of cell shape and motility. They are present in nearly all eukaryotic cells and are made of subunits of tubulin assembled into long tubular structures. Electron microscopy reveals some variation in the fine structure of microtubules depending on their origin. However "simple" microtubules have a median diameter of 23nm and an indefinite length although this is generally longer than the diameter. The tubular wall is composed of 13 parallel protofilaments which are in turn formed by beaded or globular subunits approximately 4nm apart and having a diameter of 5nm. (Dustin, 1984).

The tubulin subunits are dimers of two very small proteins, α and ß tubulin each of approximately 50,000 molecular weight but differing in electrophoretic mobility. They assemble in a helicoidal structure of alternating α and ß units. Their precise location is not known but most data from simple microtubules indicate an alternating pattern both longitudinally and laterally (Dustin, 1984). Microtubule assembly is thought to occur by two distinct steps (Fig. 1.2). Firstly there is the formation of the tubulin dimers from a cytoplasmic pool of tubulin and their association into intermediate structures, either rings or spirals. Secondly these structures develop into protofilaments which curl up and form a shorter version of assembled microtubule. These then lengthen to form complete microtubules by the addition of tubulin dimers, often predominately at one end of the tubule only (Dustin, 1980).

These polymerised tubules are thought to exist in a state of dynamic equilibrium with the addition of tubulin at one end of the microtubule and loss at the other. This unidirectional flux of tubulin was defined as "treadmilling" by Margolis and Wilson (1981). An alternative theory of "dynamic instability" has also been proposed by Mitchison and Kirschner (1984) to explain the dynamic state of polymerised microtubules. This involves a model where equilibrium is maintained by the balance between a small number of rapidly depolymerising microtubules and a large number of slowly polymerising microtubules.

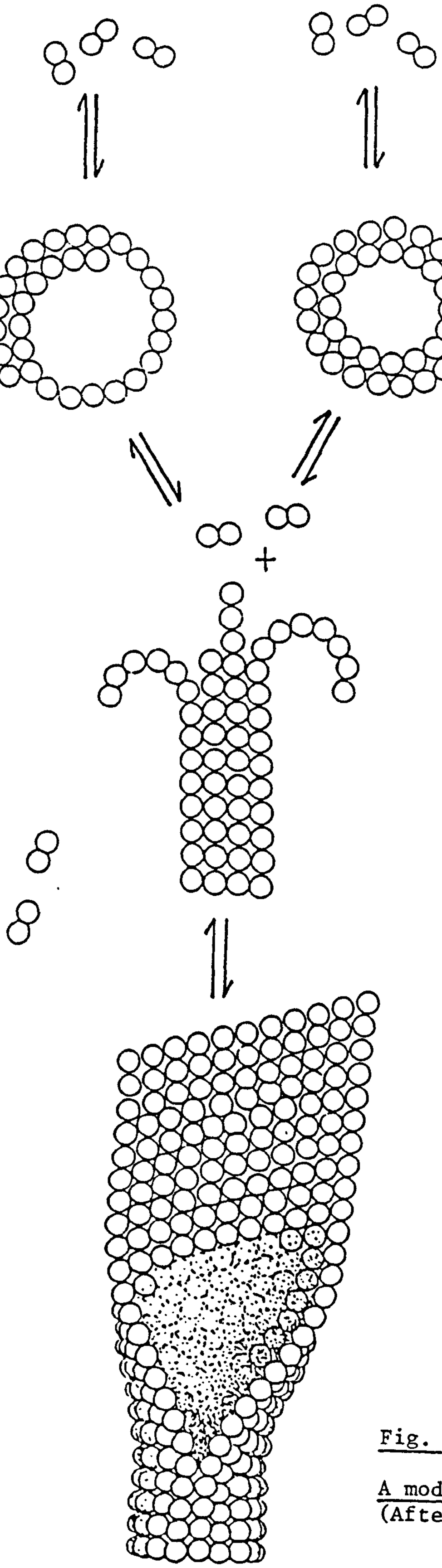


Fig. 1.2

A model for tubulin polymerisation
(After Somers, 1986; Dustin, 1980).

With either model it is obvious that the dynamic state of polymerised microtubules is directly related to its function. It is known that most microtubules in eukaryotic cells are labile and that depolymerisation can occur at low temperatures, in high calcium ion concentrations and polymerisation can be prevented by the action of drugs such as colchicine and vinblastine (Margolis and Wilson, 1981). The lability of the microtubules in the mitotic spindle, in so far as it reflects the rates of assembly and disassembly of the microtubules, seems to be linked with chromosome movement.

The mitotic spindle is formed by bundles of microtubules lying parallel and overlapping between the two poles formed by the centrioles. It often appears barrel shaped and symmetrical about the equator. The alignment of the microtubules and their associated proteins accounts for the structure's property of birefringence. Prior to the onset of cell division in the nucleus, the cytoplasmic microtubule complex breaks down and liberates free tubulin dimers into a cytoplasmic pool. Following initiation by microtubule organising centres (MTOC) found at the centrioles and kinetochores, tubulin polymerisation occurs. Microtubules grow out from the centrioles, forming the poles of the spindle, and towards the opposing pole. These microtubules are called polar microtubules (PMT). Kinetochore microtubules (KMT) grow from the kinetochores of each chromosome towards the centrioles. PMT are involved in elongation of the spindle at anaphase by pushing the poles apart, and KMT decrease in length to draw the chromatids towards the poles.

The microtubule associated proteins (MAP's) are thought to be of three types, namely a, b and c (De Brabander, 1986). Whilst they seem not to be an absolute requirement for microtubule assembly, experiments <u>in vitro</u> suggest that they aid polymerisation and help to stabilize the spindle. Some MAP's actually appear to be competitive to colchicine for binding to tubulin, others may increase stability towards low temperature and calcium ions. They have also been found to bind readily to satellite deoxyribonucleic acid (DNA) and it may be that they mediate the binding of tubulin to the kinetochore region, known to be rich in satellite DNA (Önfelt, 1986).

The microfilaments Actin, Myosin and Dynein have been shown to be present in the mitotic spindle, alongside the MAP's. Actin has been visualised between the spindle poles and the chromosomes at anaphase and may play some role in the shortening of KMT. However the exact functions of all three microfilaments are unknown although they may be involved in the contractile movements at cytokinesis (Dustin, 1984).

The assembly and disassembly of the microtubules throughout the mitotic cycle are thought to be under regulatory control. This is thought to be the function of calcium ions and a regulatory protein called calmodulin. Calcium binds to tubulin; there is one high affinity and several low affinity sites and the high

affinity site is inhibited by Mg^{2+}. In preparations of brain tubulin microtubule assembly is favoured by μM amounts of Ca^{2+} while larger concentrations (10μM) are inhibitory, suggesting that variations of Ca^{2+} could physiologically control microtubule assembly. Calmodulin, on the other hand, has a potent inhibitory action on microtubule assembly in the presence of Ca^{2+} (Dustin, 1984).

It is proposed that calmodulin, present in the polar regions of the spindle regulates the release of calcium ions, stored in vesicles, to trigger anaphase with a rapid disassembly of the microtubules (Dustin, 1984). Recently a protein called caldesmon has been described (Gratzer, 1987) which appears to bind calmodulin and actin and there is a likelihood that it exerts some form of calcium dependent control over actomyosin activity. Thus it appears that the regulatory proteins and the microfilaments may be interrelated in their activity although the mechanisms are unknown.

Electron microscopy reveals two types of membranes from within and around the mitotic apparatus. The first type are called vesicles and are derived from the Golgi apparatus which disintegrates at cell division. Large numbers of vesicles are found spread over the spindle during metaphase and anaphase. In addition to sequestering and storing calcium ions they may also contain other mitotic essential compounds and act as transport channels for them. The second type of membranes are

called cisternae and are derived from the nuclear envelope endoplasmic reticulum complex. They form a membranous sheath around and along the spindle. Their function is unknown although they may be involved with chromosome movement at anaphase (Paweletz and Schroeter, 1986).

In the non dividing cell there is one centrosome which appears as an electron dense cloud close to the nuclear membrane. This centrosome replicates just before cell division to form two centrioles which have the same structure as microtubules. The centrioles separate and move away from each other to form the poles of the mitotic spindle. Their separation is essential in maintaining the bipolarity of the spindle and their presence protects against its disassembly (De Brabander, 1986).

1.2.2 The chromosomes and kinetochores

In non dividing nuclei DNA is present in a very loosely structured form called chromatin. This chromatin replicates during 'S phase' in preparation for cell division. This is initiated in the nucleus by the condensation of the chromatin, firstly into fine filaments and then to form the chromosomes. Each chromosome consists of two identical sister chromatids which are joined at a constriction called the centromere. The position of the centromere on a chromosome forms the basis for the classification of the chromosome for karyotyping. For

example, a metacentric chromosome has a centromere mid way along its length and a telocentric chromosome has the centromere situated at one end. Other constrictions can sometimes be seen along the chromosome and are called secondary constrictions. These are the nucleolar organising regions and encode the ribosomal genes. In the interphase state the nucleolar organising regions form the nucleolus and are the site of active transcription of ribosomal ribonucleic acid (rRNA) (Oshimura and Barrett, 1986).

Superimposed over the centromeres of each chromosome are the kinetochores. Visualised by electron microscopy these structures are disc shaped with a diameter of between 0.2-0.8μm and with a thickness of between 60-150nm (Somers, 1986). They are seen to be composed of three layers and may be formed from the saturation of centromeric and repetitive DNA by proteins (Vig, 1986). They act as the point of attachment of the chromosomes to the spindle.

Since the major components for cell division have now been discussed it is possible to briefly describe the eukaryotic cell cycle. This can be broadly divided into three stages, namely interphase, division and cytokinesis. Interphase is the period between successive cell divisions when the cell recovers from division, performs its normal functions, and prepares for the next. These states are called G1 and G2 (G = gap), between them is S phase. This is where new DNA is synthesised by replication from the existing DNA. Interphase is also the

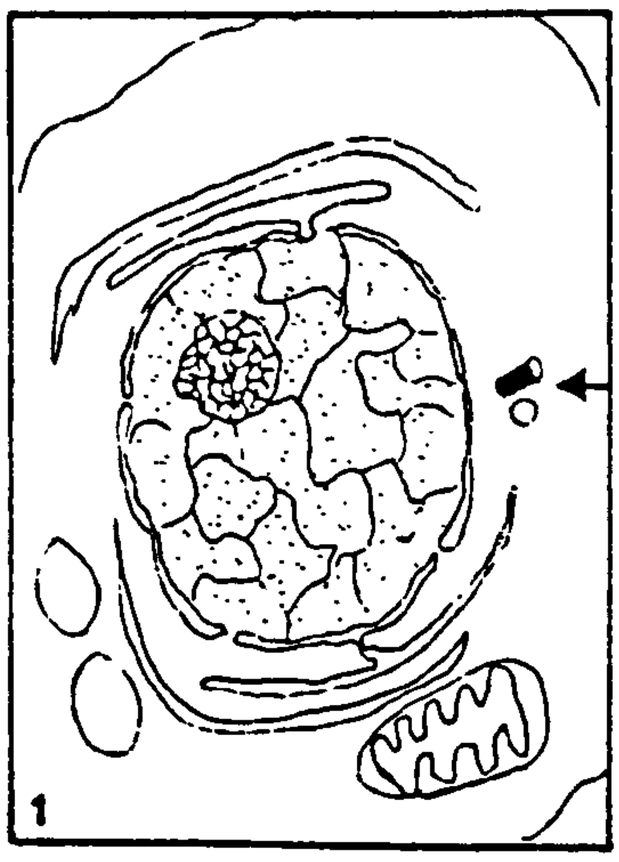
time when various macromolecules and cell organelles are synthesised for division. The precise sharing of the DNA and associated components into daughter nuclei occurs during the division stage. These proceed to form two independent cells following cytoplasmic contraction and separation at cytokinesis.

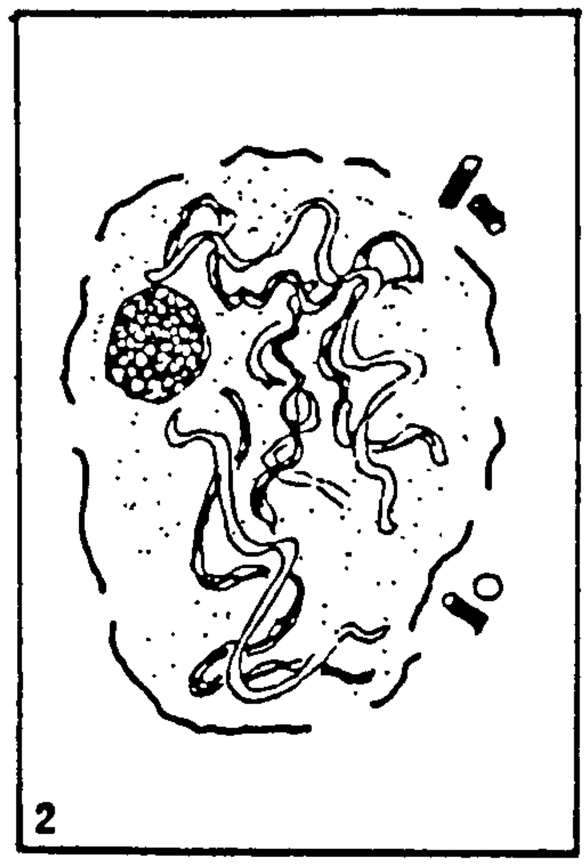
1.3 Mitotic division

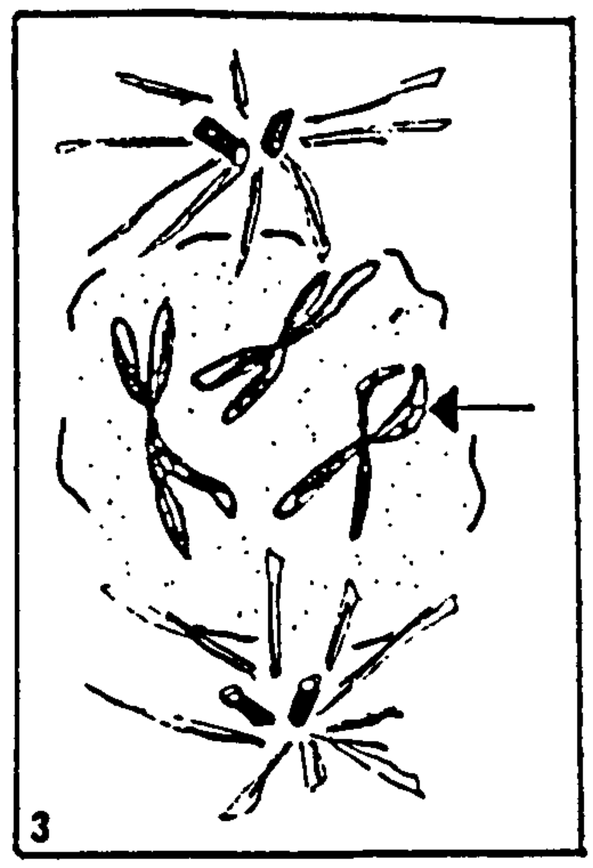
This is a continuous process although specific division phases can be identified. Fig. 1.3 illustrates these stages. 1; Interphase cell. The nucleus is contained within a membrane and the centrioles (arrowed) lie in the cytoplasm. 2-4; Early, middle and late Prophase. The nuclear membrane breaks down and the centrioles migrate to opposite sides of the cell, the chromosomes (arrowed) condense and the spindle begins to form. 5; Metaphase. The chromosomes are aligned at the equator of the spindle. 6; Anaphase. The centromeres divide and the chromatids move towards the poles. 7-8; Telophase. The spindle disappears all except for a few microtubules which form the telophasic bundle (arrowed) in the mid body. The daughter nuclei resume their interphasic pattern. Separation of the daughter cells involves contraction and sliding of the cytoplasm on the telophasic bundle (Dustin, 1984).

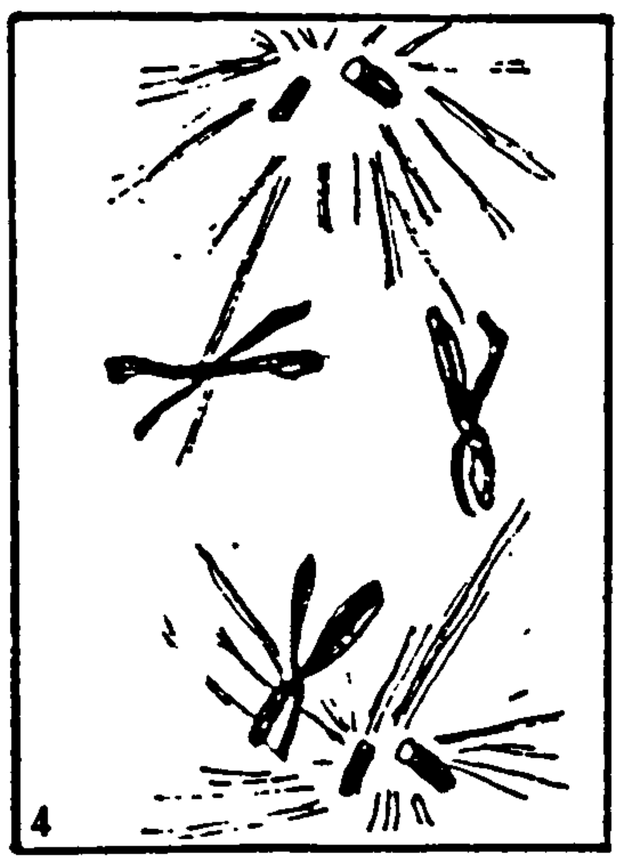
1.4 Chromosome movement in mitosis

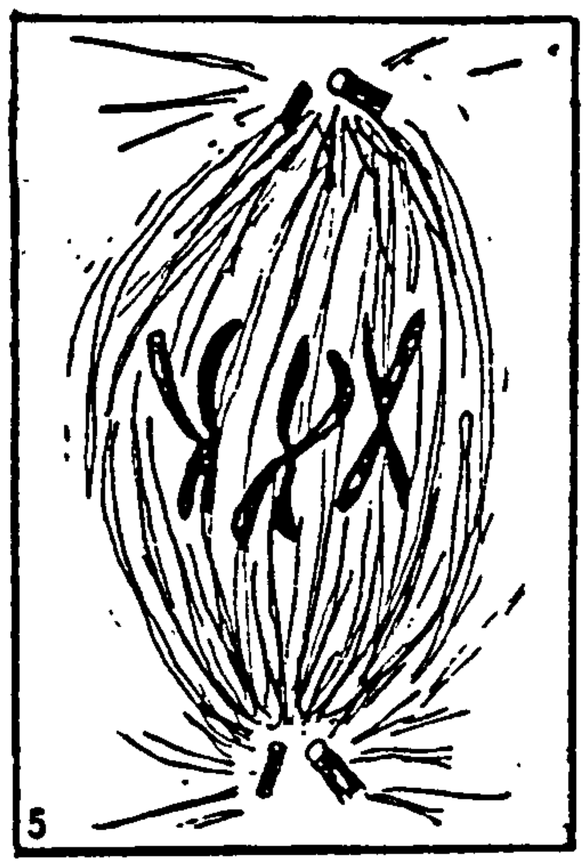
The actual mechanisms bringing about chromosome movement is of great interest and various theories have been proposed. They all involve the microtubules in an active role and the chromosomes in a passive role. Three theories will now be discussed briefly.

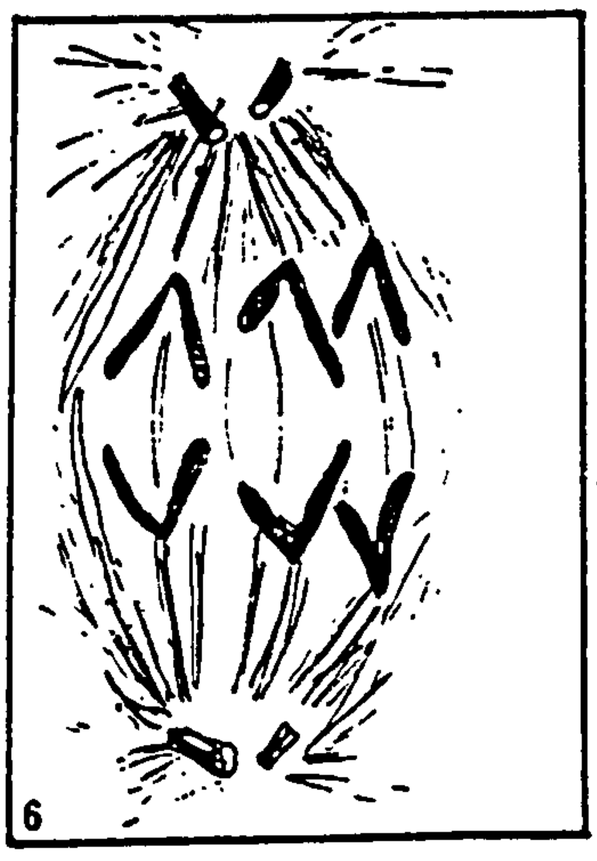


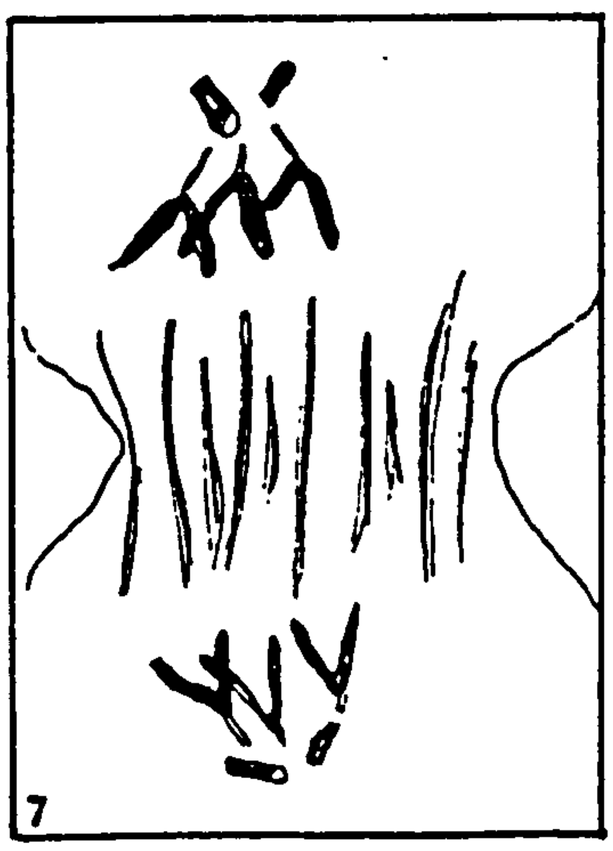












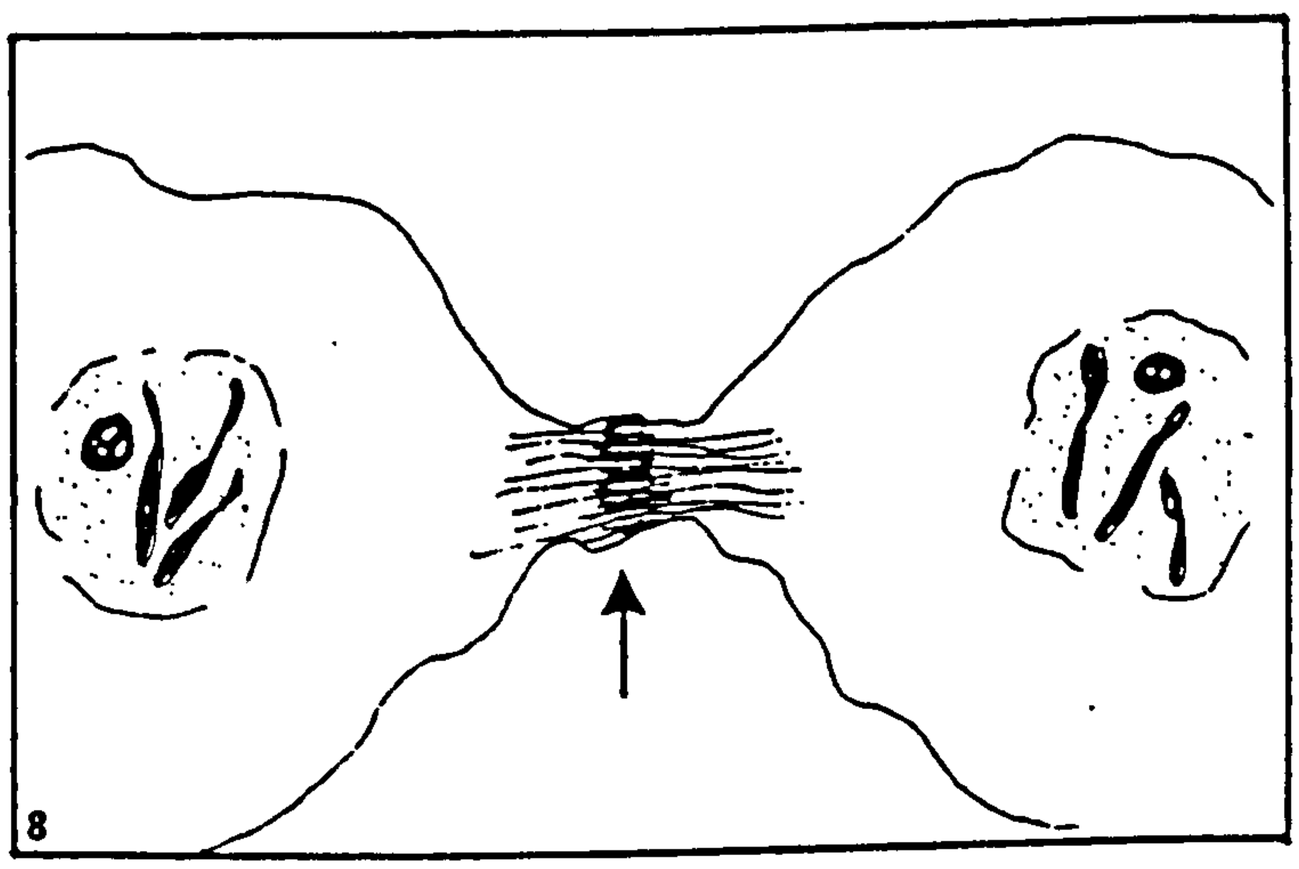


Fig. 1.3 Mitosis

1. Interphase
2-4. Early, middle and late prophase. → see text for explanation
5. Metaphase.
6. Anaphase.
7-8. Telophase.

(Adapted from Dyson, 1978).

1.4.1 The role of assembly - disassembly.

During mitosis the assembly and disassembly of microtubules may proceed side-by-side. Microtubules grow during mitosis, from a pool of tubulin originating from the disassembly of interphasic microtubules, and are in a dynamic condition as demonstrated by the elongation of the PMT whilst the KMT shorten during anaphase. The assembly-disassembly theory proposes that the elongation of the PMT and KMT pushes the chromosomes on to the equatorial plate at metaphase and then the shortening pulls the chromatids apart at anaphase (Fig. 1.4). This theory however does not take into account the elongation of the spindle and therefore microtubule growth at anaphase (Dustin,1984).

1.4.2 The sliding filament theory

An alternative theory, that involves sliding filaments (Fig. 1.5). has been proposed by McIntosh et al (1969). In this model there is interdigitation between KMT and PMT at the equator of the spindle. The opposing polarities of each microtubule type allow a sliding action of the microtubule which move the chromatids to the poles and the poles apart at anaphase. The sliding continues until telophase when the telophase bundle and its mid body become apparent. This theory however, has one weakness since it has been shown that the zone of overlap does not shorten at ana-telophase and therefore cannot explain the great elongation of the spindle (Dustin, 1984).

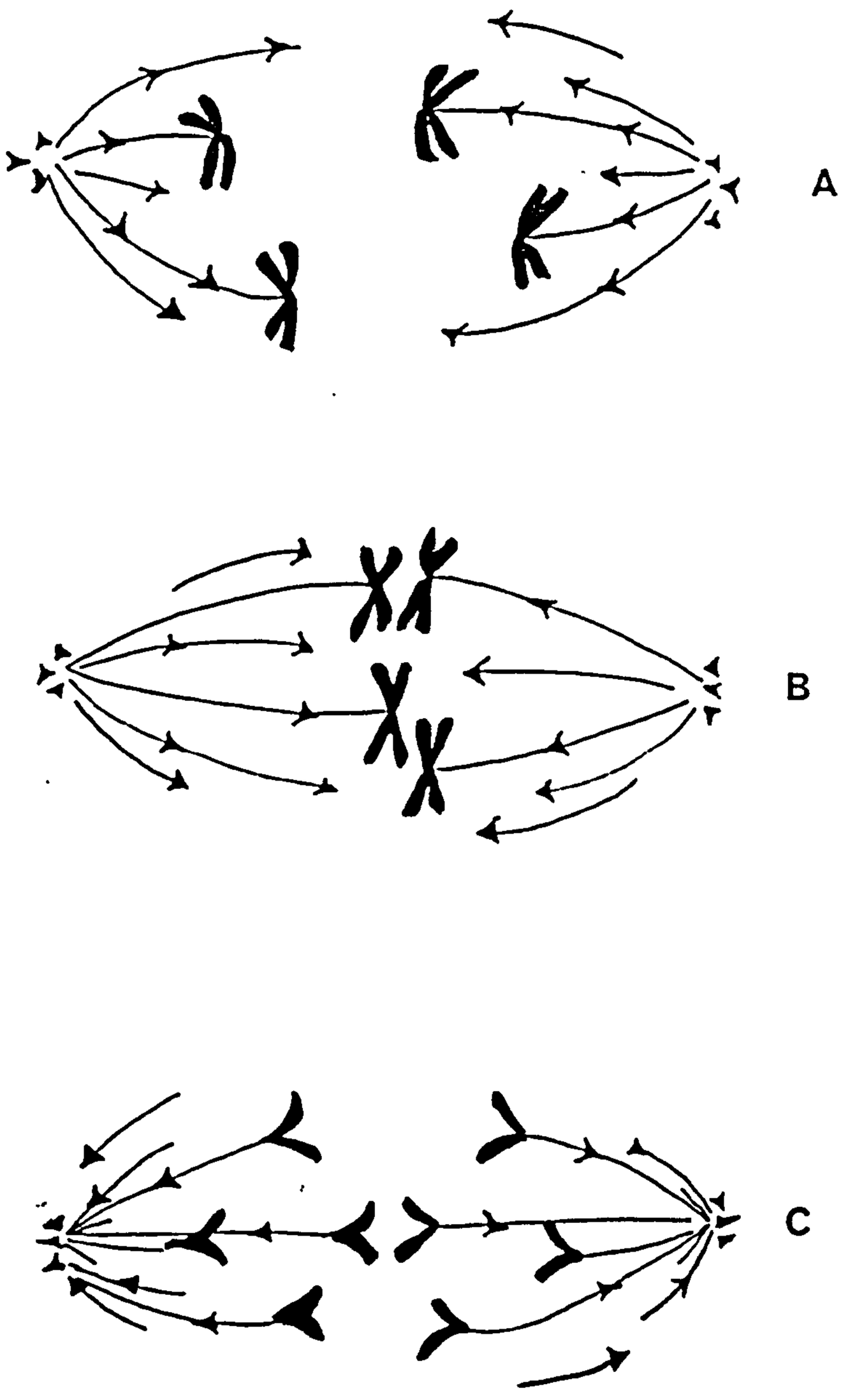


Fig. 1.4

The assembly-disassembly theory for chromosome movement; (A,B) tubulin dimers are added to the spindle at the poles pushing the chromosomes onto the metaphase plate. (C) tubulin dimers are removed from the spindle poles at anaphase and the sister chromatids separate (after Somers, 1986).

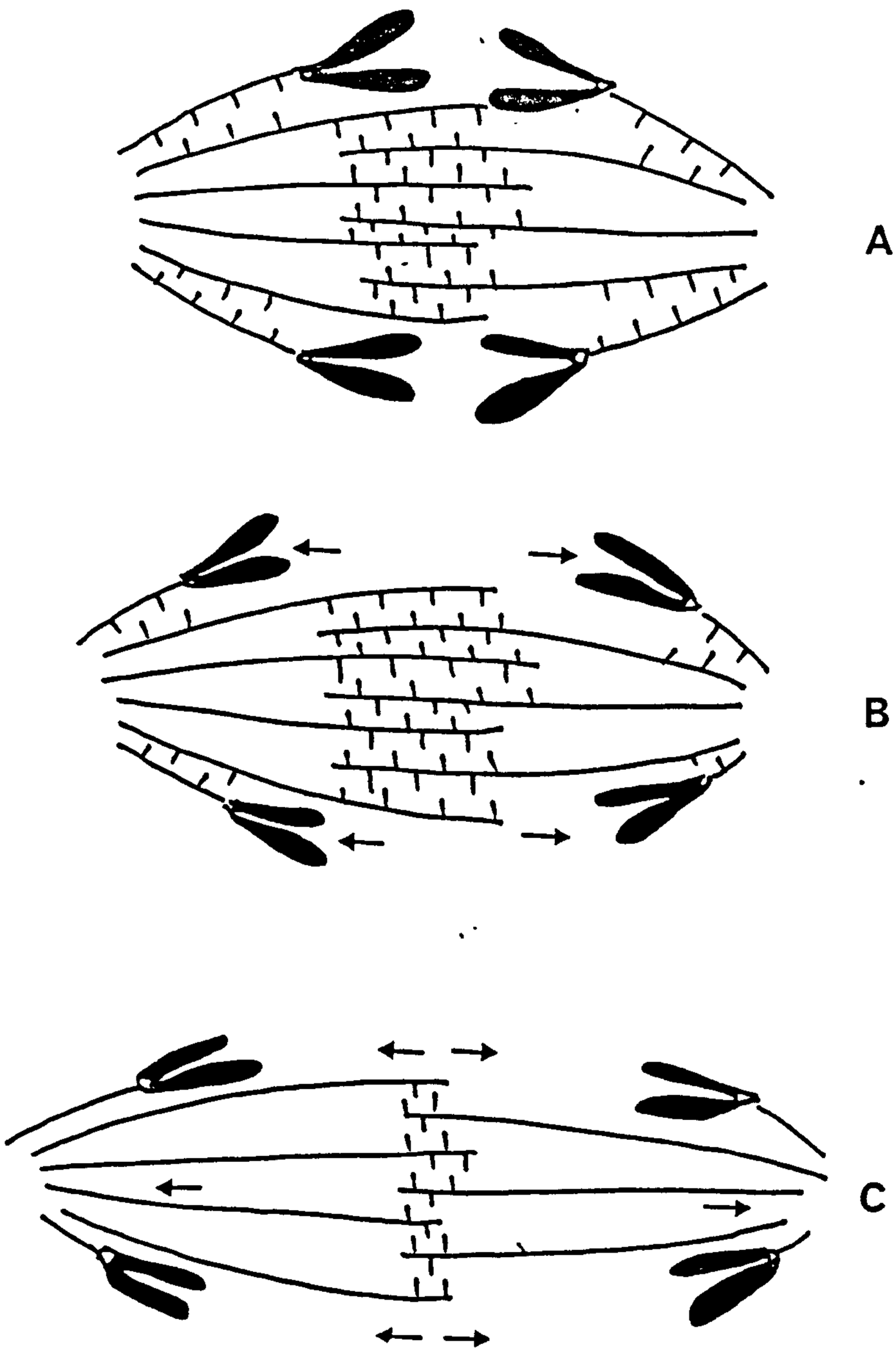


Fig.1.5

The sliding filament theory for chromosome movement: (a) arrangement of spindle microtubules before anaphase; (b) sliding between the KMT and PMT spindle fibres causes the chromosomes to move to opposite poles; (c) sliding between the PMT spindle fibres pushes the poles further apart (After Wolfe, 1981).

1.4.3 Treadmilling

A combination of these two models forms a third called treadmilling (Margolis and Wilson, 1981) which has been mentioned previously in relation to the dynamics of polymerised microtubules. It implies the assembly of tubulin guanine triphosphate at the (+) end of a microtubule, and the liberation of tubulin guanine diphosphate at the other or (-) end. As this has been calculated to take place at a rate of about 1μm/hr. it could be related to the slow movement of chromosomes at mitosis. The thoery is expanded to suggest an interaction and sliding of antiparallel microtubules for chromosome movement to the equator and a sliding of polar microtubules only at anaphase (Fig. 1.6). This suggests a constant assembly of microtubules at or near the mid plane of the spindle with an accompanying disassembly at the poles.

This theory however, contradicts the results observed when microtubules grow after their destruction by nocodazole since they appear to be assembled at the centrosomes and kinetochores only (Dustin, 1984).

All three models stress the importance of the mobility of the spindle throughout mitosis in relation to the function of chromosome movement. Although there is disagreement between workers as to the exact mechanism of chromosome movement two facts are certain. Firstly, mitosis is not possible without microtubules and any disruption of their proper assembly or disassembly prevents cell division. Secondly, the chromosome

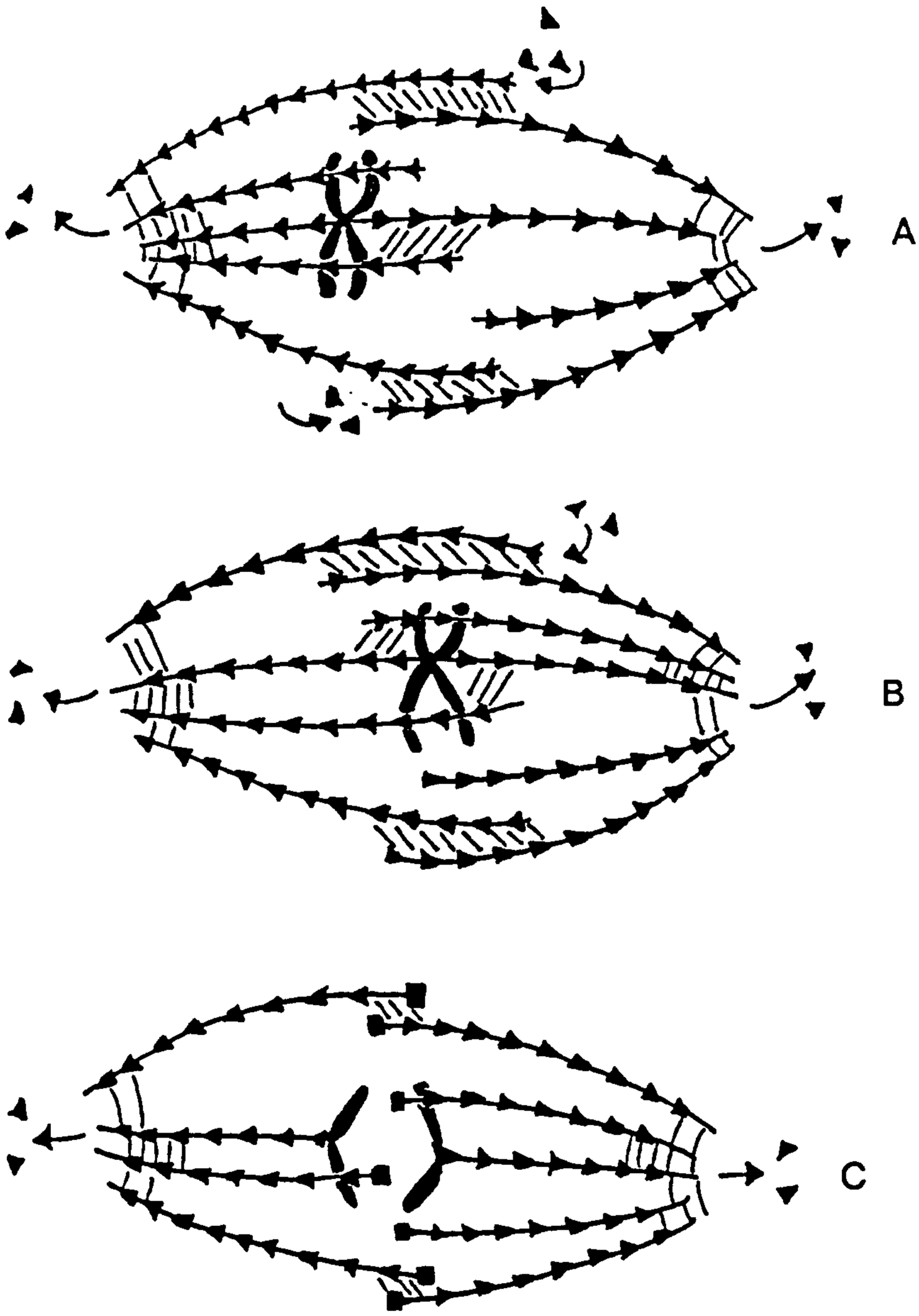


Fig. 1.6

The microtubule treadmilling theory for chromosome movement; (a) antiparallel microtubule sliding pushes chromosomes onto metaphase plate; (b) metaphase structure is maintained by the force generated by antiparallel sliding of KMT and PMT spindle fibres; (c) at anaphase microtubule assembly is blocked at the kinetochores but antiparallel sliding of microtubules continues (After Margolis and Wilson, 1981).

movements require microtubules which are of two types PMT and KMT which may have differing roles in mitosis. These facts have come to light by the use of certain chemical or physical factors which induce metaphase arrest either by acting as microtubule poisons or by causing alteration to the spindle apparatus.

1.5 The action of microtubule poisons on mitosis

The appearance of arrested mitoses is similar after most chemical poisons and after disassembly of the microtubules by cold or high hydrostatic pressure. Dustin (1984) grouped the morphological characteristics into three types.

1.5.1 The star metaphase

This appearance followed partial microtubule disassembly. The chromosomes surrounded a zone of cytoplasm in the centre of which were found the centrioles, which if replication had occurred, would be in a group of four. A few microtubules extended from this region towards the kinetochores which were turned towards the centre. No other microtubules were seen in the cell.

1.5.2 The exploded metaphase

This was seen when there had been total disassembly and the chromosomes became scattered throughout the cytoplasm.

1.5.3 The pseudo spindle

This described the disassembled microtubules which was found in the centre of the cell as an amorphous mass around which the chromosomes were scattered.

If the arrested metaphases did not die, Dustin (1984) notes that they may recover and form two daughter cells with a normal number of chromosomes or recover without cytoplasmic division leading to the formation of cells with polyploid nuclei or multiple micronuclei.

Although the morphological appearances of arrested metaphases induced by a variety of chemical or physical factors may look the same, it is known that the mechanisms involved can be very different, some of which have now been identified.

1.6 Mechanisms of microtubule inhibition

1.6.1 The colchicine type

Colchicine is an alkaloid extracted from the plant Colchicum and was one of the first mitotic arrestants found. Colchicine and its derivative colcemid bind to a 6S tubulin dimer and prevent the polymerisation process but do not depolymerise microtubules already in existence. This suggests that the binding site for colchicine is not accessible to the compound in intact microtubules which infers that the site may be in the region of tubulin-tubulin (or tubulin-MAP) interactions (Onfelt 1986). Following binding the formation of the spindle apparatus is inhibited and the cells entering mitosis are blocked at metaphase but cells already at metaphase or anaphase are unaffected. The chromosomes are often clumped in the

cytoplasm forming what is described as a C-mitosis. If arrest is prolonged the chromosomes continue to condense and eventually the sister chromatids separate and lie side by side (c-anaphase). The chromatids finally despiralize as if they were in telophase (c-telophase) and a diploid cell enters the next interphase as a tetraploid (Hsu and Satya-Prakash, 1985).

The effects of colcemid treatment on mitosis in Chinese hamster cells has been examined using electron microscopy. This demonstrated the appearance of metaphases very similar to Dustin's (1984) "star metaphase" and confirmed the presence of two pairs of centrioles in the centre (Hsu and Satya-Prakash, 1985).

When colcemid is removed from the medium, recovery quickly ensues. The two pairs of centrioles migrate away from each other and the chromosomes reorientate their position and form a metaphase plate with microtubules emanating from the kinetochores to the poles. Anaphase movement and cell division follows (Hsu and Satya-Prakash, 1985).

As far as the mode of action is concerned, probably most microtubule inhibitors belong to the colchicine type : interfering with polymerisation but not depolymerisation. This group includes podophyllotoxin, the benzimidazole derivatives and griseofulvin. Agents that bind to the sulphydryl residues of protein molecules have been shown to interfere with tubulin

polymerisation eg. mercury and arsenic compounds and both diethylstilboestrol (DES) and p-fluorophenylalanine (pFPA) appear to affect tubulin polymerisation but not depolymerisation although the exact mechanism is unclear.

1.6.2 The Vinca alkaloids

Vinblastine and vincristine are two well-known Vinca alkaloids. Superficially their effects on cell division are not dissimilar from colchicine however their binding sites on tubulin are different from that occupied by colchicine and their mechanism of action is unique. At high concentrations ($\geq$ $x10^{-5}M$) these compounds bind at two sites in such a way that the tubulin molecules aggregate and precipitate into "paracrystals". Affected cells arrive at metaphase but are unable to organise a spindle, they are unable to recover because there are no free tubulin molecules available and eventually will re-enter interphase without anaphase movement and become polyploid cells.

However at lower concentrations, vinca alkaloids do not induce tubulin crystallisation but metaphase arrest still occurs. It is thought that there may be two mechanisms of action and the second may be similar to that used by colchicine, especially as anaphase recovery is possible after treatment with the lower concentrations only (Hsu and Satya-Prakash, 1985). This effect may be due to the compounds binding to a set of low affinity binding sites which are primarily responsible for the depolymerisation of microtubules at intermediate concentrations (Somers, 1986).

1.6.3 Taxol

Taxol is isolated from the plant Taxus brevirolia and is used as an anti-tumour drug. Taxol affects microtubules and tubulin in the opposite way to colchicine; since it enhances the polymerisation process and inhibits depolymerisation. Long exposure of cells to taxol results in an accumulation of polymerised microtubules in the cytoplasm leaving no tubulin available for spindle formation and metaphase arrest occurs.

1.6.4 The action of physical factors on microtubules and mitosis

The alterations of spindle microtubules by physical means has been used to investigate the mechanisms of mitotic movement and the differences between microtubule types. These physical factors include low temperature treatment, heat shock, irradiation by ultra violet light and high hydrostatic pressure. The arresting properties of low temperature (3°C) have been known since 1890 and a relationship exists between low temperature and the loss of birefringence of the spindle (due to distruction of its fibrillar structure). Heat shock was one of the first techniques used in the production of polyploid plant cells and it is known that heating to 45.5°C disassembles the spindle of Chinese hamster cells in vitro causing mitotic arrest. High hydrostatic pressure affects the spindle by the binding of water molecules to the microtubules (Dustin, 1984).

1.7 Mitotic poisons and the induction of aneuploidy

To ensure the exact distribution of the genetic material at cell division all the division related components have to

function in a strictly controlled fashion. Exposure to a mitotic poison may interrupt the correct sequence of events which could lead to the maldistribution of the chromosomes and aneuploidy induction in the daughter cells. For example, of the compounds that affect the polymerization of tubulin, colchicine, organic mercury compounds and DES are all aneugenic in mammalian cells and the benzimidazole compound nocodazole and pFPA can induce aneuploidy in lower eukaryotes (Liang and Brinkley, 1985). Agents that crystallize tubulin eg. vinblastine and agents that enhance polymerisation of tubulin eg. Taxol have also been shown to induce aneuploidy in mammalian cells. It is now known that tubulin and microtubules are not the only targets of chemical attack which could potentially lead to the development of aneuploidy. Many of the division related components can be specifically affected by compounds and cause their dysfunction via a variety of mechanisms (Oshimura and Barrett, 1986) (Table 1.1,1.2) and some chemicals have been shown to induce aneuploidy (either meiotic or mitotic) in a variety of test sytems.

Agents which act on the mitotic apparatus or division related structures tend to induce chromosomal aneuploidy rather than segmental aneuploidy. It is the agents which act directly on the genetic material which may induce segmental aneuploidy due to chromosome breakage or an increase in chromosome stickiness. These effects may be due to crosslinking or intercalation of the DNA resulting in separation difficulties and breakage of the chromatids at anaphase (Somers, 1986, Oshimura and Barrett, 1986).

Table 1.1 Examples of compounds that have been shown to affect microtubules

Target	Chemical	Effect	Reference
Tubulin	Colchicine Colcemid Podophyllotoxin Benzimidazole derivatives Griseofulvin Sulphydral reagents Diethylstil-boestrol p-fluorophenyl-alanine	Affect the polymerisation of tubulin.	Liang and Brinkley (1985)
	Vinca alkaloids	Crystallize tublin	Liang and Brinkley (1985)
	Taxol	Enhance polymerisation of tubulin.	Liang and Brinkley (1985)
	Carbaryl	May decrease cellular glutathione and protein SH groups.	Oshimura and Barrett (1986)
Anaphase Spindle	Taxol	Inhibits chromosome-to-pole movement.	Liang and Brinkley (1985)
	Chloral hydrate	Blocks pole-to-pole elongation.	Liang and Brinkley (1985)

Table 1.2 Examples of compounds that have been shown to affect targets other than microtubules

Target	Chemical	Effect	Reference
Centromeres/ Kinetochores	Colcemid, Formaldehyde	Premature movement of chromosomes-to-pole, dislocation of chromosomes from spindle.	Gaulden (1985)
	Colchicine	Affects trilamellar structure of kinetochore.	Liang and Brinkley (1985)
	Mitomycin C	Remove kinetochores from chromosomes.	Liang and Brinkley (1985)
Centrioles/ Centrosomes	Colcemid	Prevents full growth of centrioles.	Dustin (1984)
	Griseofulvin	Formation of electron dense material around centrioles.	Dustin (1984)
	Diazepam	Blocks centriole separation.	Oshimura and Barrett (1986)
	Diethylstilboestrol	Elongation of centrioles.	Brinkley (1985)
	Ethidium Bromide	Structural damage to centrioles.	Liang and Brinkley (1985)
	Actinomycin D	Inhibits RNA synthesis and therefore inhibits centriole duplication.	Liang and Brinkley (1985)
	Ethylene glycol Halothane	Premature centriole division.	Bond (1986)

Table 1.2 cont.

Target	Chemical	Effect	Reference
Calmodulin Actin	Chlorpromazine	Induces metaphase arrest.	Liang and Brinkley (1985)
Cell membranes	Amphotericin B	Alters distribution of chromosomes.	Liang and Brinkley (1985)
	Fenariomil	Affects ergosterol synthesis.	
	Miconazole	Affects membrane structure.	
Nuclear envelope	Formaldehyde	Accelerated breakdown of nuclear membrane and premature separation of centrioles.	Gaulden (1985)

Chromosomal aneuploidy may also be induced by agents which act on the genetic material when the damage occurs at an important functional site of the chromosome such as the centromere or telomere (Somers, 1986; Oshimura and Barrett, 1986).

1.8 Test systems

Thus, it can be seen that there are several potential targets for chemical or physical action and as many possible mechanisms for the induction of aneuploidy in dividing cells. In man there is a high spontaneous frequency for aneuploidy and it is feared that this may be further elevated by the action of chemical hazards in the environment. Geneticists are well aware of the dangers of environmental mutagens and clastogens and the possible role that they might play in the development of human cancers. For this reason, mutagenicity screening has been introduced over the past 10-12 years to detect potential mutagenic and clastogenic chemicals which may be produced by the medical, chemical and agricultural industries. The role played by environmental aneugens with regard to any effect upon human health is not so clear, therefore it can only be postulated that exposure to these agents may increase meiotic and mitotic aneuploidy. For these reasons a variety of test systems supplementary to those already in use for the detection of mutagens and clastogens have been developed solely for the detection of aneugens (Table 1.3). This was prompted by the

TABLE 1.3 Approaches to detect aneuploidy

Test systems	Cell type	Aneuploid type*	Method
Fungi			
Aspergillus	Mitotic	Loss/gain	Genetic
Neurospora	Meiotic	Gain	Genetic
Saccharomyces	Mitotic/meiotic	Loss/gain	Genetic
Sordaria	Meiotic	Gain	Genetic
Higher plants			
Allium	Mitotic	Loss/gain	Cytological
Vicia	Mitotic	Loss/gain	Cytological
Hordeum	Mitotic/meiotic	Loss/gain	Cytological
Pennisetum	Mitotic/meiotic	Loss/gain	Cytological
Insects			
Drosophila	Meiotic	Loss/gain	Genetic
Mammalian cells in culture (established and primary)	Mitotic	Loss/gain	Cytological
Whole mammalian somatic cells (rodent or human)	Mitotic	Loss/gain	Cytological
Whole mammalian female germ cells (rodents)	Meiotic	Loss/gain	Cytological
Whole mammalian male germ cells (rodents)	Meiotic	Loss/gain	Cytological genetic

*Chromosome gain is considered to be the critical diagnostic aneuploid type, because loss may be due to artifacts of clastogenic effects.
From Dellarco et al (1985).

discovery that some chemicals eg. DES, could be human carcinogens but pass undetected in the standard assays for mutagenicity and clastogenicity. It is thought that aneuploidy induction may be the sole end point of their activity. None of the tests devised for the detection of mitotic and meiotic aneuploidy have been sufficiently investigated to meet the accepted standards of test validation and an ideal system has yet to be identified.

The test systems usually detect both chromosome loss and gain although a few of the fungal systems detect chromosome gain only. The methods of detection are either genetic or cytological. The genetic methods rely on the phenotypic expression of the chromosome imbalance in the aneuploid daughter cells and the cytological methods require microscopic examination and/or karyotypic analysis.

1.8.1 Genetic tests in fungi

These are capable of detecting aneuploidy in mitotic and meiotic cells. The Aspergillus, Neurospora and Saccharomyces test systems have been reviewed by Kafer et al (1986); Griffiths et al (1986) and Resnick et al (1986) respectively and all the systems were previously reviewed by Griffiths (1982). The fungal systems are reportedly simple and quick for the detection of chemically induced aneuploidy and have the advantage they they can be used both in the presence and in the absence of exogenous metabolic activation. However the differences between fungal and mammalian tubulin result in some species specificity, for example both colchicine and

vinblastine are very active in mammalian cells but are almost totally ineffective against fungal microtubules whilst the reverse is found with pFPA. This casts doubt on the results obtained in these systems with regard to their relevance to man.

1.8.2 Cytological tests in higher plants

These are also capable of detecting both meiotic and mitotic aneuploidy. A number of tests have been reviewed by Sandhu *et al* (1986) who concluded that they have the advantages of being more precise than mammalian meiotic cells and are less expensive than most of the *in vitro* and *in vivo* systems. However none of the plant test systems have been sufficiently utilized to warrant judgement for its sensitivity and specifity for detecting induced aneuploidy.

1.8.3 *Drosophila*

Drosophila offer an alternative to microbial systems and is used for the detection of meiotic nondisjunction. Test systems in *Drosophila* have been reviewed by Zimmering *et al* (1986) who concluded that they have not yet met the commonly accepted criteria for validation of a screening procedure ie. (1) reproducibility of results within a given laboratory in tests with a large number of compounds, (2) agreement between different laboratories testing a common set of chemicals with the same protocol and (3) an acceptable level of concordance with test systems in other organisms. Apart from the lack of system validation, the tests are usually tedious and time consuming which are disadvantages to a routine screening procedure.

However the advantages of Drosophila are that they are sensitive to colchicine (unlike fungi) and that the female germ cell stages are similar to those in human females. Their metabolic activation and deactivation processes are similar to human and a wide range of genetic markers are available for use (Foureman, 1986).

1.8.4 The grasshopper embryo system (Laing, 1983)

This system uses exposure to test chemicals via inhalation, a route which is very difficult to use in whole animals or for cells in culture. The embryos are exposed directly to the chemical in sealed containers and are then examined cytologically. The embryos are large and many are found in each egg case and they have a cell cycle time of <4 hours at 37°C. However only two generations are produced a year as the supply of eggs is seasonal. This problem can be helped by storage of the eggs at low temperatures which can be used when required (Hsu et al, 1986).

1.8.5 Cultured mammalian cells

The induction of chromosome aneuploidy by environmental chemicals in cultured mammalian cells has been studied using a number of different techniques (reviewed by Galloway and Ivett 1986). These range from the observation of abnormalities of cell division indicative of damage to the mitotic spindle (Parry et al, 1982) assessment of chromosome number by karyotypic analysis (Parry et al, 1984) the detection of double Y chromosome (Tenchini et al, 1983) and the detection of micronuclei (Viaggi et al, 1987). Flow cytometry has been used in an attempt to automate the screening of large numbers of cells for changes in DNA content in aneuploid cells (Barlogie et al, 1983; Rabinovitch et al, 1982). However flow cytometry

may not be able to distinguish aneuploidy from clastogenic events. The majority of the mammalian cell culture systems are based on metaphase chromosome counts. This technique is time consuming and susceptible to artifacts arising during slide preparation although Danford (1984) has developed a procedure to minimise the occurrence of chromosome loss during hypotonic treatment. Cells must be examined for aneuploidy induction after sufficient time has elapsed after treatment for one intervening round of cell division, yet before death of many aneuploid progeny has occurred. Second division cells can be detected by their differential staining following incorporation into the growth media of bromodeoxyuridine (BrdUrd) and a subsequent harlequin banding technique (Rainaldi et al, 1987). Established cell lines (particularly Chinese hamster CHO or V79) are often used which have a high and variable background frequency for aneuploidy and this may reduce the sensitivity of the system. The use of a primary cell culture might eliminate this problem (Dellarco et al, 1985). Assays in mammalian cells in culture like those in fungi can be used both with and without external metabolic activation.

A recent paper by Danford and Balfe (1987) investigated two different methods of scoring for aneuploidy induction in mammalian cells in culture which were a) counting all the chromosomes and b) counting only the chromosomes of a selected group within the karyotype. The results were then analysed by a variety of statistical methods. They concluded that whilst

counting of selective chromosomes may be advantageous in certain circumstances the former method was more sensitive for the detection of weak inducers of aneuploidy.

1.8.6 Mammalian somatic cells in vivo

As with mammalian cells in culture, the most common approach used to detect aneuploidy in somatic cells in vivo has been by direct cytological analysis of the chromosome complement of metaphase cells. Such studies have been conducted primarily in rodents using bone marrow cells and peripheral lymphocytes. Aneuploidy in somatic cells has also been inferred from the induction of micronuclei in rodent bone marrow (Schmid, 1975) and liver cells (Ashby and Braithwaite, 1987). However micronucleus analysis cannot discriminate between aneuploidy and chromosome breakage unless centromeres or kinetochores can be identified within the micronuclei by the use of specialist techniques (Vig, 1988).

1.8.7 Mammalian germ cells in vivo

Various in vivo analyses have been used for detecting aneuploidy in mammalian germ cells and the majority use chromosomal analysis. The analysis can be carried out at any stage from meiotic metaphase I and II, through gestation to birth. Based on known developmental effects of different trisomies, gross observation of day 12-19 mouse foetuses followed by cytological examination of the abnormals may be useful as an aneuploidy screen (Dellarco et al, 1985).

Genetic methods are confined to detecting aneuploidy in liveborns, and the majority of the work has used mice. The sex chromosome anomaly test detects chromosome loss at meiosis and a method using Robertsonian translocations can detect autosomal meiotic aneuploidy (Dellarco et al, 1985). These genetic tests in mice are favoured as they represent a system close to man, however they are still not well enough developed for general use and the cost of animal care makes the test system expensive. Reviews of mammalian in vivo assays for aneuploidy in male and female germ cells have been carried out by Allen et al (1986) and Mailhes et al (1986) respectively.

There is an urgent need for the development of an assay for aneuploidy which is simple, quick and relatively inexpensive and can be used as a screening procedure for environmental aneugens. Bearing this in mind, the aim of this present experimentation was to investigate an assay using cultured muntjac fibroblasts in conjunction with a novel staining technique for its potential as a screening procedure.

1.9 The muntjac spindle staining assay

In 1981, Wissinger et al published a method for the simultaneous staining of the mitotic spindle and chromosomes in mammalian cells in culture. They suggested that the technique would be useful in studies where alterations of chromosome number were due to aberrant spindle function and where the

effects of mitotic poisons were being analysed. The technique was modified by Parry et al (1982) to form the basis of an assay to investigate spindle synthesis and fidelity. The morphology of the mitotic spindle was maintained by the use of appropriate fixation procedures and the chromosomes were visualised with the stain Safranin O and the spindle with the stain Brilliant Blue R. The assay was used to determine the effects of colcemid and DES upon mitosis in cultured human fibroblasts. Since this time the spindle assay has been used by several workers and has demonstrated an important feature, that is its ability to be applied to a variety of cell lines [eg. Chinese hamster liver and lung cells and human fibroblasts (Parry et al, 1985)]. The muntjac fibroblast line has an advantage over several of these cell lines because the karyotype consists of seven chromosomes only. This means that the chromosomes do not obscure the spindle at metaphase and so small abnormalities in the structure can be observed. Initial experimentation with the muntjac spindle assay gave encouraging results so it was proposed to develop this system to screen several classes of chemicals known to have differing effects on the mitotic spindle and attempt to categorise the chemicals according to their mode of action.

1.10 The chemicals examined in the assay

The chemicals chosen and the reasons for their inclusion in the muntjac spindle assay will now be discussed;

1.10.1 Colcemid

Colcemid is an antineoplastic alkaloid, structurally similar to colchicine but less toxic. It induces mitotic arrest and aneuploidy in higher eukaryotes (Önfelt, 1986) and cell transformation in mammalian cells in culture (Tsutsui, et al, 1984). Recovering cell populations demonstrate an increase in the frequency of multipolar divisions which is related to the length of treatment time (Hsu and Satya-Prakash, 1985).

Colcemid was evaluated in the muntjac spindle staining assay for comparison with published data and used with a recovery protocol for the demonstration of its potential aneugenic activity.

1.10.2 Vinblastine

Vinblastine is also an antitumour alkaloid. It binds to tubulin in higher eukaryotes only, at a specific site different to that occupied by colchicine, inducing mitotic arrest and aneuploidy (Waters et al, 1985). The unusual mechanisms of action of vinblastine have already been discussed and it is the aim of this present study to investigate the effects induced in muntjac fibroblasts.

1.10.3 Diethylstilboestrol

DES has been used clinically to prevent abortion in early pregnancy, for hormone treatment in breast and prostatic cancer (Sawada and Ishidate, 1978) and up to 1979 it was also used as a growth promoter in cattle (Tsutsui et al, 1983). DES was implicated as a transplacental human carcinogen in 1971 by

Herbst et al. However, by this time DES had been prescribed to approximately three million women in the USA alone (Buitendijk, 1984). This has been a major disaster, with an estimated 1% of the cases progressing to cancer (Melnick et al, 1987) and an unknown number of both male and female offspring displaying congenital and behavioural changes (Ehrhardt, 1987). DES inhibits mitosis and induces aneuploidy and cell transformation in mammalian cells in culture (Tsutsui, et al 1983). DES is often used in other test systems for aneuploidy and so is included here for comparison with published data.

1.10.4 Nocodazole

Nocodazole is a synthetic drug with antitumour activity and is a derivative of the active product of the fungicide Benylate. In contrast to colchicine, nocodazole is a strong inhibitor of fungal tubulin and cell growth and is aneugenic in the lower eukaryotes. The evidence that nocodazole is aneugenic in higher eukaryotes is sparse, however its similarity to colchicine with regard to its binding site and mechanism of action suggests that its effects on chromosome number may be the same.

1.10.5 p-Fluorophenylalanine

pFPA is an amino acid analog which is incorporated into proteins, thus producing direct and indirect effects on mitotic as well as meiotic systems (Waters et al, 1985). It is

thought to be incorporated into tubulin in the place of phenylalanine affecting post-translational modification and leading to defective microtubule function and aneuploidy (Bond and Chandley, 1983). pFPA has been found positive for aneuploidy induction in fungal systems (Griffiths, 1979) and in male mammalian germ cells (Tates, 1979). It has also been shown to induce mitotic delay and some spindle abnormalities in mitotic cells in culture (Bond and Chandley, 1983). The proposed mechanism of action is unusual and was thought worthy of investigation in the muntjac spindle assay.

1.10.6 Acenaphthene

Acenaphthene is an insecticide and a member of the naphthalene series used by Levan and Ostergnen (1943) when they established the relationship between the solubility of a compound in water and its c-mitotic activity. They demonstrated that the activity of the naphthalene derivatives increased when their solubility decreased and suggested that it was a physical rather than chemical property of the substances to interfere with the spindle when it is exposed to concentrations above a threshold value (Bond and Chandley, 1983). Acenaphthene is a polycyclic hydrocarbon and is therefore only slightly soluble in water. Using a saturated water solution with an excess of undissolved crystals present, Kostoff (1938) demonstrated the induction of meiotic and mitotic aneuploidy in the seedlings of various grasses and the floral buds of Nicotiana. Kostoff observed that the formation of the spindle was inhibited so that the chromosomes did not divide and this resulted in the

formation of polyploid or polynucleate cells. Pathak and Hsu (1977) reported the induction of aneuploidy by acenaphthene following injection of the compound into the testes of Chinese hamsters and then examination of cells in second meiotic metaphase.

Thus acenaphthene was included in this experimentation since it has been shown to have colchicine like activity both <u>in vitro</u> and <u>in vivo</u> although structurally the two compounds are quite different. It is was hoped to demonstrate that the activity of acenaphthene is due to an entirely different, possibly physical mechanism, rather than due to a chemical mode of action as found with colcemid.

1.10.7 <u>Hydroquinone</u>

Hydroquinone is a metabolite of benzene, it is a known mitotic poison and a potent inducer of micronuclei in mammalian cells (Jenssen and Ramel, 1980). Parmenter and Dustin (1948, 1953) reported scattered groups of chromosomes in mouse cells following hydroquinone injection which under certain circumstances could move to the poles. They suggested that hydroquinone was a mitotic poison affecting pre-metaphase movement. The exact mechanism of action is still unknown and it is hoped that inclusion in the muntjac spindle assay could help in its identification.

The chemical structures for these compounds are given in Fig. 1.7.

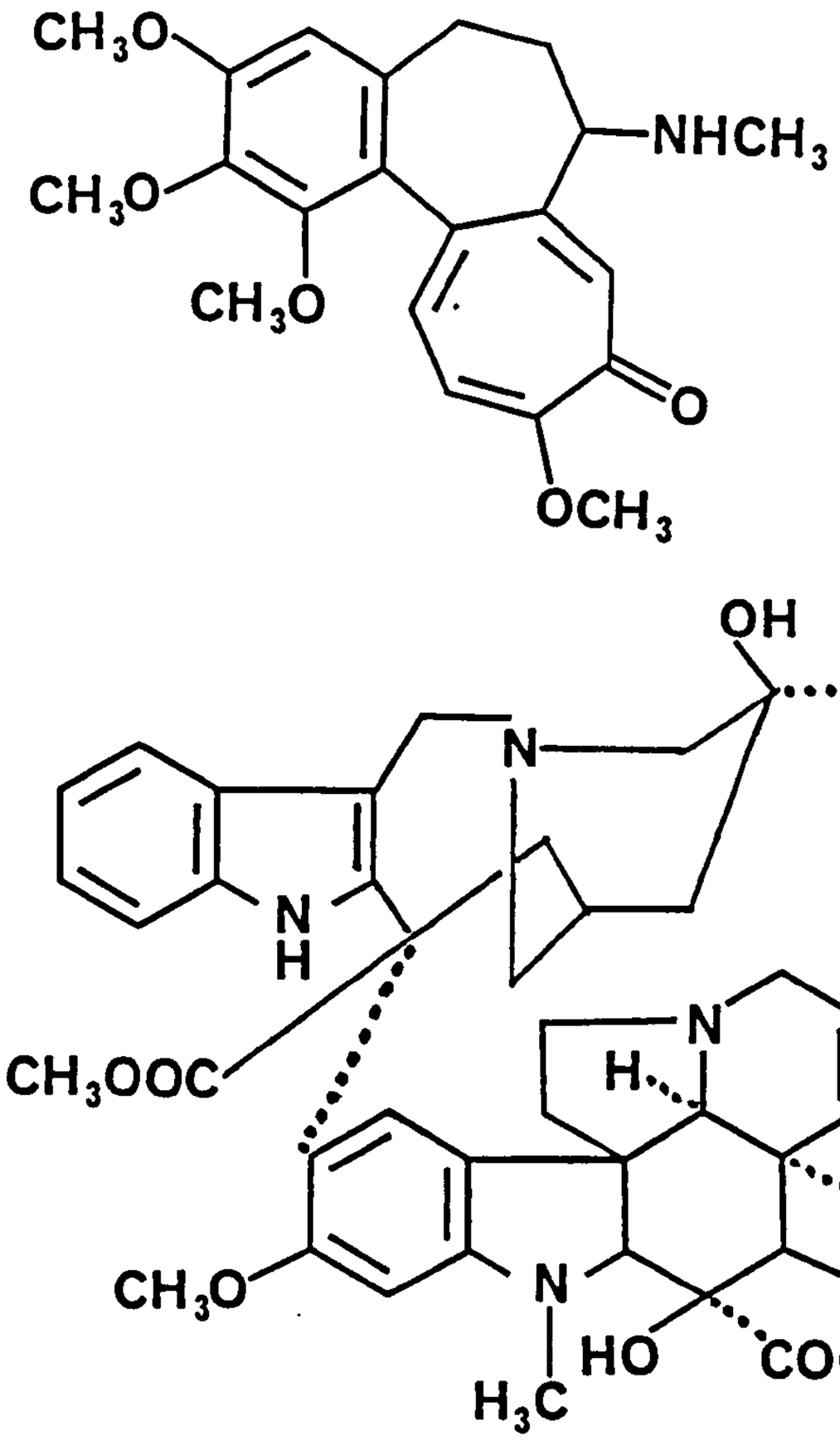
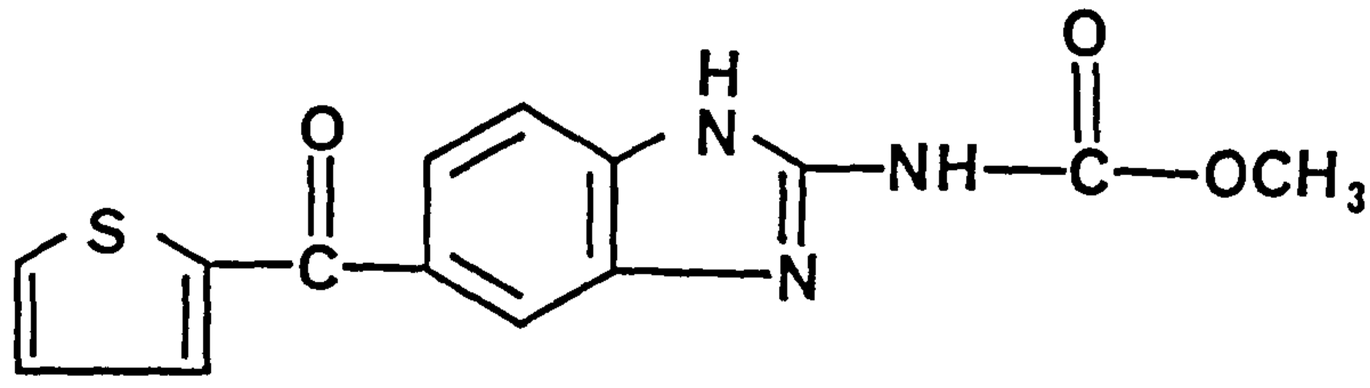


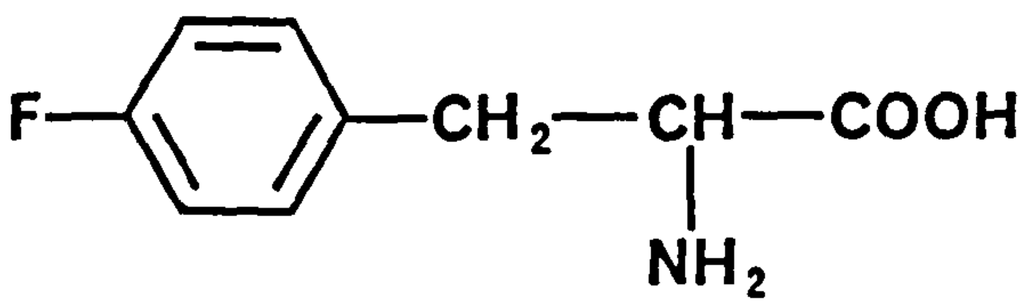
Fig. 1.7

Chemical structures of the compounds under investigation.

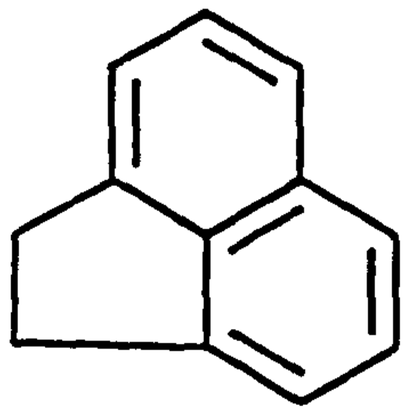
A Colcemid
B Vinblastine
C Diethylstilboestrol



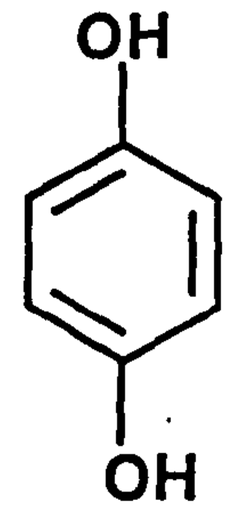
D



E



F



G

Fig. 1.7 Cont.

D Nocodazole
E p-Fluorophenylalanine
F Acenaphthene
G Hydroquinone

1.11 Indian muntjac fibroblast cell line

The Indian muntjac (Muntiacus muntjak) cell line was originally initiated by Wurster and Benirschke in November 1969, from a skin biopsy from an adult male muntjac. Muntjac, members of a primitive subfamily of the deer family, Cervidae, have a diploid chromosome number of seven in the male and six in the female. It is thought that the karyotype evolved through a series of translocations from a related deer, the Chinese Muntjac (Muntiacus reevesi), whose diploid chromosome number is 46 and consists of small telocentric chromosomes (Brinkley et al, 1984).

1.11.1 Karyotype

The chromosomes of the Indian muntjac may be unique among mammals due to their low diploid number, large size and unusual centromeres. The karyotype (Fig 1.8, Plate 1.1) consists of one pair of large metacentric and two pairs of acrocentric autosomes. There is an X-autosome translocation with the short arm being the actual X and the long arm the translocated autosome. The whole chromosome is usually referred to as the X chromosome and is characterised by an unusually long and segmented centromeric neck. The female of the species has two such X-autosome translocated chromosomes. The male has a sex chromosome complement of XY_1Y_2 with the true Y chromosome (Y_1) being a tiny metacentric and Y_2 being the normal homolog of the autosome with the X translocation (Brinkley et al, 1984).

Both the X chromosome and its homologue Y_2 have conspicuous secondary constrictions in the proximal region of the long arms. These are associated with nucleolus organizing centers, the chromosomal locations for rDNA, and have been demonstrated in the muntjac chromosomes by <u>in situ</u> hybridization techniques (Pardue and Hsu, 1975). In addition, a cluster of rDNA is found near the tip of one arm on the large metacentric chromosomes. This however does not usually express as a secondary constriction at metaphase.

A final morphological characteristic for the Indian muntjac chromosomes is a pronounced telomere formation (Plate 1.1).

1.11.2 <u>Giemsa banding</u>

The muntjac chromosomes are further characterised by their giemsa (G) banding patterns following trypsin treatment (Fig. 1.9 Plate 1.2).

Chromosome pair number 1: Four bands (the second much darker) on the upper arm, a small dark band near the centromere and 4 bands (the third much darker) on the lower arm.

Chromosome pair number 2: Banding in the long arm only with one central band and two or three less distinct bands either side.

Chromosome pair number 3: A distinct dark band just below the centromere and two lighter bands below the secondary constriction in the long arm. The elongated neck of the X

chromosome is lightly stained and a single band is seen in the centre of the short arm.

The single Y chromosome appears lightly stained but this may be due to its very small size.

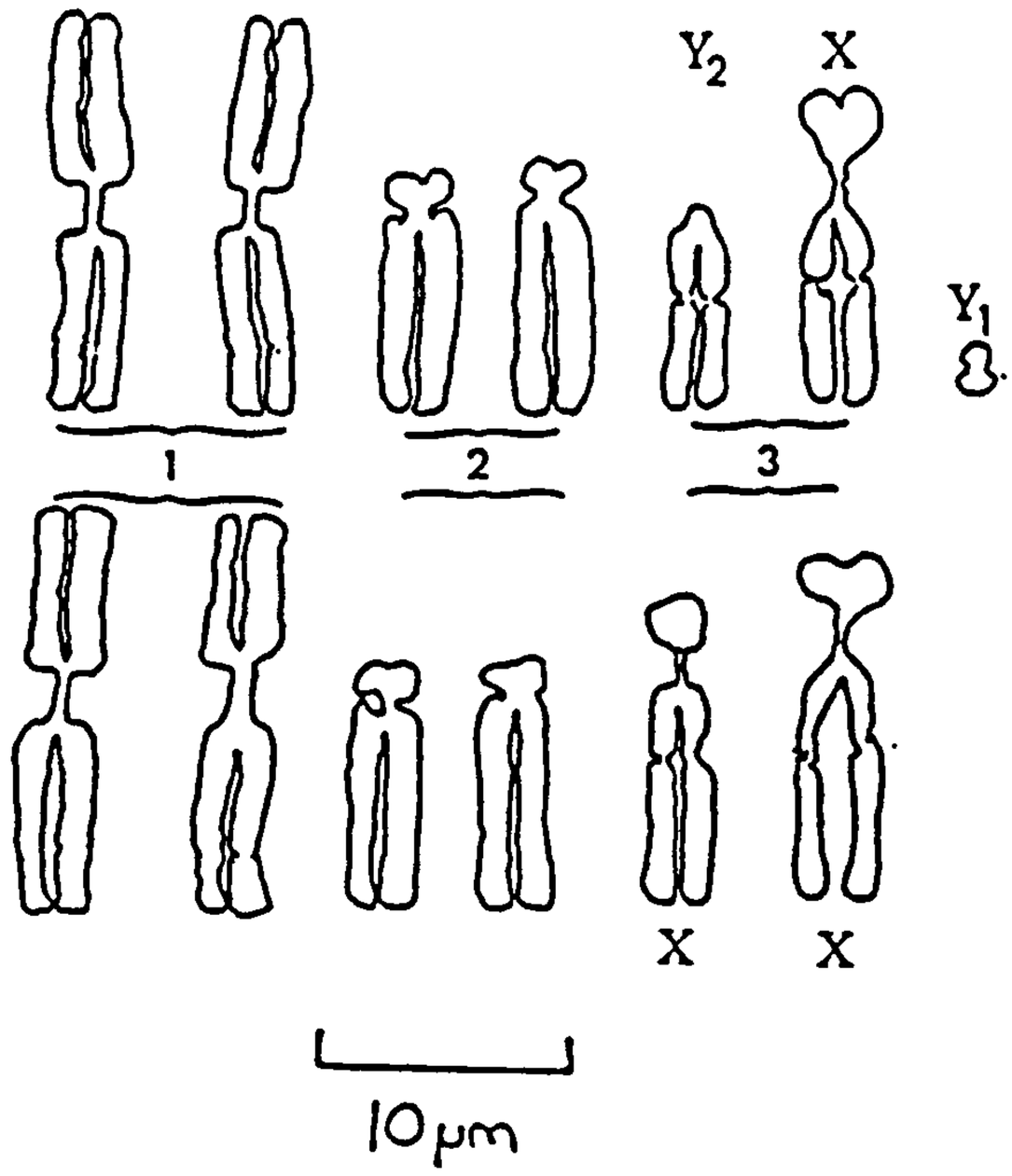


Fig.1.8

Indian muntjac chromosomes (after Wurster and Benirschke, 1970).
Top row ♂
Bottom row ♀

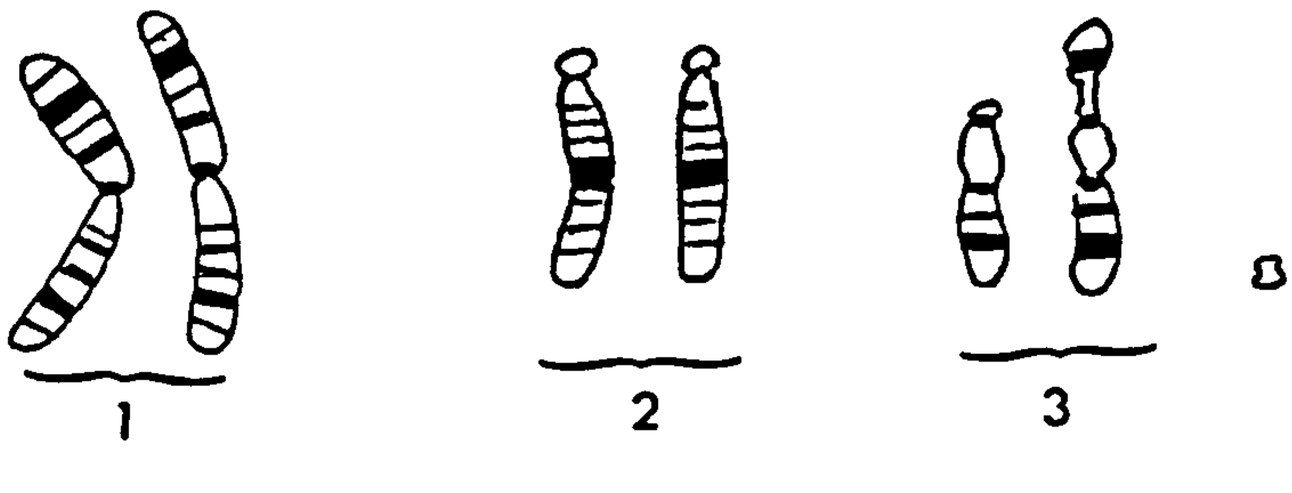


Fig. 1.9

Diagrammatic representation of
G banded Indian muntjac chromosomes (♂)

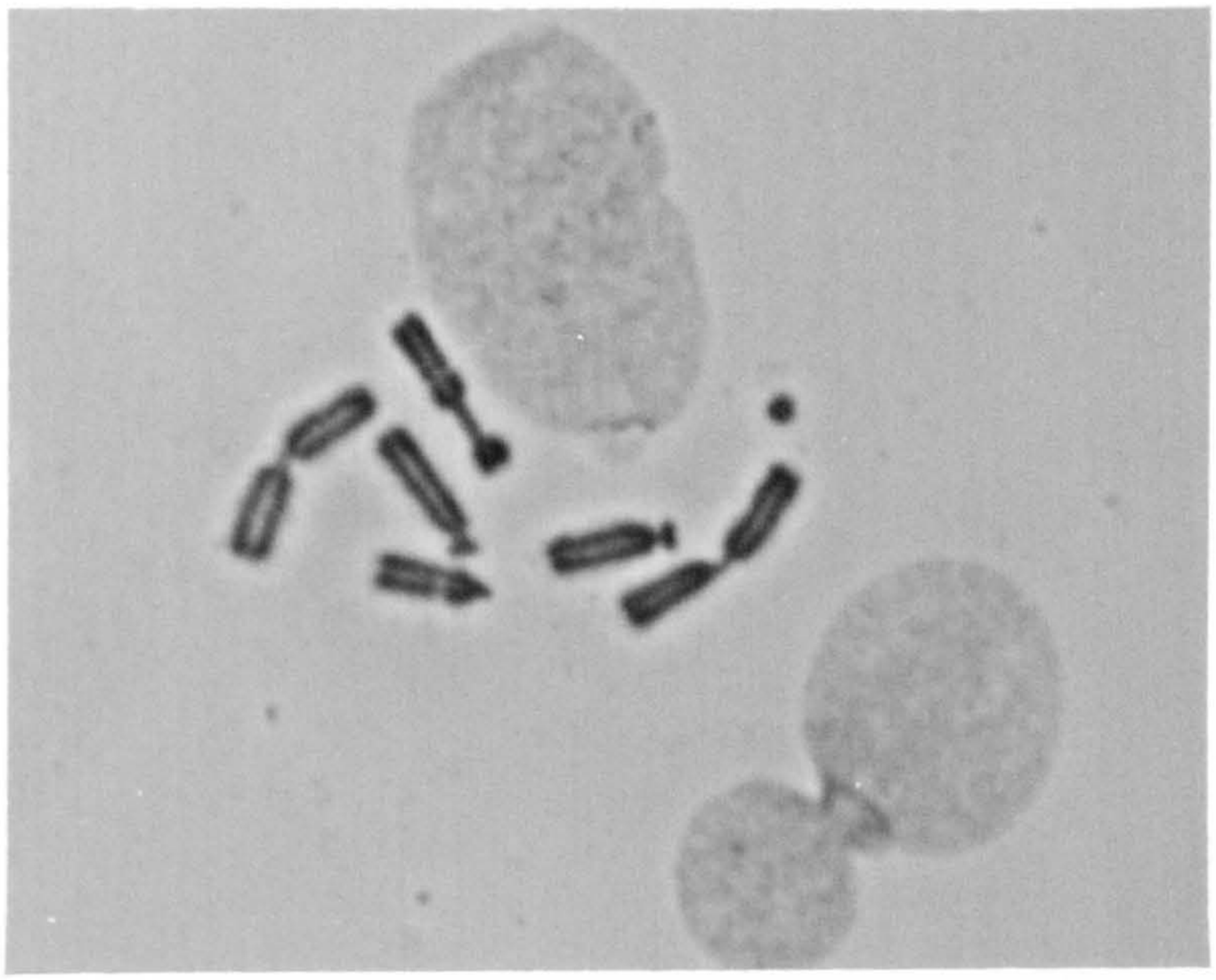


Plate 1.1

Muntjac chromosomes ♂
Aceto-orcein/phase contrast

Plate 1.2

Muntjac chromosomes G banded,
normal light microscopy

2. PRELIMINARY INVESTIGATIONS

2.1 Cell culture

The actual passage number of the cell line could not be specified by the supplier, however the cell lines obtained for the following experiments were at the lowest passage number available.

The cells were grown in Ham's F10 medium supplemented with L-glutamine, foetal calf serum and antibiotics. The latter were omitted regularly to confirm the absence of low levels of suppressed bacterial or mycoplasmal contamination. The percentage of foetal calf serum in the growth medium varied depending on its toxicity towards the cells. This was determined by monitoring the cell generation time each time a new batch of serum was obtained. This method was also used to determine the suitability of the medium from different suppliers and to check for the optimal incubation conditions.

When sufficient cells had been cultured they were preserved in liquid nitrogen until required. Different cryopreservation media [containing either dimethylsulphoxide (DMSO) or glycerol] and cryopreservation techniques were evaluated to determine the optimum conditions for preservation and recovery of cell stocks.

2.2 Characterization of the cell line

2.2.1 Cell generation time.

The generation time was calculated frequently to monitor cell growth at the passage numbers between which the experiments were carried out. If the generation time was too long ie. cell growth was slow, the cells were discarded and more removed from frozen storage. The cell generation time for muntjac fibroblasts reported in the literature varied between 12 and 24 hours (Ved Brat et al, 1979; Huttner and Ruddle, 1976; Carrano et al, 1976). This sort of variation was experienced with the cells used in this laboratory but was regarded as acceptable providing that it did not exceed 24 hours. The cell generation time was determined by seeding $5x10^4$ cells into pairs of T25 flasks and then harvesting and counting the cells at various intervals after culture initiation. (Plotting the total cell number against time resulted in the production of a growth curve). The cell generation time was calculated over the period of logarithmic growth an example of which is illustrated in Fig. 2.1. The cells exhibited a lag phase for approximately 24 hours after culture initiation whilst they attached to the new culture flask and prepared for cell division. This lag phase has also been reported by other workers (Patterson and Petricciani, 1973). Frequently there was a drop in total cell number in flasks harvested after the first 24 hours of incubation. This was due to some cells not surviving the subculture.

Fig.2.1 Growth curve for Muntjac fibroblast cells, passage 11 and determination of cell generation time

Total cell no.

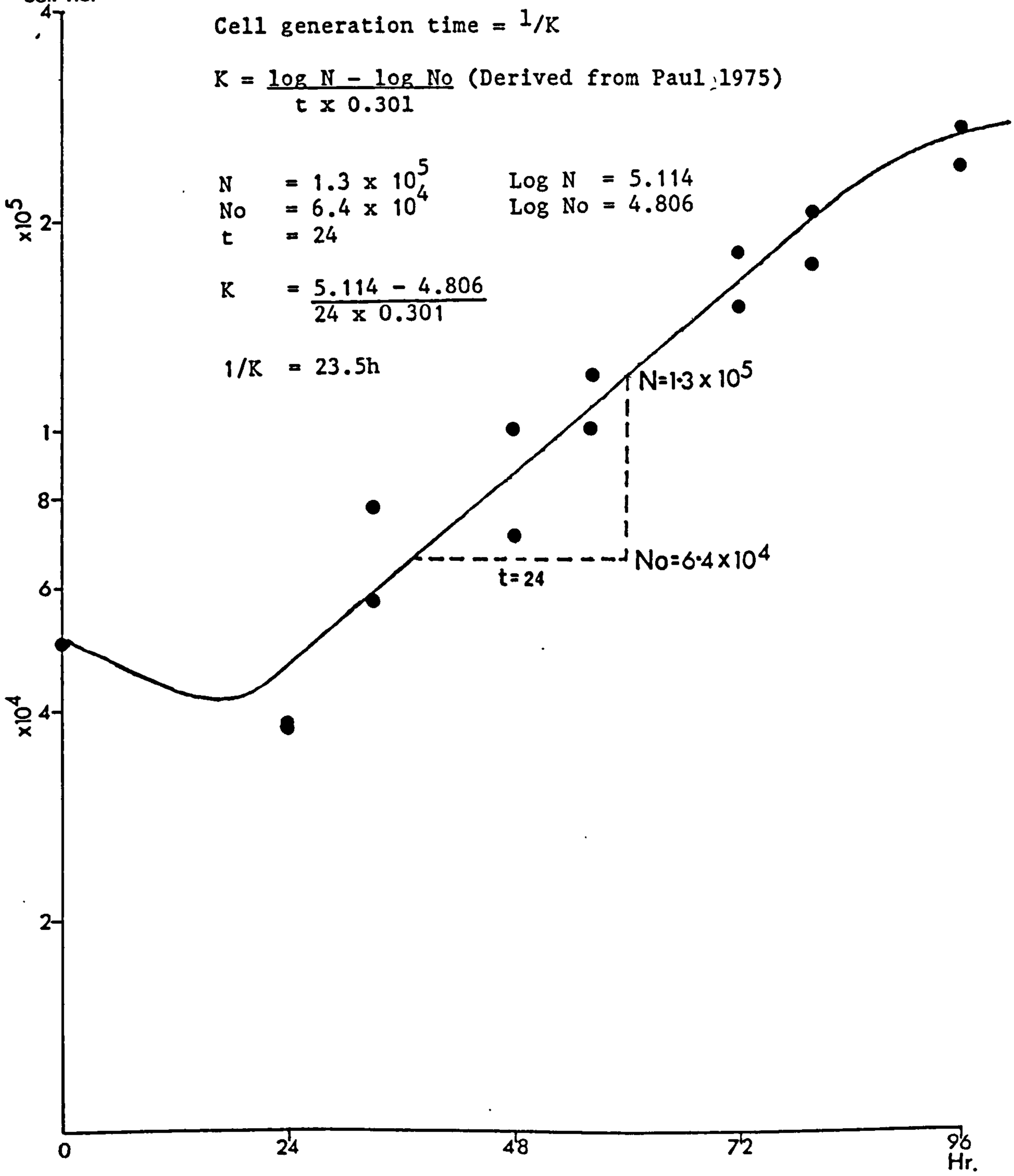
Cell generation time = $^1/K$

$K = \frac{\log N - \log No}{t \times 0.301}$ (Derived from Paul, 1975)

$N = 1.3 \times 10^5$ Log N = 5.114
$No = 6.4 \times 10^4$ Log No = 4.806
$t = 24$

$K = \frac{5.114 - 4.806}{24 \times 0.301}$

$1/K = 23.5h$



The lag phase would be followed by a period of rapid growth when the cells were dividing logarithmically, this phase would then begin to tail off when confluency was reached or the medium exhausted.

The mean cell generation time for the muntjac fibroblasts over the cell passages used was approximately 20 hours. This is similar to a human fibroblast line in culture (approximately 20 hours, Parry et al, 1985) but longer than a Chinese hamster fibroblast line (approximately 14 hours, Krahn 1983).

2.2.2 Metaphase analysis to determine ploidy distribution

Galloway et al (1986) recommended the use of diploid cell lines of either primary or early passage numbers in aneuploidy assays, to achieve low background rates of variability in chromosome number. This is necessary because statistical resolution of small differences is difficult when the background variability is high and because the inherent stability of heteroploid cells may be altered so that the meaning of an increase in aneuploidy is questionable. Initial experiments with the muntjac fibroblasts showed that the number of diploid cells decreased when the cells were

grown for many passages (>15). Therefore by limiting the number of passages and closely monitoring the ploidy distribution, the cells were only used for the spindle assay and recovery protocol when the karyotype was stable (table 2.1) Galloway et al (1986) recommended a suitable background rate of 0-5% cells with 2n+1 chromosomes and considered unsuitable, for example a Chinese hamster fibroblast cell line in which about 25% of the cells were 2n+1 (Danford, 1984) and an ascites line in which 36% of the cells were hypodiploid.

The male muntjac fibroblast line was shown to have a mean number of 70.9% diploid cells (2n=7) and 6.4% of hyperdiploid cells in the cell populations between passages 9 and 16 (Table 2.1). The hyperdiploid count included all chromosome numbers between 8 and 13, had it been confined to just 2n+1 chromosomes, the frequency would have been lower and probably well within the limits suggested by Galloway et al (1986) ie. 0-5%. The mean hypodiploid count in the muntjac fibroblasts was 19.2% and was mainly due to the loss (probably artifactual) of the tiny Y chromosome during slide preparation.

Preparation of muntjac fibroblasts for metaphase analysis

Cells were seeded in either T125 tissue culture flasks or 500cm^2 roller bottles from a split ratio of 1:2 or 1:3. The spindle poison vinblastine was added at 1µg/ml (final concentration) after 42 hours of incubation and then the cells were harvested by "mitotic shake off" technique (Carrano et al,

Table 2.1

Ploidy distribution of the muntjac fibroblast line

Passage Number	Number of cells	Number of Chromosomes							
		6		7		8-13		14	
		No.	%	No.	%	No.	%	No.	%
9	99	23	23	71	71	3	3	2	2
10	102	13	12.7	74	72.5	7	5.0	8	7.8
11	96	26	27	68	71	0	0	2	2
12	100	22	22	67	67	10	10	1	1
13	114	11	9.6	90	78.9	10	8.8	3	2.6
14	119	16	13.5	90	75.6	11	9.2	2	1.7
15	79	19	24	49	62	11	14	0	0
16	105	26	24	73	69.5	1	1	4	4.8
TOTAL	814	156		582		53		23	
$\bar{x}$%			19.5		70.9		6.4		2.7
sd			±6.5		±5.1		±4.9		±2.5

1976) 6 hours later. This technique relies on the cells in mitosis being less firmly attached to the substratum than the interphase cells and can therefore be selectively harvested from monolayers by mechanical action. Approximately 90% of the cells on the resulting slide preparations were in mitosis. The cells were stained using aceto-orcein and examined using phase contrast microscopy.

2.2.3 The background level of spindle dysfunction

This was monitored by examining the normal and abnormal division stages in vehicle control cell cultures. The percentage of cells in each stage in the total cell population are presented graphically in Fig 2.2 as the historical vehicle control data mitotic profile. The frequency of other types of aberrations are also expressed as a percentage of the total cell population and are presented in Table 2.2.

2.3 Cytotoxicity test

This test was carried out in 24-well Linbro plates and the optimum seeding density for muntjac fibroblasts in these plates was determined by observing the growth of untreated cells over a range of concentrations 24 and 48 hours after culture initiation. The wells were seeded with cell numbers between $1x10^4$ and $3x10^5$ cells/well in duplicate. Optimum density was that which produced sub-confluent growth over 48 hours and this was found to be between $5x10^4$ and $9x10^4$ cells per well.

Fig. 2.2 Historical vehicle control data. Represented as a mitotic profile of the normal and aberrant (-spindle) division stages.

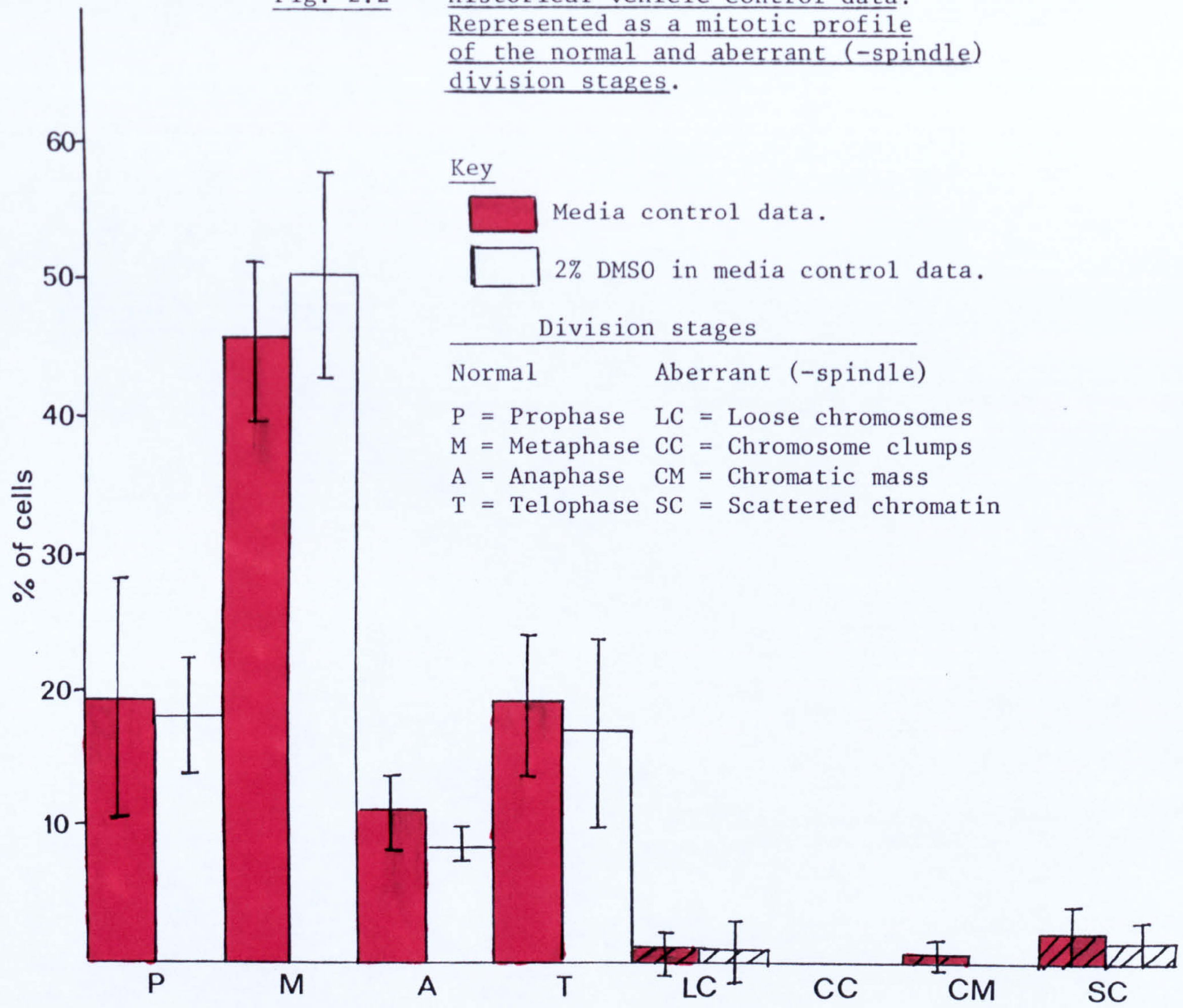


Table 2.2
Muntjac spindle assay: historical vehicle control data

No. of dividing cells scored	% metaphases with chromosome dislocation from the spindle (MDC) *1	% Anaphases and telophases with chromosome bridges and lagging *2	% Polyploid metaphases *3	% Multipolar metaphases	Anaphase, Telophase/ Metaphase Ratio (AT/M)	Mitotic Index (MI)
	x ± sd	x ± sd	x ± sd	x ± sd	x + sd	x ± sd
10, 910	0.14±0.23	1.8±1,8	0.4±0.4	0.08±0.2	0.670±.173	1.8±0.8
I	(0.3)	(6.0)	(0.85)			
2,328	0.12±0.2	1.62±1.68	0.7±0.7	0.04±0.09	0.530±.198	1.9±1.0
II	(0.26)	(3.36)	(0.95)			

I = Media vehicle control data

II = 2% DMSO in media vehicle control data

*1 = Figure in brackets is % of MDC in total metaphase population only

*2 = Figure in brackets is % of cells with bridging and lagging in total anaphase and telophase population only

*3 = Figure in brackets is % of polyploid metaphases in total metaphase population only

2.4 Muntjac spindle staining assay (24 hour protocol) Initial experiments involved growing cells on sterile glass slides or coverslips in sterile petri dishes. However superior results were obtained using chamber-slide "Flaskettes" and these were used for the subsequent experiments. Seeding densities were utilized which produced a cell population in log phase growth for the period 24-48 hours after initiation. This allowed the cells to be exposed to the test agent for at least one cell cycle. The seeding density needed to be great enough to produce a large number of analysable cells but not so large that confluency was reached before 72 hours. This extended incubation time was required for the recovery protocol.

Investigation of the staining procedure demonstrated that the intensity of the stained components was affected by exposure time and the concentration of acetic acid within the staining solution. Staining solutions were prepared in 10, 15 and 20% acetic acid in distilled water and slide preparations were stained for between 16 and 24 hours. Wissinger et al (1981) reported that uptake of the Safranin O predominates during the first 10 hours of staining resulting in distinct red chromosomes with little evidence of cytoplasmic staining. This appeared to be the case since the Brilliant Blue R component of the staining solution was taken up into the cells in preference to the Safranin O between 16 - 18 hours. The intensity of both stains increased with time but it was also dependent upon the

acetic acid concentration since Brilliant Blue R is more soluble in acid than it is in water. The danger however, of increasing the solubility of this dye was the possibility of overstaining with the blue and masking of the red components. Thus, a balance was required between these two variables in order to optimise the staining procedure for the muntjac fibroblast cells. This was achieved by using 10% acetic acid in the staining solution and staining the preparations for 24 hours.

2.5 Incorporation of a harlequin banding technique into the recovery protocol

Removal of the treatment medium after 24 hours, followed by a period of recovery and growth in fresh medium is necessary for the expression of any non-lethal genetic damage. This recovery period must be long enough to allow the cells to undergo at least one mitotic division. This can be confirmed by the incorporation of a harlequin banding technique (Perry and Wolff, 1974) which enables differentiation between first, second and third division cells post treatment. A variation of the original Perry and Wolff technique, developed in this laboratory, was used and the test incorporated into the recovery protocol along with metaphase analysis for the detection of aneuploidy.

3. MATERIALS AND METHODS

3.1 Materials

Muntjac fibroblasts

The first cell line was obtained from the American Tissue Type Collection (ATTC); Maryland, USA. Subsequent to this, three more cell lines were obtained from Flow Laboratories Ltd., Irvine, Scotland. The preliminary investigations of the test system were carried out using the first three lines but the majority of the spindle assay and recovery work was carried out with the most recent cell line. All of the lines were derived from the cells originally isolated by Wurster and Benirschke (1970) and all have shown the same characteristics eg. karyotype, cell generation time, background level of spindle dysfunction etc.

Tissue culture medium

The culture medium used throughout this study was prepared as follows:-

For 1 litre,

840ml 1x Ham's F-10	(Imperial Laboratories (Europe) Ltd.)
150ml Foetal Calf Serum	(Gibco Ltd.)
10ml 200mM L-Glutamine	(Flow Laboratories Ltd.)
20,000 IU Penicillin	(Gibco Ltd.)
20,000 µg Streptomycin	(Gibco Ltd.)

Once prepared the medium was stored at 4°C and used within seven days.

Phosphate Buffered Saline

(Dulbecco's formula) without calcium and magnesium (PBS $-Mg^{2+}Ca^{2+}$). This was made up in 1 litre of deionised water using the following chemicals obtained from BDH Chemicals Ltd.

8.0g	NaCl
0.2g	KCl
1.15g	Na_2HPO_4
0.2g	KH_2PO_4

Once prepared this solution was heat sterilized and stored at +4°C until use.

Versene Trypsin

This solution was made up in 1 litre deionised water using the following chemicals obtained from BDH Chemicals Ltd.

40.0g	Sequestric Acid (versene)
800.0g	NaCl
20.0g	KCl
115.0g	Na_2HPO_4
20.0g	KH_2PO_4
500.0g	Trypsin
1.0g	Phenol Red
20.0g	D-Glucose
200.0g	$NaHCO_3$
2.068g	Neomycin 677IU/mg
0.195g	Polymyxin 7704IU/mg

Once prepared this solution was filter sterilized and stored at -20°C until use.

Fixative for spindle stain

One litre of fixative was prepared just prior to use, using the following reagents and chemicals obtained from BDH Chemicals Ltd.

750ml	Methanol, Analar
250ml	Acetic Acid, Analar
0.8g	$NaCl_2 6H_2O$
0.1g	$CaCl_2 2H_2O$

Differential spindle stain

A solution of 0.5% Brilliant Blue R (Sigma Chemical Co. Ltd.) and 0.5% Safranin O (Sigma Chemical Co. Ltd.) in 10% acetic acid (BDH, Analar) was prepared on the day of use. This solution was stirred for one hour, filtered and stirred for a further hour being filtered once more before use.

Aceto-orcein staining solution

A solution of 1% orcein synthetic (BDH Chemicals Ltd.) in 450ml acetic acid (BDH, Analar) was refluxed for 20 minutes, allowed to cool and then was made up to 1 litre with 550ml distilled water. This solution had a shelf life of one year stored at room temperature and was filtered just before use.

2 x SSC

This was prepared in one litre of distilled water using the following chemicals from BDH Chemicals Ltd.

17.53g	NaCl
8.82g	$Na_3C_6H_5O_7 2H_2O$

The solution had a shelf life of seven days at room temperature.

Test compounds (suppliers)

1. Colcemid (demecolcine) crystalline; Sigma Chemical Co. Ltd.
2. Vinblastine sulphate salt, crystalline; Sigma Chemical Co. Ltd.
3. Diethylstilboestrol; a gift from D. Paton, Imperial Chemical Industries Ltd.
4. Nocodazole, crystalline; Sigma Chemical Co. Ltd.
5. DL-p-fluorophenylalanine (97%); Aldrich Chemical Co. Ltd.
6. Acenaphthene (99%); Aldrich Chemical Co. Ltd.
7. Hydroquinone (99+%); Aldrich Chemical Co. Ltd.

3.2 Methods

3.2.1 Culture conditions

Cells were grown in either plastic tissue culture flasks (Gibco Ltd.) or tissue culture roller bottles (Gallenkamp). Tissue

culture flasks, loosely capped, were incubated at 37°C in an atmosphere of 5% CO_2 in air (Heraeus Equipment Ltd.). Roller bottles were gassed with CO_2 to give a 5% concentration in air. The caps were tightened and incubation took place on a roller machine (Bellco Ltd.) at 1rpm. contained within a walk-in 37°C incubator.

Subculturing procedure

1) The culture medium was removed from the tissue culture vessel and the sub-confluent monolayer rinsed with warm PBS-$Ca^{2+}Mg^{2+}$.
2) The cells were then rinsed with the versene-trypsin solution and all but 1ml of the solution decanted off.
3) The culture vessel was placed at 37°C until the cell sheet began to detach (2-5 minutes).
4) The enzymic action of the trypsin was halted by the addition of fresh culture medium which was aspirated vigorously to obtain a single cell suspension.
5) The cell suspension was diluted as required with medium and decanted into new culture vessels.
6) The cells were then incubated as described previously. The subculturing ratio was between 1:2 and 1:4 at an interval of 6-8 days and the medium was renewed twice weekly.

Storage of cells

Reserve cells were stored frozen in liquid nitrogen at a temperature of -196°C. They were frozen down in growth medium supplemented with 10% Dimethylsulphoxide (DMSO) (Aldrich Chemical Co. Ltd.) which acts as a cryopreservative (Freshney, 1983).

Freezing

1) Sub-confluent monolayers were trypsinised as for subculturing and a single cell suspension prepared in fresh, chilled cryopreservation medium.
2) The cell titre was adjusted to between 2 and $4x10^6$/ml and the suspension dispensed into 1ml cryotubes (Gibco Ltd.).
3) The tops of the cryotubes were sealed with cryoflex (Gibco Ltd) and the vials placed in a polystyrene igloo box (Jencons Scientific Ltd.) and frozen at -70°C for 2 hours.
4) The vials were then transferred for storage into liquid nitrogen.

Recovery

1) When the cells were required for use vials were removed from the liquid nitrogen and thawed quickly in a water bath (Grant Instruments Ltd.) set at 37°C.
2) Without breaking the seal, the vials were centrifuged (Heraeus Equipment Ltd.) at approximately 800rpm. for 5 minutes.
3) The seal was then broken, the preservation media removed and the cells were resuspended gently in fresh culture media prewarmed to 37°C.

4) The cells were dispensed into new culture vessels with just enough medium to cover the bottom of the vessel.

5) The cells were incubated for 24 hours or until they had attached to the surface of the vessel and then the medium was replaced with fresh medium to the normal volume and the cells reincubated.

3.2.2 Test and control materials;- formulation

Diethylstilboestrol and acenaphthene were both dissolved in DMSO before dilution with culture medium. The final concentration of DMSO in the treatment medium was 2%. Colcemid, vinblastine, nocodazole, pFPA, acenaphthene and hydroquinone were all soluble in growth medium at the doses used. The vehicle controls were either 2% DMSO in medium or culture medium only. A positive control was not used in these experiments as there was no well established positive control for the following types of assay at the beginning of this study.

3.2.3 pH and osmolality measurements

The pH and the osmolality of the treatment medium (final highest concentration) and vehicle media was determined using a pH stick meter (Gallenkamp) and a Wescor vapour pressure osmometer (Chemlab Instruments Ltd.) respectively. It has been reported by Galloway et al (1985) and Galloway and Ivette (1986) that the pH and osmolality in vitro should be maintained as close as possible to a normal physiological environment in order to avoid the possible induction of aneuploidy and/or chromosome structural aberrations.

3.2.4 Cytotoxicity Assay

The purpose of a cytotoxicity assay is to establish the

appropriate concentrations of a test agent to be investigated in a subsequent short term assay, since many chemicals exert their effects at concentrations which are cytotoxic to the cells involved. A number of cytotoxicity assays can be used in tissue culture and these include growth inhibition, cell counts and plating efficiency assays (Dean and Danford, 1984). Cytotoxicity was determined in this investigation by the observation of growth inhibition, monolayer detachment, cellular vacuolation or lysis. The procedure was as follows:-

1) A single cell suspension was obtained following trypsinisation and 1ml of medium containing between $5x10^4$ and $9x10^4$ cells was added to each well of a twenty four well Linbro plate (Flow laboratories Ltd.).

2) After 24 hours incubation in an atmosphere of 5% CO_2 in air at 37°C the medium was carefully removed and replaced with 1ml of vehicle in each well.

3) 1ml of the treatment medium containing the test compound at double the required concentration was added to well 1 of rows A and C (Fig. 3.1). The media were mixed and then 1ml removed and added to the second well. This double dilution technique was continued until all but the last well in rows B and D were filled. These were treated with the vehicle only.

4) The plate was then incubated at 37°C for 24 hours after which time the cell monolayers were examined for cytotoxicity using an inverted microscope (E. Leitz Ltd.) with phase contrast illumination (x 10 objective). The cells were then fixed with spindle fixative and stained with the Brilliant Blue R and Safranin O spindle stain (Plate 3.1, 3.2).

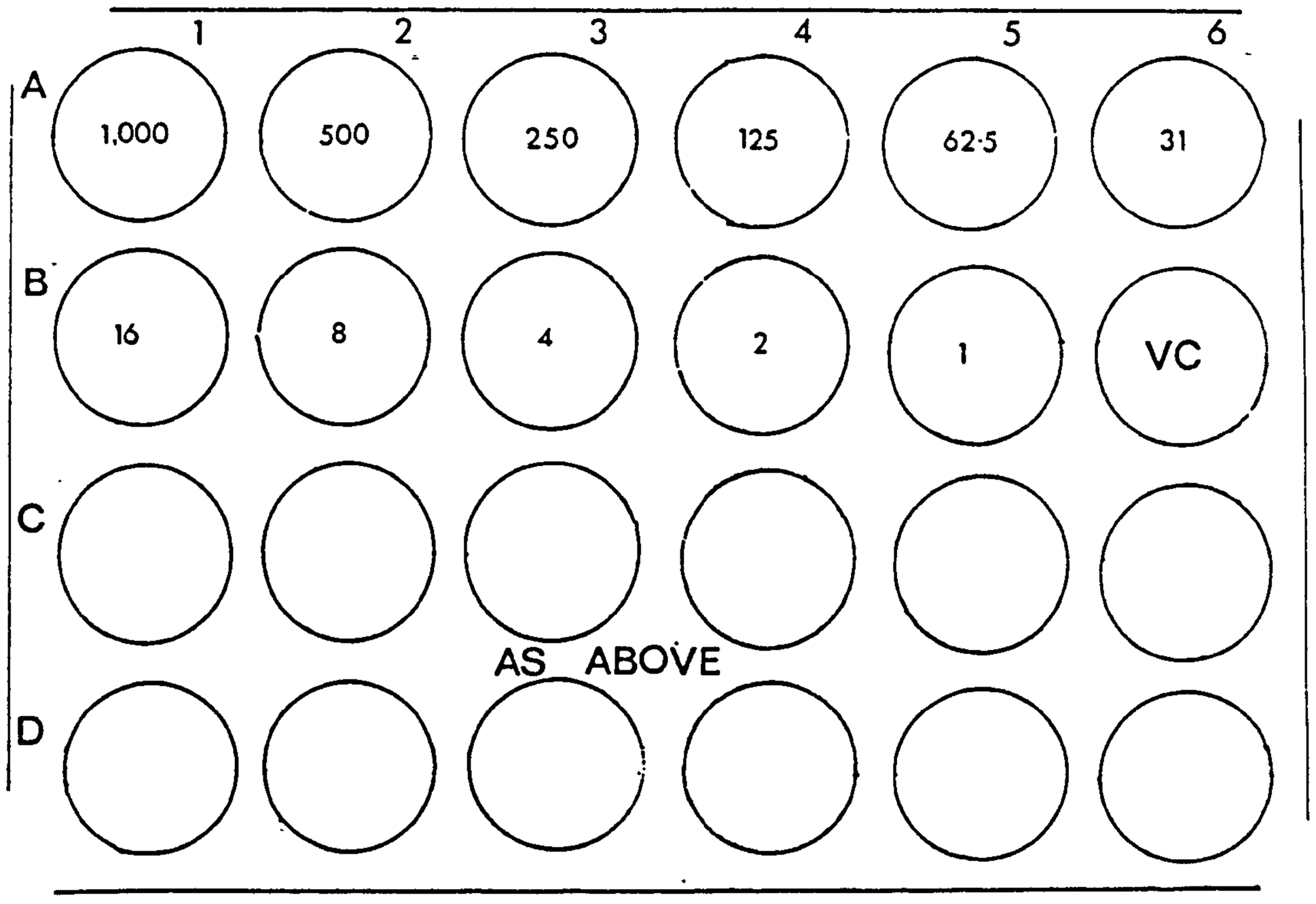


Fig. 3.1

24-well linbro plate, dilution pattern

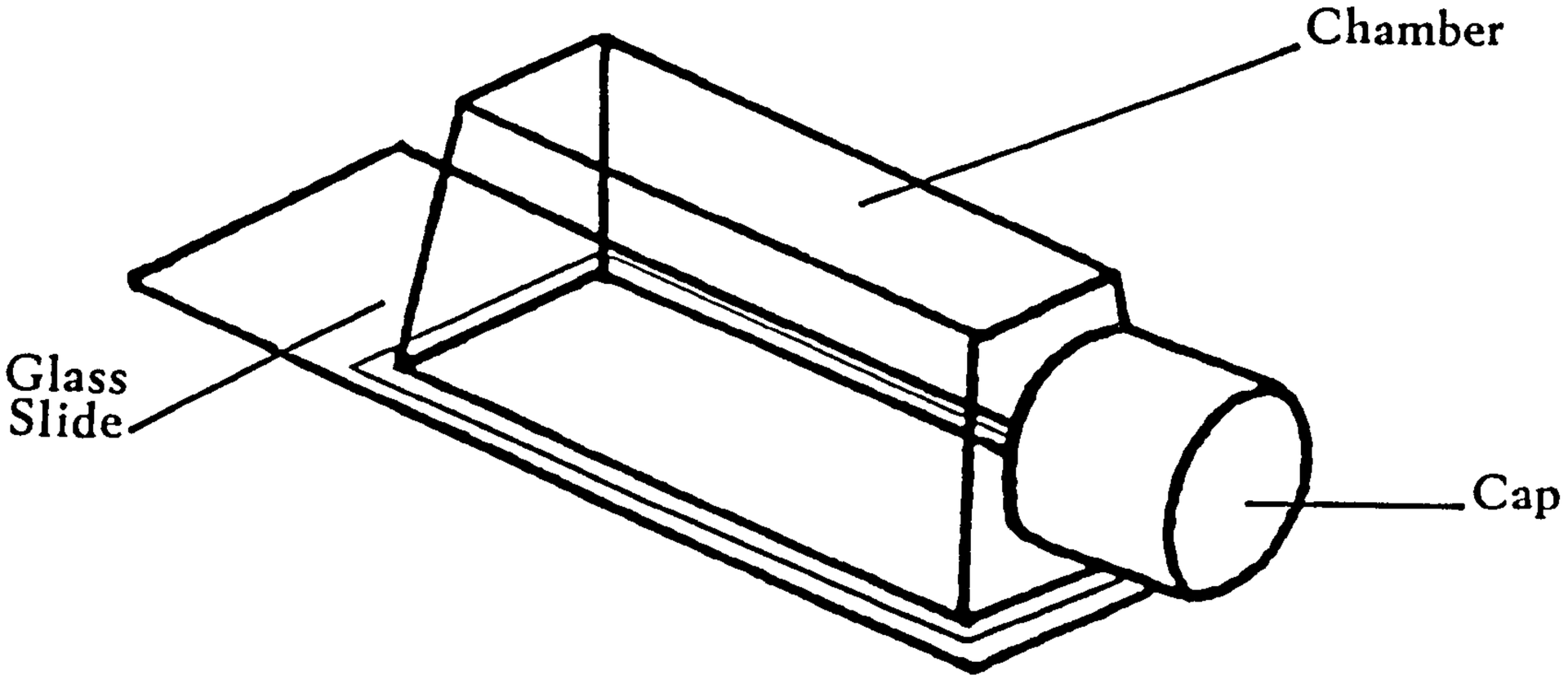


Fig.3.2

"Flaskette" tissue culture chamber slide.

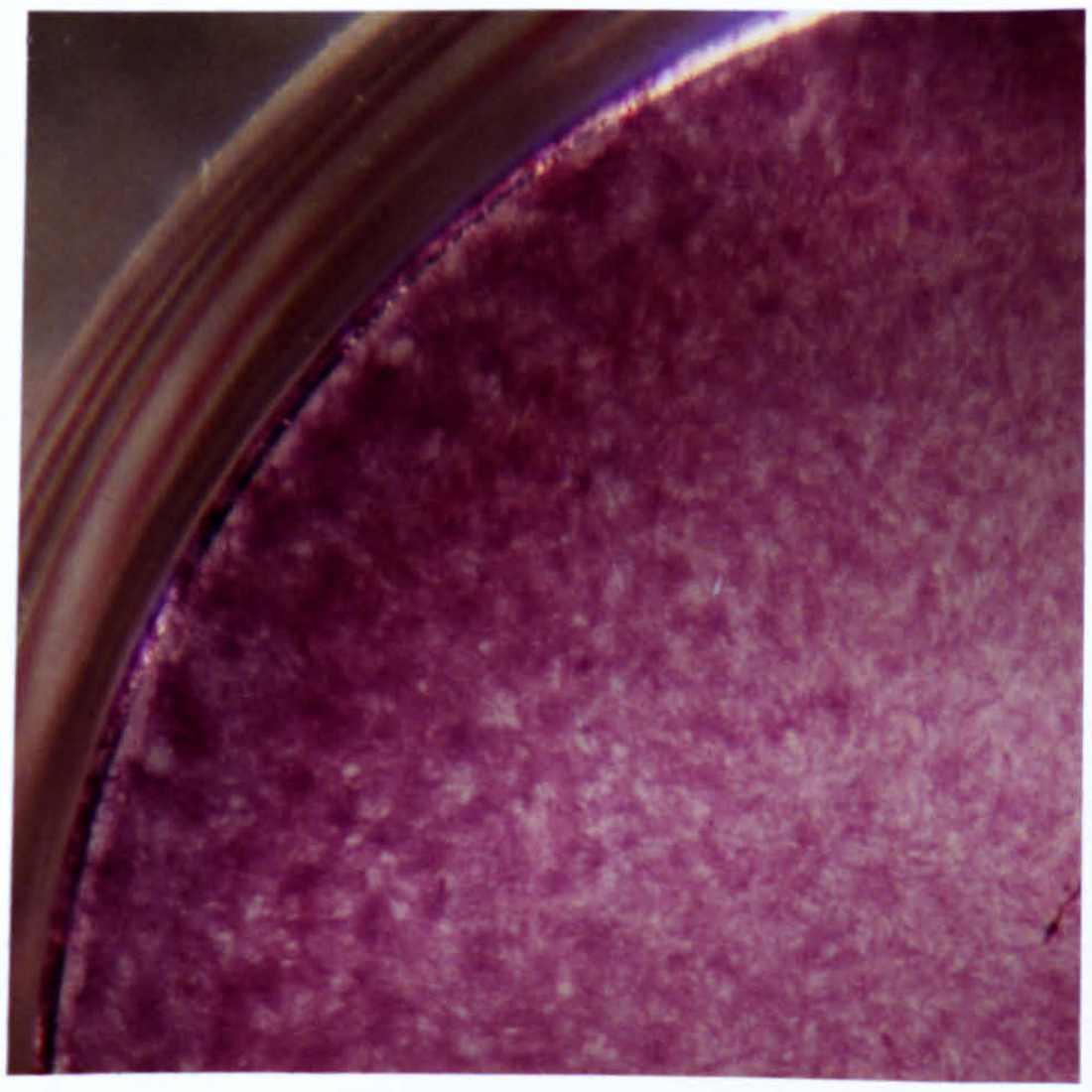


Plate 3.1

Muntjac cytotoxicity test.
No toxicity.

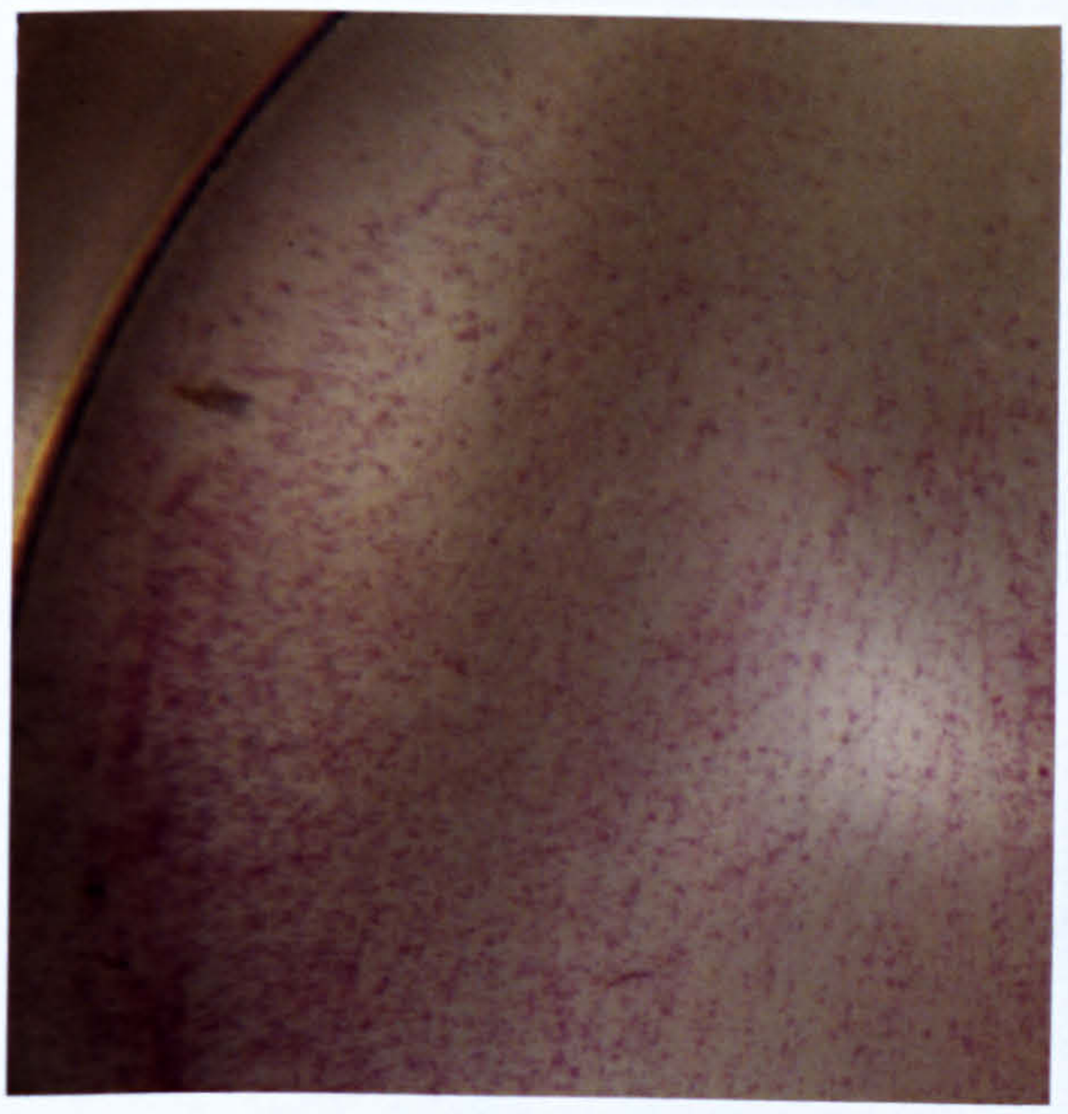


Plate 3.2

Muntjac cytotoxicity test.
Toxicity.

Choice of test concentration range

The initial test concentration ranges were chosen to include a dose where there was some evidence of cytotoxicity in an attempt to confirm that the compound had entered the cells. However, it was desirable to include non toxic doses as well. It is necessary to take toxicity into account when interpreting the results because of non-specific effects of cytotoxicity. It has been reported by Parry et al (1985) that aberrant division stages can be induced at toxic levels by generalised effects on protein metabolism and may not be due to true spindle inhibition.

A second confirmatory experiment was carried out, often over a narrower dose range, so that small changes could be observed in the hope of identifying specific mechanisms of action. It was necessary to have at least 3 dose points, and in some of the experiments there were 4 or 5 if the compound appeared inactive, or if the activity occurred over a narrow dose range.

DES and hydroquinone were the first compounds to be tested using the muntjac spindle assay. In both cases only one flaskette slide culture (Fig. 3.2) was used per dose point. In the repeat and in all of the subsequent experiments duplicate cultures were examined.

3.2.5 Spindle staining assay (Fig. 3.3)

1) Cells were trypsinised as before and the cell suspension adjusted to a titre of between $3.5x10^4$ and $6.5x10^4$/ml of culture medium.

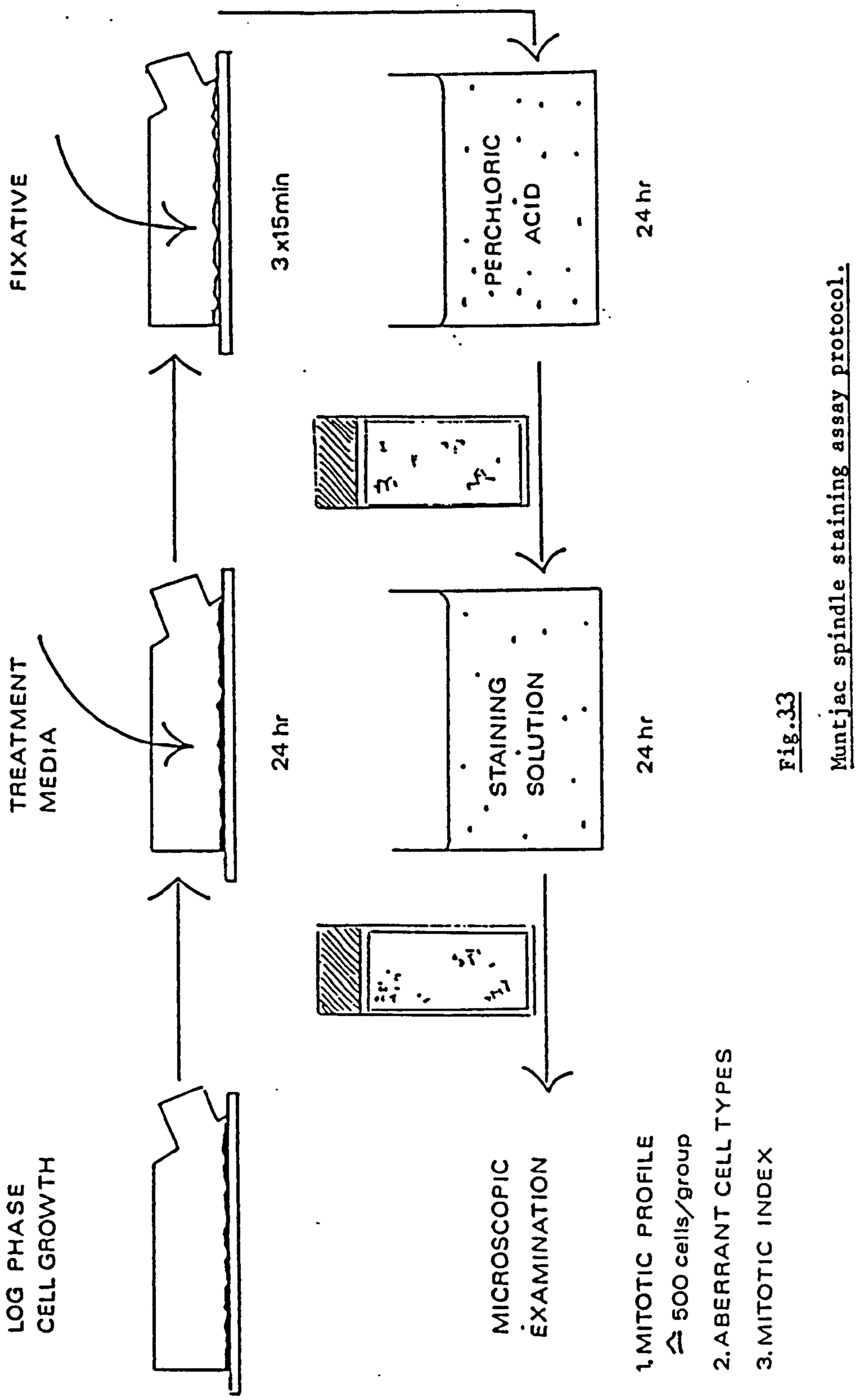


Fig.33

Muntjac spindle staining assay protocol.

2) 3ml of the cell suspension (ie. approximately 1-2x10^5 cells) was added to each flaskette which was capped loosely and incubated for 24 hours at 37°C. During this time the cells would attach to the bottom of the flaskette and begin to divide.

3) The medium was then removed and replaced with 3ml of either the vehicle control or treatment medium as necessary. The flaskettes were incubated as before for 24 hours.

4) The treatment and control medium was then removed and the cells fixed <u>in situ</u> using 3 changes of spindle fixative each exposure lasting 14 minutes. The chambers were removed from the slides taking care not to damage the cell monolayer and the cells allowed to air dry overnight at room temperature.

5) The slides were then placed in chilled 5% v/v perchloric acid (Sigma Chemical Co. Ltd.) in distilled water for 24 hours at 4°C. This treatment removed the RNA from the cells.

6) The slides were rinsed for 10 minutes in several changes of distilled water and allowed to air dry before being placed in the Brilliant Blue R/Safranin O staining solution for 24 hours at room temperature.

7) The slides were washed in distilled water, blotted and allowed to air dry before clearing in xylene (BDH, Analar) and mounting in Eukitt (R.A. Lamb).

8) The slides were screened using a Zeiss photomicroscope and normal transmitted light microscopy (x16 objective) and individual cells examined under higher magnification (x63 objective).

Scoring Procedure

The slides were coded and scored blind.

1. When possible at least 250 dividing cells/slide (2 replicates/dose) were classified as to their division stage. Division stages were either normal (prophase(P), metaphase(M), anaphase(A), telophase(T)) or aberrant. The aberrant division stages (spindle absent) were grouped under the following phenotypes;

(i) Loose chromosomes (LC)

(ii) Chromosome clusters (CC)

(iii) A chromatin mass (CM) and

(iv) Scattered chromatin (SC) (Appendix, Plates 1-7)

The aberrant division stages (spindle present) were classified as:

(i) Chromosome dislocation from the spindle (MDC).

(ii) Anaphase or telophase chromosome fragment or laggard and chromosome bridge (A/T FL/B).

(iii) Multipolar divisions (MP).

(iv) Polyploid metaphases (Poly. Mets.) (Appendix, Plates 8-11)

2. Observed effects only;

(i) Alteration to spindle morphology ie. shape and size.

(ii) Alteration to chromosome morphology ie. extreme condensation and stickiness.

(iii) Alteration to cell morphology ie. loss of normal shape.

3. Following decoding of the slides, the data from the duplicate treatment and vehicle control groups were combined. By expressing the results as a % of the total dividing cell population, the normal and aberrant (-spindle) division stages were demonstrated graphically as a mitotic profile for each experiment.

The remaining data for the aberrant division stages (+ spindle) was expressed both as a % of the total dividing cell population and as a % of the total number of cells in a specific division stage eg. the number of metaphases with chromosome dislocation; (MDC)/total metaphase population (normal and aberrant) x 100.

4. The anaphase and telophase/metaphase (AT/M) ratio was used to indicate metaphase arrest. This was total when the ratio was zero.

5. The percentage of dividing cells in a total of 1000 cells was determined to produce the Mitotic Index (MI) for each slide.

6. By examination of the mitotic profile, the (AT/M) ratio and the MI it was possible to determine if the test agent was disturbing cell division to a significant extent.

Observation of the types of damage indicated the possible mechanisms involved eg. by a direct effect of the spindle.

Statistical analysis

Statistical analysis was carried out on the data obtained for the normal and aberrant (spindle absent) division stages in each experiment. In the analysis, each dose group was compared with the negative control using Dunnett's (1955) multiple comparison test. A one-sided 5% significance level was used. This test assumes that replicate data would fit binomial distributions. However, if this was not the case a second analysis was carried out which uses a weighted model to take into account extra-binomial variation (Williams, 1982).

This model was reasonable when all doses were similarly affected by the problem of extra-binomial variation. However if the problem was due to just one of the doses having extra binomial variation this dose was omitted and the data re analysed using the first test. This was the case for only two experiments. The first with diethylstilboestrol (experiment 2) when the 10µg/ml dose group was omitted and the second was with acenaphthene (experiment 2) when the 80µg/ml dose group was omitted. Both groups represented the highest dose in each experiment and the extra-binomial variation was probably due to the extreme treatment conditions.

3.2.6 The treatment and recovery protocol

The aim of this experimentation was firstly, to see if the muntjac fibroblasts were capable of recovery from drug treatment and secondly to determine whether aneuploid cells or spindle disturbances could be observed in the recovered population. The protocol development occurred in 3 phases. (Fig 3.4)

Two compounds, colcemid and pFPA were tested once only with this protocol.

Muntjac spindle assay; cells were examined after 24 hours treatment with a) no recovery (as before), and following removal of the treatment medium b) 24 hour recovery, or c) 48 hour recovery in drug free medium.

Harlequin banding; this technique was used to identify cells in second division following treatment, indicating recovery of the exposed population. Cells were harvested for examination at the same times at those for the spindle assay ie. directly following treatment or after 24 or 48 hours recovery.

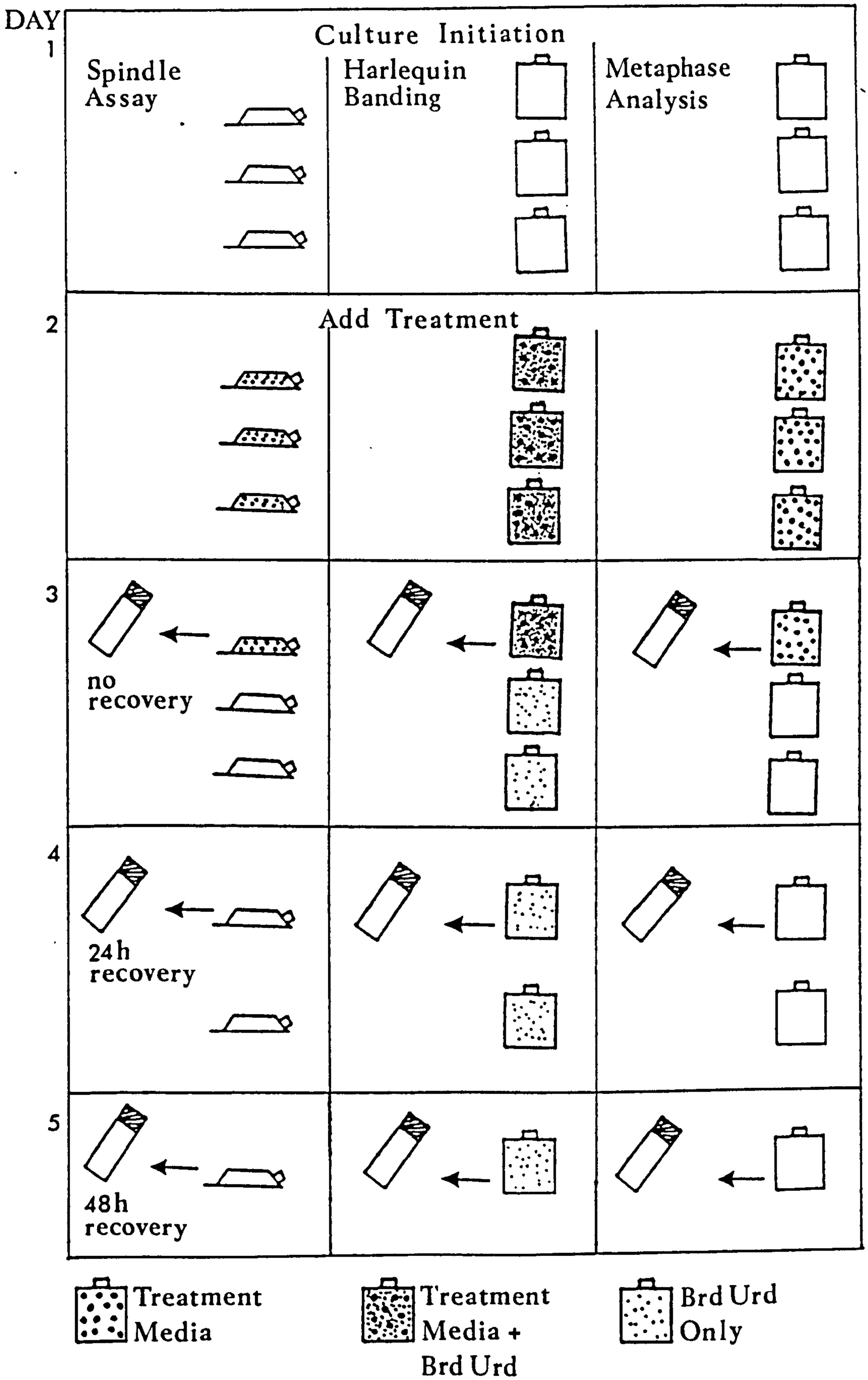


Fig.3.4

Treatment and recovery protocol.

Metaphase analysis; this was carried out on the treated cell population with the largest proportion of second division cells, to determine the aneuploid frequency. This frequency was compared to that obtained from a control culture containing an equivalent number of second division cells. The cells were harvested at the same time as those in the spindle assay and harlequin banding methods.

Spindle staining assay

1) Cells were trypsinised as before and the cell suspension adjusted to a titre of between 3.5 x 10^4 and 6.5 x 10^4/ml of culture medium.
2) 3ml of the cell suspension (ie. approximately 1-2 x 10^5 cells) was added to 3 sets of flaskettes. Each set consisted of treatment group(s) and vehicle control in duplicate. The flaskettes were capped loosely and incubated for 24 hours.
3) The media was then removed and replaced with 3ml of either the vehicle control or treatment media as necessary and the flaskettes were incubated as before for 24 hours.
4) The treatment and control media was then removed and one set of flaskette cultures fixed in situ using 3 changes of spindle fixative, each lasting 14 minutes and then air dried with the chambers removed. The remaining two sets of cultures were washed free of the treatment medium and reincubated as before in fresh culture medium.
5) The second set of flaskette cultures were fixed (as above) after 24 hours incubation and the third set after 48 hours.

6) The staining and scoring procedures for all 3 sets of cultures were the same as described previously for the spindle staining assay.

Harlequin banding metaphase preparations

1) Cells were trypsinised as before and the cell suspension adjusted to a titre of between 3.5 x 10^4 and 6.5 x 10^4/ml of culture medium.

2) 50ml of the cell suspension (ie. 1.75-3.25 x 10^6 cells) was added to 3 sets of 500cm^3 roller bottles. Each set consisted of treatment group(s) and vehicle control. The bottles were then gassed with CO_2 and incubated for 24 hours at 37°C.

3) The medium was then removed and replaced with 50ml of either the vehicle control or treatment medium both containing bromodeoxyuridine (BrdUrd) (Sigma Chemical Co Ltd.) to a final concentration of 3µg/ml. The bottles were then regassed and incubated as before for 24 hours.

4) Vinblastine was added to the first set of cultures [to a final concentration of 1µg/ml] for the last 6 hours of incubation. The mitotic cells were then harvested using a "mitotic shake off" technique and the resulting cell suspension decanted into 25ml universal containers (Sterilin Ltd.) and centrifuged for 5 minutes at 1000rpm.

5) The supernatant was removed and the cells resuspended in a few drops of media. The volume was made up to 10ml with the addition of 0.075M potassium chloride (BDH Chemicals Ltd.) and the cells remained in this hypotonic solution for 15 minutes at room temperature.

6) Following centrifugation (as before), the cells were fixed with three changes of Carnoy's fixative (methanol:acetic acid, 3:1) and metaphase preparations were made by dropping the cell suspension onto clean slides and air drying.

7) The treatment and control medium in the remaining two sets of cultures was decanted off and fresh growth medium containing BrdUrd (3µg/ml) was added. The bottles were gassed and incubated for an additional 24 hours at 37°C.

8) Vinblastine (1µg/ml) was added to the second set of cultures for the last 6 hours of incubation and then the mitotic cells were harvested as described above.

9) The remaining set of cultures was harvested after a further incubation period of 24 hours (total recovery period of 48 hours).

Harlequin banding staining technique

1) The slides were placed on a glass tray, cells uppermost and covered with pH 7.2 phosphate buffered saline (PBS. Oxoid Ltd.). They were then exposed to ultra violet light (254nm) from a Hanovia low pressure mercury lamp (Hanovia Lamps Ltd.) for 60 minutes.

2) The slides were removed from the PBS., drained and placed in a solution of 2 x SSC at 60°C for 60 minutes.

3) They were rinsed in tap water followed by a rinse in pH 6.8 Sörrensen's buffer (RA Lamb) and stained in a 2% solution of Giemsa (BDH Chemicals Ltd.) in buffer for 8 minutes.

4) The slides were blotted and air dried before clearing in xylene and mounting in Eukitt.

Harlequin banding scoring procedure

The slides were screened using x16 objective and individual cells examined with a x63 objective. At least 100 metaphases from each treatment were classified by their staining patterns into 1st, 2nd and 3rd division cells. 1st division cells (M1) stained uniformly dark (Plate 3.3) 2nd division cells (M2) showed complete harlequin banding (Plate 3.4) and 3rd division cells (M3) contained approximately 50% harlequin banding (Plate 3.5). These values, were then expressed as a percentage of the dividing cells in each population.

C. Metaphase preparations for chromosome analysis

The method was identical to that used to obtain metaphase preparations for Harlequin banding except that BrdUrd was not included in either the treatment or control media.

Metaphase preparation staining technique

1) The metaphase preparations were stained in a filtered aceto-orcein solution for 20 minutes.
2) They were rinsed in a 45% acetic acid solution and then in two changes of industrial methylated spirit alcohol to remove excess stain.
3) Following blotting and air drying the slides were cleaned in xylene and mounted in Eukitt.

Metaphase preparation scoring procedure

The metaphase preparations were examined using phase contrast microscopy. The slides were screened using a low power

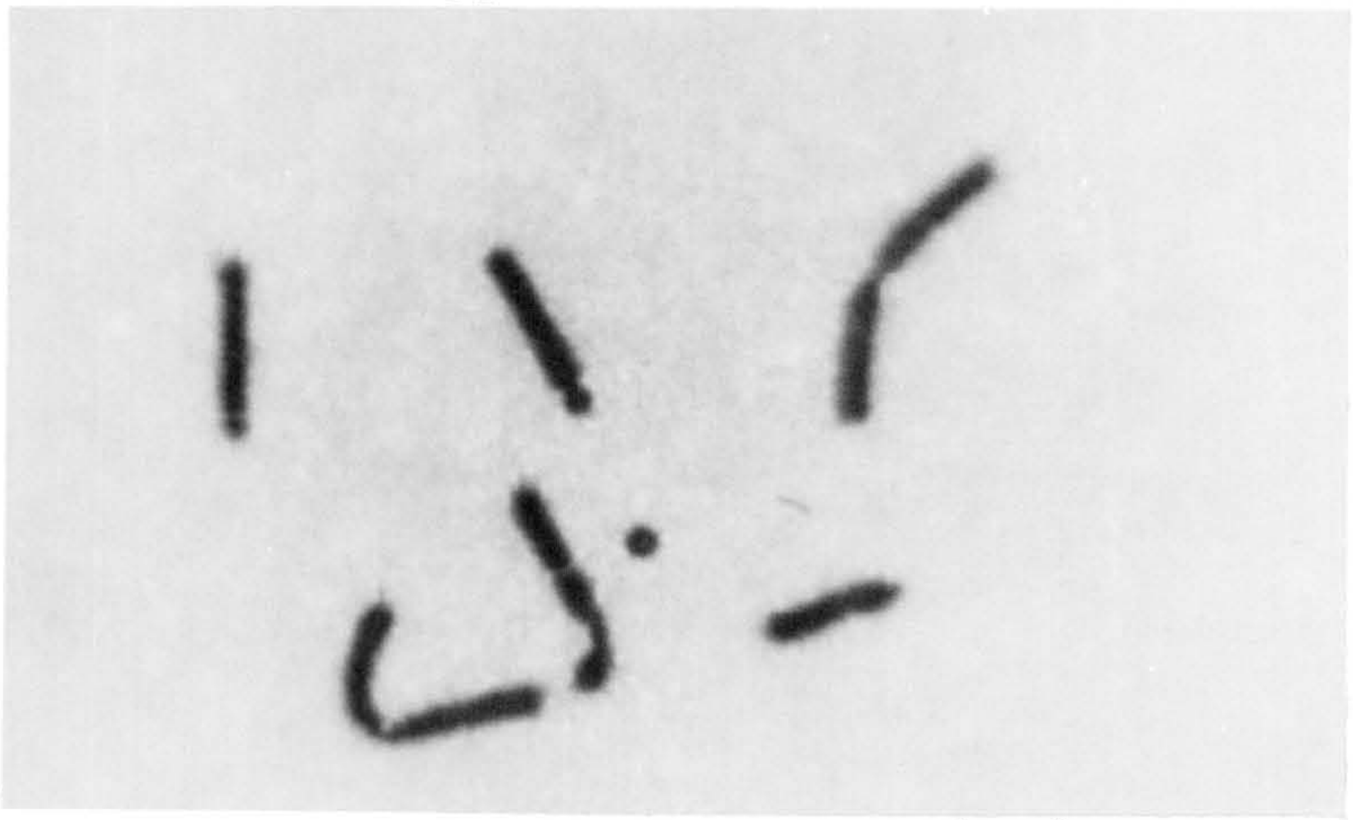


Plate 3.3

Muntjac chromosomes : first division cell.(M1)

Plate 3.4

Muntjac chromosomes : second division cell.(M2)

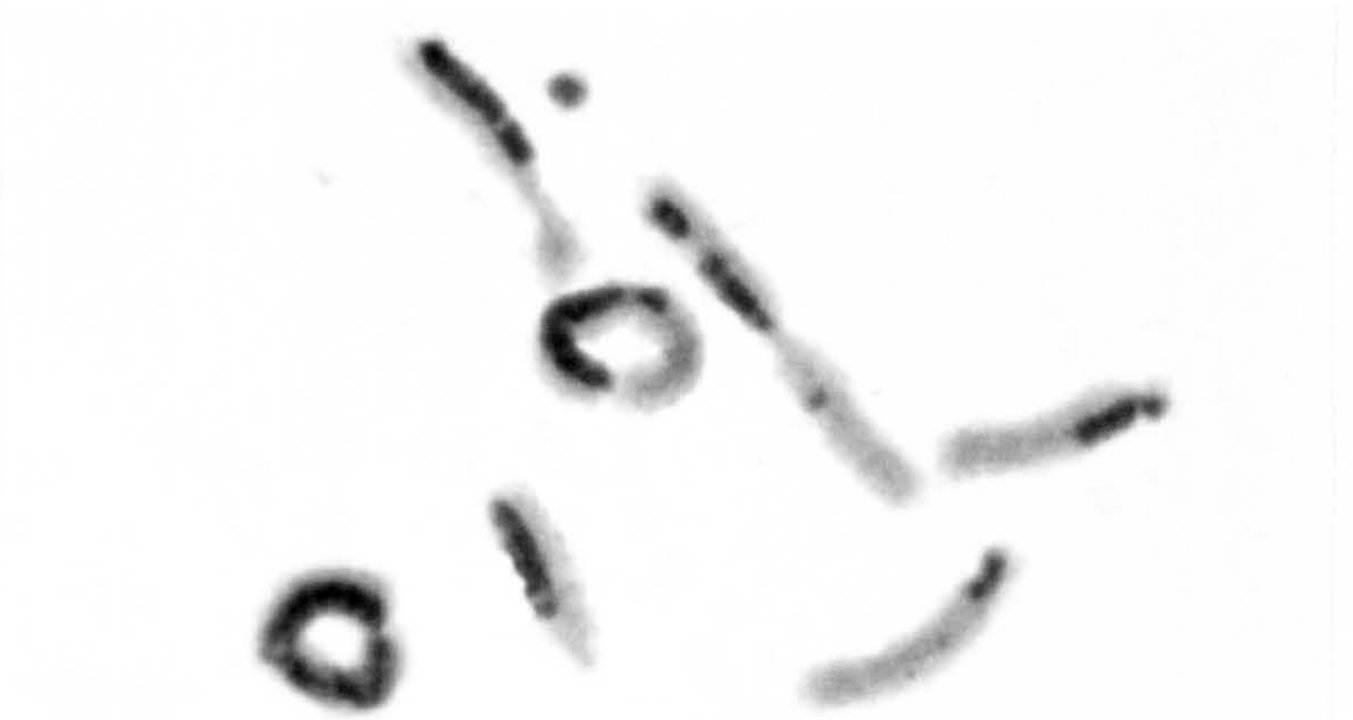


Plate 3.5

Muntjac chromosomes : third division cell.(M3)

objective (x16) and individual cells examined with a higher power objective (x63). Only individual metaphases having a good spread of chromosomes, but not in close proximity to other chromosome spreads, were analysed for numerical aberrations. It was desirable to score cells in which the cytoplasmic membrane was still visible since this ruled out the possibility of artificial hypodiploidy occuring as a result of chromosome loss during slide preparation. If possible 100 metaphases per replicate (2 replicates/dose) were analysed .

4. RESULTS

4.1 pH and osmolality of treatment media

The pH and osmolality of the highest concentration of each treatment medium was monitored for variation from the vehicle control medium (Table 4.1). The pH readings for both the treatment and control media were above the expected value for normal tissue culture media (pH 7.0 - 7.2). This was due to storage of the solutions at room temperature without a 5% CO_2 atmosphere. The increased osmolality of the DES and acenaphthene solutions were due to the presence of 2% DMSO in the treatment media

Shimada and Ingalls (1975) reported the induction of various chromosomal anomalies including aneuploidy in human peripheral lymphocytes following culture in acid (6.5 to 6.9) or alkaline (8.4 to 8.8) pH equilibra. Chromosome aberrations have also been induced in mammalian cells in vitro by hypotonic (<100mOsm/kg H_2O) (Nowak,1987) and hypertonic (>430mOsm/kg H_2O) Albanese, Mutagenesis In press) culture conditions.

None of the treatment media pH or osmolality readings were significantly different from the vehicle control levels. It is therefore unlikely that the pH or the osmolality of the treatment media contributed to the effects seen in the muntjac assay and recovery experiments.

Table 4.1 pH and osmolality
reading taken at room temperature, ungassed solutions

Compound	Concentration per ml	Vehicle	pH	Osmolality mmol/kg*
Colcemid	30ng	Media	7.8	291/298
Vinblastine	30ng	Media	7.6	295/289
DES	10µg	2% DMSO	7.9	547/545
Nocodazole	40ng	Media	7.9	312/331
pFPA	60µg	Media	7.4	297/303
Acenaphthene	90µg	2% DMSO	7.7	565/554
Hydroquinone	18µg	Media	7.5	287/291
Vehicle	-	Media	7.5	294/293
Solvent vehicle	-	2% DMSO	7.7	553/545
Media gassed to 5% CO_2 in air at 37°C			7.0	290/293

* Readings in duplicate

Table 4.2 Muntjac cytotoxicity test

Compound	Estimated Maximum nontoxic dose/ml	Estimated Boarderline toxic dose/ml	Highest dose analysed in subsequent assays
Colcemid	5.0ng	10.0ng	30.0ng
Vinblastine	5.0ng	10.0ng	30.0ng
DES	1.6µg	3.1µg	10.0µg
Nocodazole	10.0ng	20.0ng	20.0ng
pFPA	31.0µg	63.0µg	60.0µg
Acenaphthene	63.0µg	125.0µg	80.0µg
Hydroquinone	4.0µg	8.0µg	18.0µg

4.2 The muntjac cytotoxicity test

The results obtained in the muntjac cytotoxicity tests (Table 4.2) were used to identify a dose range for compound testing in the spindle assay. The differences between these values and the actual highest dose tested in the spindle assay indicates the limitations of the cytotoxicity test. With four of the compounds the borderline toxic dose was over estimated, with one it was underestimated and for two, the dose was correct. The error was usually ± 2-3 times the highest dose obtained in the spindle assay. Once the potential error had been identified allowances were made when setting the dose range for the following assays.

4.3 Colcemid

This compound was tested three times, twice with the standard protocol and once with the recovery protocol (Tables 4.3-4.11, Figs. 4.1-4.3). The compound was tested over a narrow dose range, 1-30ng/ml. On each occasion it was shown to be a potent spindle inhibitor, causing a dose related mitotic block and significant increases in aberrant division stages. These effects were accompanied by a decrease in the AT/M ratio and an increase in the mitotic index.

After 24 hours recovery, none of the treated cultures exhibited mitotic arrest and all had mitotic indices comparable to control levels. There was however, a small but significant increase in spindle damage at the 24 hour recovery time point. After 48 hours this effect had disappeared.

In the first two experiments negative results were obtained for the induction of MDC, chromosome bridging and lagging, multipolar divisions or polyploidy. However a dose related increase in MDC was seen in the third experiment as was an increase in chromosome bridging and lagging and multipolar divisions (Appendix, Plates 10,12) after colcemid treatment (20ng/ml) and 48 hours recovery.

Colcemid induced dose related effects on the spindle morphology before the structure was totally destroyed at 10-20ng/ml. Firstly, at 5ng/ml the spindle began to lose its rigid shape as the fibres appeared to be loosely arranged and perhaps fewer in number, with the metaphase chromosome attached randomly along its length. This was followed by the appearance of "star metaphases" as described by Dustin (1984) (see introduction) and/or reduction in spindle length. At doses where spindle destruction occurred in some cells only (10ng/ml, first experiment) very small dark metaphases, having the appearance of only one central pole (monopolar metaphases) could also be seen. This type of metaphase could be a later stage of the star metaphase following shortening of the pole to chromosome spindle fibres. The metaphase cells in which the spindle was

totally absent usually contained chromosomes scattered randomly throughout the cytoplasm (the "exploded metaphase" Dustin, 1984) although some cells contained chromosome clumps and even fewer chromatin masses or scattered chromatin.

4.3.1 Harlequin banding of cells treated with 20ng/ml colcemid (Table 4.10)

Very few second division cells were seen in the treated cultures harvested directly or after 24 hours recovery. Approximately 42% harlequin banded cells appeared in the 48 hour recovery cultures. This dividing cell population was comparable with the 24 hour recovery vehicle control cultures (46% second division cells) and illustrates a lag period of approximately 24 hours between removal of the treatment media and commencement of mitosis.

4.3.2 Metaphase analysis (Table 4.11) (Appendix, Plates 13,14)

Examination of the two sets of cultures indicated a small (3.2%) increase in cells containing between 8-13 chromosomes in the colcemid treated cultures when compared to the control level. The incidence of endoreduplication appeared to be raised and also some chromosome aberrations were seen in the colcemid treated cultures (observation only).

Table 4.3 shows the mitotic profile of Colcemid treated muntjac cells (experiment 1) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. ng/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	259	16	131	41	51	1	0	0	6	0	2	3	4	1	1	2	0.9
	231	20	130	25	47	0	1	0	5	0	0	0	1	1	0	1	1.5
1	210	35	119	12	31	2	0	2	4	1	2	1	0	0	1	0	0.6
	206	27	115	24	36	3	0	0	0	0	1	0	0	0	0	0	0.7
3	238	39	140	18	32	1	0	0	1	0	3	2	1	1	0	0	0.6
	240	48	130	16	36	3	0	2	0	1	2	0	1	0	1	0	0.6
10	208	10	160	5	1	13	6	6	4	3	0	0	0	0	0	0	4.4
	202	11	155	2	4	16	5	4	5	0	0	0	0	0	0	0	4.3
30	208	3	0	0	0	120	60	16	9	0	0	0	0	0	0	0	5.7
	226	6	0	0	0	145	43	26	6	0	0	0	0	0	0	0	5.5

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

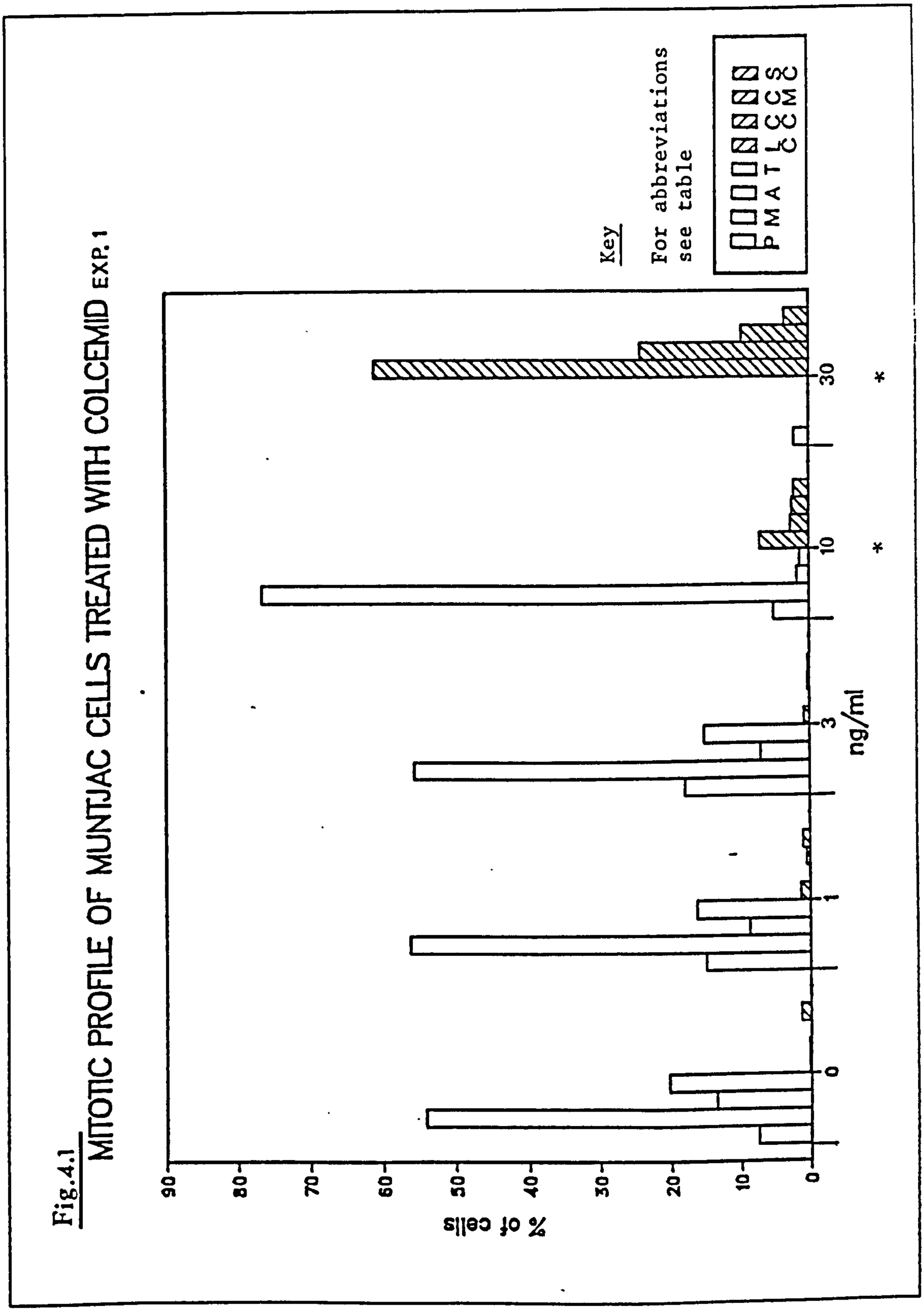

MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH COLCEMID EXP.1
Key
For abbreviations see table
PMATLCCMC
* Difference is significant at p <0.01
% of cells
ng/ml
0
1
3
10
30
90
80
70
60
50
40
30
20
10
0
*
*

Fig.4.1

Table 4.4 shows the mitotic profile of Colcemid treated muntjac cells (experiment 2) All data are actual cell number except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. ng/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	280	74	136	29	36	2	0	0	0	0	0	2	0	1	0	0	2.8
	255	76	130	18	21	0	0	0	2	3	1	0	2	2	0	0	2.7
5	316	114	161	14	13	3	0	0	1	2	1	4	0	0	0	3	2.3
	273	94	142	11	19	5	0	0	0	1	0	0	0	1	0	0	2.9
10	308	10	90	0	0	176	5	8	17	2	0	0	0	0	0	0	3.1
	245	38	70	0	1	130	5	0	1	0	0	0	0	0	0	0	3.3
20	515	8	0	0	0	415	78	5	9	0	0	0	0	0	0	0	14.3
	453	2	0	0	0	339	108	4	0	0	0	0	0	0	0	0	10.5
30	426	0	0	0	0	360	61	4	1	0	0	0	0	0	0	0	16.6
	396	2	0	0	0	314	67	10	3	0	0	0	0	0	0	0	16.2

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

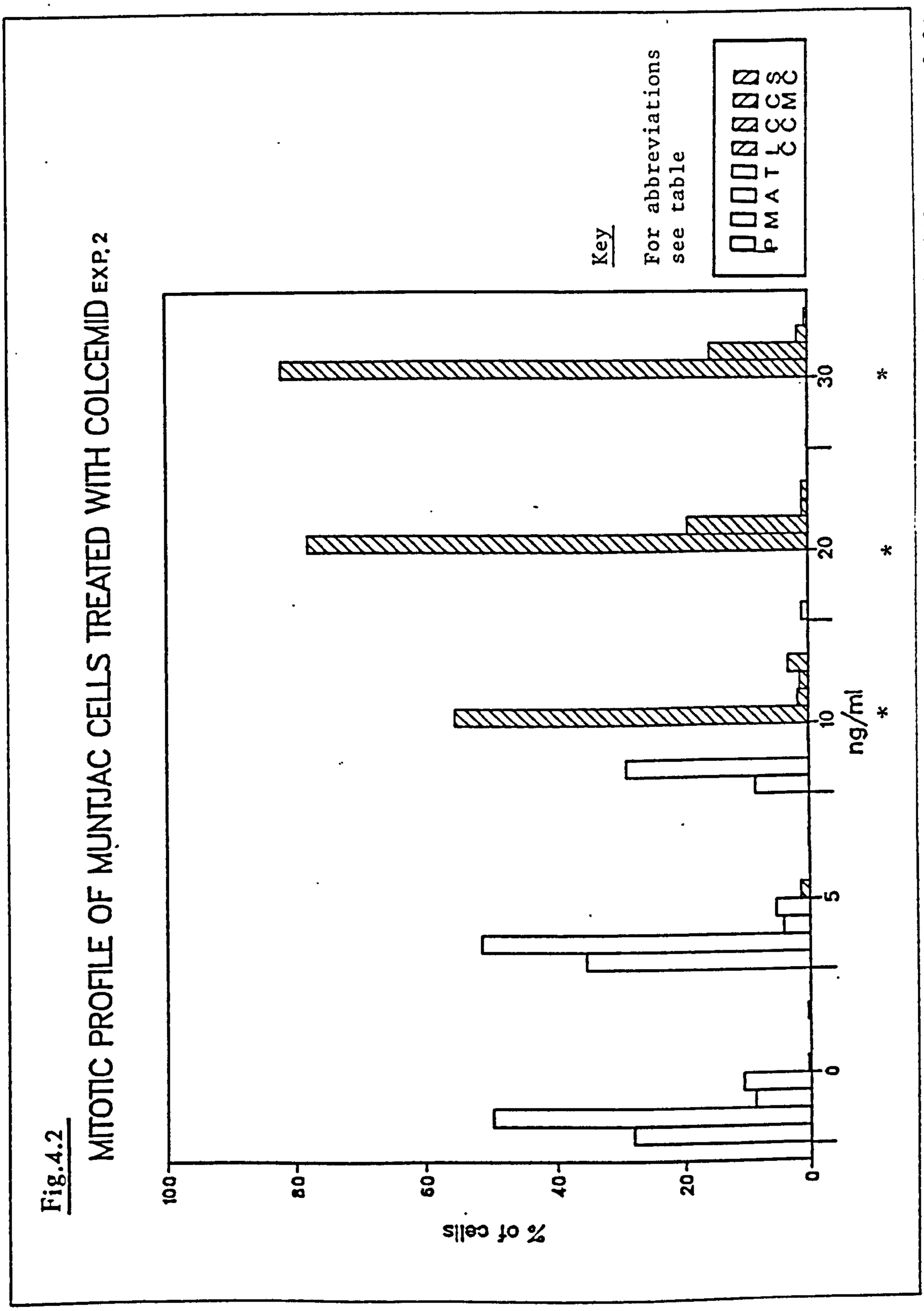

MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH COLCEMID EXP.2
% of cells
100
80
60
40
20
0
0
5
10
ng/ml
20
30
* Difference is significant at p <0.01
Key
For abbreviations see table
PMAT CCMS

Fig.4.2

Table 4.5
Effects of Colcemid on cell division
Experiments 1 and 2

	Conc. ng/ml	No. of dividing cells scored	Spindle	% Metaphases with chromosome dislocation from the spindle (MDC) *1	% Anaphases and telophases with chromosome bridges and lagging *2	% Polyploid Metaphases *3	AT/M Ratio	MI $\bar{x}$
Exp 1	0	490	+	0	2.5 (6.8)	0.6 (1.1)	0.628	1.2
	1	416	+	0.2 (0.4)	1.2 (4.7)	0	0.440	0.7
	3	478	+	0.2 (0.4)	2.3 (9.7)	0	0.378	0.6
	10	410	+	0.7 (0.9)	0	0	0.034	4.4
	30	434	-	0	0	0	0	5.6
Exp 2	0	535	+	0.6 (1.1)	1.5 (7.1)	0	0.394	2.8
	5	589	+	0.5 (1.0)	1.0 (9.5)	0.5 (1.0)	0.187	2.6
	10	553	+	0.4 (1.2)	0	0	0.007	3.2
	20	968	-	0	0	0	0	12.4
	30	822	-	0	0	0	0	16.4

*1 Figure in brackets is % of MDC in total metaphase population only.
*2 Figure in brackets is % of cells with bridging and lagging in total anaphase and telophase population only.
*3 Figure in brackets is % of polyploid metaphases in total metaphase population only.

Table 4.6 shows the mitotic profile of Colcemid treated muntjac cells, (experiment 3) no recovery. All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. ng/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	690	156	344	68	101	3	0	0	2	4	0	2	2	1	0	7	2.6
	397	119	188	33	40	6	0	0	0	1	3	0	0	2	0	5	3.5
5	391	126	168	42	34	11	0	0	0	2	1	1	0	3	0	3	2.2
	470	126	233	48	45	4	0	0	2	1	0	0	0	4	0	7	2.8
10	313	69	152	27	15	33	4	3	3	2	0	2	0	1	0	2	2.3
	354	76	183	17	20	39	1	0	3	10	0	0	0	0	0	5	3.0
20	290	6	0	0	0	172	92	15	5	0	0	0	0	0	0	0	7.0
	324	10	4	0	0	162	110	22	15	0	0	0	0	0	0	1	5.4

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

Table 4.7 shows the mitotic profile of Colcemid treated muntjac cells (experiment 3), after a 24 hour recovery period. All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. ng/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	569	118	332	34	83	1	0	0	0	0	0	0	0	1	0	0	2.4
	649	148	352	55	80	1	0	0	0	2	0	4	2	3	1	1	2.7
5	663	140	360	58	93	0	0	0	0	1	0	1	1	4	0	5	1.6
	520	101	300	46	69	0	0	0	0	0	0	1	0	0	0	3	1.9
10	450	129	221	27	61	1	0	0	0	1	2	0	1	2	2	3	2.5
	561	148	284	45	69	0	0	0	0	1	0	2	0	4	0	8	2.1
20	224	54	127	12	16	2	0	0	1	3	0	3	0	0	0	6	0.8
	204	46	112	10	23	4	0	0	1	3	0	0	0	1	1	3	1.0

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

Table 4.8 shows the mitotic profile of Colcemid treated muntjac cells (experiment 3), after a 48 hour recovery period. All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

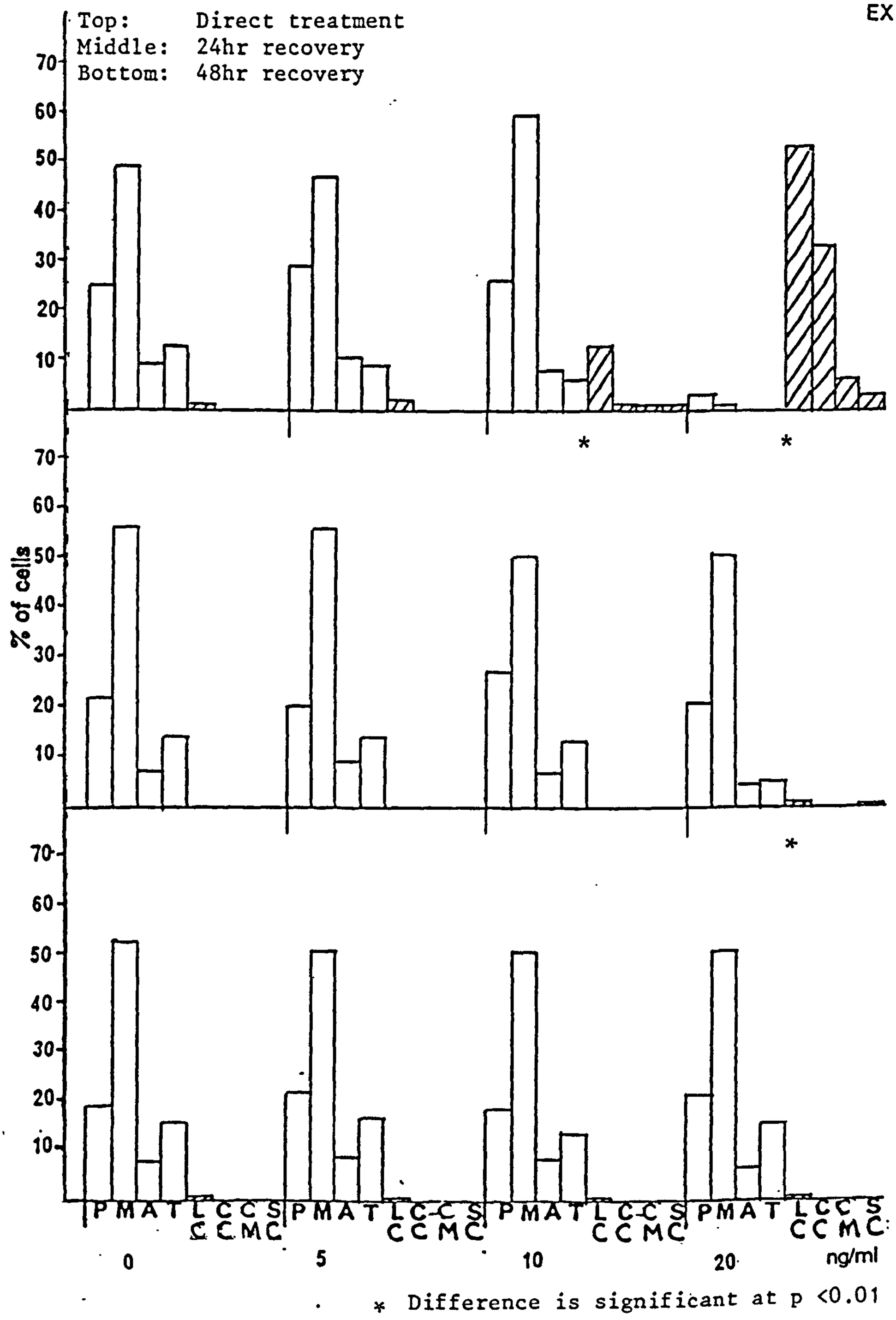
TEST CONC. ng/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	456	76	254	34	77	4	0	0	0	3	0	2	1	0	1	4	2.2
	678	144	356	62	104	3	0	0	0	3	0	0	0	2	1	3	2.2
5	445	108	219	33	74	3	0	0	2	0	0	2	0	1	0	3	2.0
	688	141	359	64	115	1	0	0	0	0	1	1	1	1	0	4	2.6
10	422	73	235	23	74	0	0	0	1	3	0	3	0	3	1	6	2.4
	468	102	237	55	56	4	0	0	1	0	0	3	2	3	0	5	1.9
20	559	133	275	38	81	3	0	0	0	6	4	6	1	5	3	4	2.1
	543	112	278	41	88	3	0	0	0	5	4	2	2	3	2	3	1.2

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

Fig.4.3

MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH COLCEMID

EXP.3



* Difference is significant at p <0.01

Table 4.9

Effects of Colcemid on cell division with recovery

Experiment 3

	Conc. ng/ml	No. of dividing cells scored	Spindle	% Metaphases with chromosome dislocation from the spindle (MDC) *1	% Anaphases and telophases with chromosome bridges and lagging *2	% Polyploid Metaphases *3	AT/M Ratio	MI $\overline{x}$
A	0	1087	+	0.5(0.9)	1.0(4.0)	1.1(2.2)	0.456	3.1
	5	861	+	0.3(0.7)	1.0(5.1)	1.8(2.4)	0.423	2.5
	10	667	+	1.8(3.4)	0.5(3.7)	1.2(2.0)	0.237	2.7
	20	614	-	0	0	0.2(2.0)	0	6.2
B	0	1218	+	0.2(0.3)	0.8(4.5)	0.1(0.1)	0.368	2.6
	5	1183	+	0.1(0.2)	0.5(2.6)	0.8(1.2)	0.403	1.8
	10	1011	+	0.2(0.4)	1.3(6.1)	1.1(2.1)	0.400	2.3
	20	482	+	1.2(2.3)	1.0(7.7)	1.9(3.6)	0.192	0.9
C	0	1143	+	0.5(1.0)	0.4(1.8)	0.6(1.1)	0.453	2.2
	5	1133	+	0	0.6(2.4)	0.6(1.2)	0.502	2.3
	10	910	+	0.3(0.6)	1.5(6.3)	1.2(2.3)	0.441	2.2
	20	1102	+	1.0(2.0)	2.5(9.8)	0.6(1.2)	0.449	1.7

*1 Figure in brackets is % of MDC in total metaphase population only.
*2 Figure in brackets is % of cells with bridging and lagging in total anaphase and telophase population only.
*3 Figure in brackets is % of polyploid metaphases in total metaphase population only.

A No recovery
B 24 hour recovery
C 48 hour recovery

Table 4.10
Number of cell cycles, as indicated
by harlequin banding of muntjac fibroblasts
after treatment with Colcemid (20ng/ml) and recovery

Treatment	Mitosis	No. of cells	%
Vehicle control	M1	78	73
no recovery	M2	29	27
	M3	0	0
Vehicle control	M1	21	19
24hr recovery	M2	51	46
	M3	40	36
Vehicle control	M1	8	8
48hr recovery	M2	34	35
	M3	55	57
Colcemid	M1	159	98
no recovery	M2	3	2
	M3	0	0
Colcemid	M1	124	92
24hr recovery	M2	11	8
	M3	0	0
Colcemid	M1	56	56
48hr recovery	M2	42	42
	M3	3	3

Table 4.11
Metaphase analysis of muntjac fibroblasts after treatment with colcemid (20ng/ml) and recovery

Treatment	No. of Chromosomes	No. of cells (duplicate cultures)	%	$\bar{x}$ %
Vehicle Control 24hr recovery (46% second division cells)	7	A. 79	87.7	86.6
		B. 93	85.3	
	8-13	A. 9	11.4	10.8
		B. 11	10.1	
	14	A. 2	2.5	4.0
		B. 6	5.5	
	21	A. 0	0	0
		B. 0	0	
Colcemid 48 hour recovery (42% second division cells)	7	A. 82	80.4	82.3
		B. 128	84.2	
	8-13	A. 15	14.7	14.0
		B. 20	13.2	
	14	A. 5	4.9	3.5
		B. 3	2.0	
	21	A. 0	0	0.4
		B. 1	0.7	

Endoreduplications

Vehicle control = 0/200 metaphases
Colcemid = 7/254 metaphases

4.4 Vinblastine

Vinblastine was tested at dose rates between 0.3 and 30ng/ml (Tables 4.12-4.14, Figs. 4.4-4.5). Vinblastine induced a dose related metaphase arrest as demonstrated by a reduction in the AT/M ratio and an increase in the mitotic index over control levels. Significant spindle damage was found at and above 1ng/ml demonstrating a potency greater than colcemid in this cell line. The dose range between the appearance of loose chromosomes and total spindle destruction was very narrow (1-10ng/ml) illustrating the sensitivity of the assay. The second experiment contained more dose points within the active concentrations of vinblastine and demonstrated increases in MDC, bridging and lagging and polyploidy, not seen in the first experiment. These effects require further confirmation. Vinblastine caused spindle shrinkage and the appearance of small dark metaphases at 2.5ng/ml. This was followed by the appearance of larger but very disorganised spindles having one or two poles with fine threadlike microtubules radiating from them (5-10ng/ml) (Appendix, Plates 15, 16). Both of these effects on spindle morphology were seen after treatment with colcemid although the sequence of events was reversed, this may indicate a slightly different mechanism of action although the final effects were similar.

Other atypical division stages induced by vinblastine included cytoplasmic cleavage without chromosome separation and migration and cytoplasmic cleavage without decondensation of the metaphase chromosomes. (Appendix, Plates 17, 18).

Table 4.12 shows the mitotic profile of Vinblastine treated muntjac cells (experiment 1) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. ng/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	222	48	105	32	25	3	1	1	2	0	2	1	0	0	0	2	1.7
	235	44	105	40	32	1	0	2	5	0	2	0	0	3	0	1	1.3
0.3	264	41	135	30	41	5	0	0	8	0	1	0	0	1	0	2	1.1
	265	68	110	24	52	3	0	1	3	0	1	1	0	0	0	2	0.8
1	225	36	120	25	21	6	2	3	8	1	0	1	0	0	0	2	1.1
	260	71	120	20	20	8	6	5	4	0	0	0	1	1	0	4	2.0
10	207	6	1	0	0	79	90	24	7	0	0	0	0	0	0	0	4.7
	208	7	0	0	0	101	58	35	7	0	0	0	0	0	0	0	1.9
30	210	4	0	0	0	70	80	41	15	0	0	0	0	0	0	0	3.3
	217	7	0	0	0	110	54	35	11	0	0	0	0	0	0	0	5.4

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

Fig.4.4 MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH VINBLASTINE EXP.1

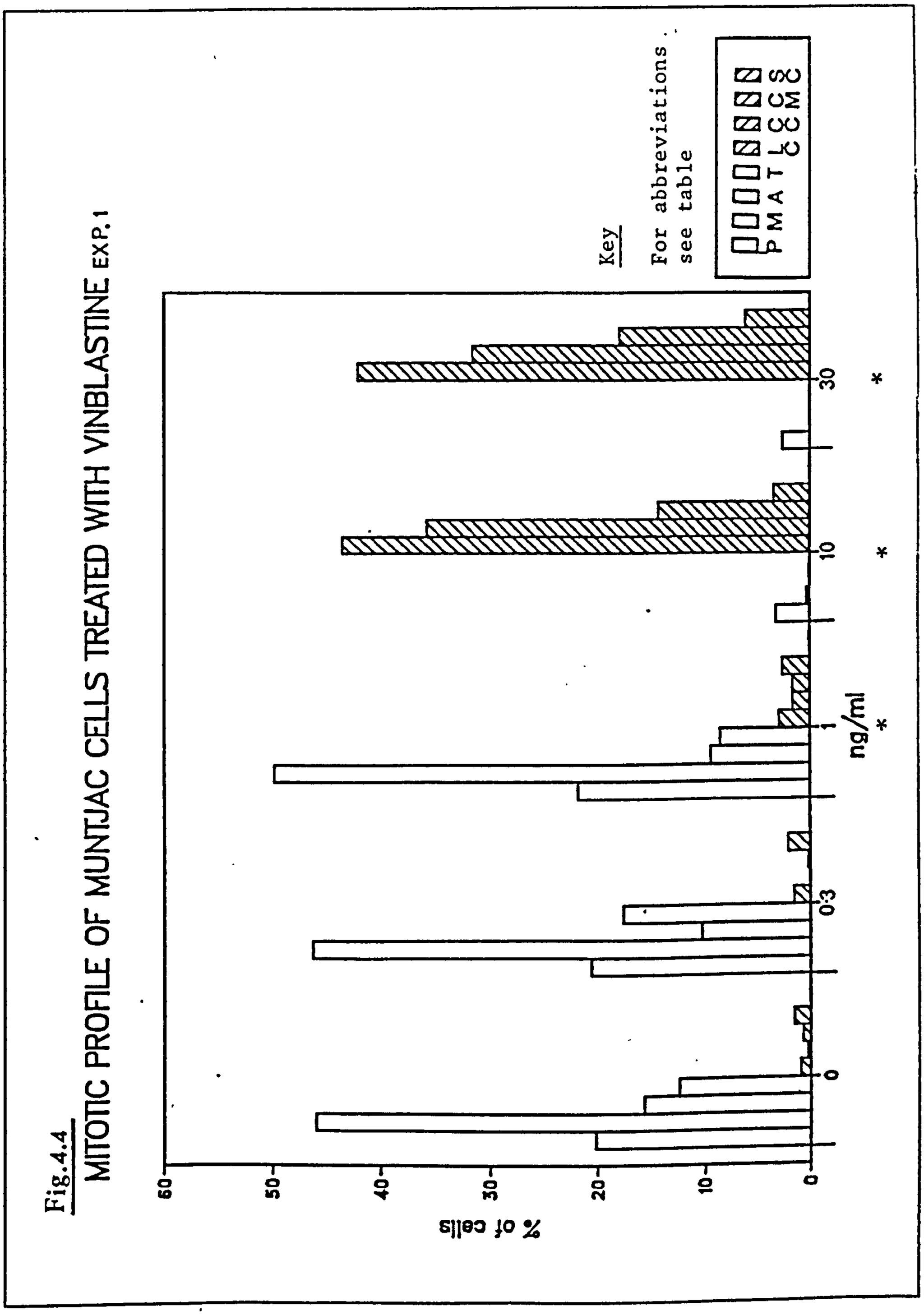


Table 4.13 shows the mitotic profile of Vinblastine treated muntjac cells (experiment 2) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. ng/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	280	74	136	29	36	2	0	0	0	0	0	2	0	1	0	0	2.8
	255	76	130	18	21	0	0	0	2	3	1	0	2	2	0	0	2.7
2.5	289	72	160	3	17	19	2	1	1	12	0	0	1	0	0	1	2.2
	277	72	160	6	18	7	4	2	2	3	0	1	1	1	0	0	2.9
5	281	42	80	3	0	125	13	2	5	9	0	0	0	0	0	2	1.7
	245	39	50	3	2	104	20	5	6	14	2	0	0	0	0	0	2.5
10	253	20	14	0	0	175	31	2	6	3	1	0	0	0	0	1	3.5
	257	16	23	0	0	195	14	2	5	1	0	0	0	0	0	1	3.0
20	240	9	0	0	0	170	40	6	15	0	0	0	0	0	0	0	5.6
	253	8	0	0	0	175	57	7	6	0	0	0	0	0	0	0	8.0

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

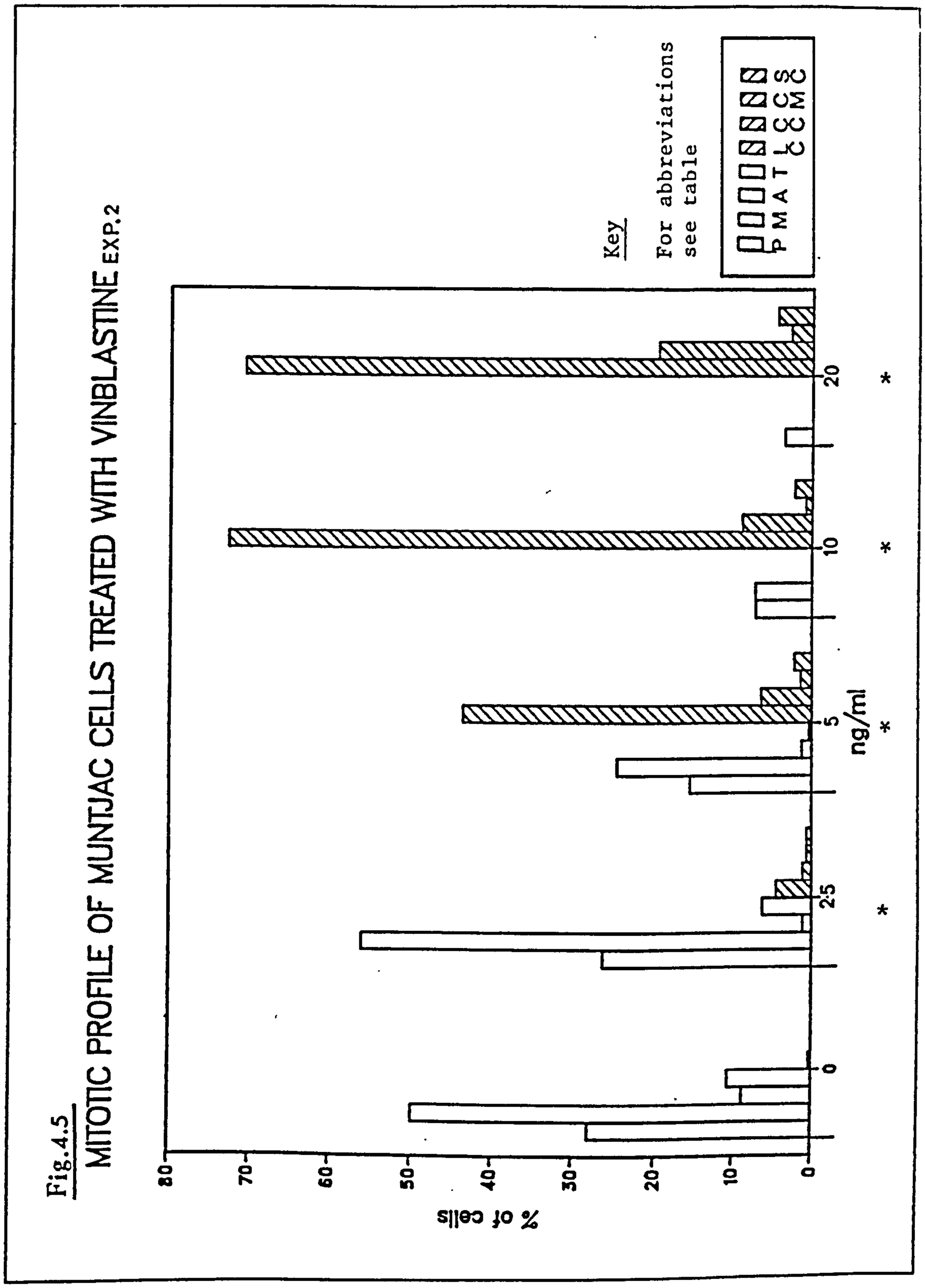


Fig.4.5 MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH VINBLASTINE EXP.2

Table 4.14

Effects of Vinblastine on cell division

Experiments 1 and 2

	Conc. ng/ml	No. of dividing cells scored	Spindle	% Metaphases with chromosome dislocation from the spindle (MDC) *1	% Anaphases and telophases with chromosome bridges and lagging *2	% Polyploid Metaphases *3	AT/M Ratio	MI $\bar{x}$
	0	457	+	0	1.8 (5.8)	0.7 (1.4)	0.613	1.5
	0.3	529	+	0	0.8 (2.6)	0.8 (1.6)	0.602	1.0
Exp 1	1	485	+	0.2 (0.4)	0.6 (3.3)	1.2 (2.4)	0.359	1.6
	10	415	-	0	0	0	0	3.3
	30	427	-	0	0	0	0	4.4
	0	535	+	0.6 (1.1)	1.5 (7.1)	0	0.390	2.8
	2.5	566	+	2.7 (4.5)	0.7 (8.3)	0.2 (0.4)	0.130	2.6
Exp 2	5	526	+	4.4 (14.8)	0.4 (20.0)	0.4 (1.3)	0.065	2.1
	10	510	-	0.8 (9.3)	0.2	0.4 (4.7)	0	3.3
	20	493	-	0	0	0	0	6.8

*1 Figure in brackets is % of MDC in total metaphase population only.
*2 Figure in brackets is % of cells with bridging and lagging in total anaphase and telophase population only.
*3 Figure in brackets is % of polyploid metaphases in total metaphase population only.

4.5 Diethylstilboestrol

DES was tested in two experiments covering in total a dose range between 0.625 and 10µg/ml (Tables 4.15-4.17, Figs. 4.6-4.7).

The first experiment with DES demonstrated that the compound was a potent mitotic spindle inhibitor, active over a narrow dose range before causing overt toxicity. At 1.3µg/ml there were no visible effects but at 10µg/ml aberrant division stages without spindles predominated. There was a significant increase in the mitotic index with increasing dose concentration, accompanied by a decrease in the AT/M ratio, indicating metaphase arrest.

In the second experiment the dose range was narrowed slightly so that the intermediate effects between the no effect and toxic levels could be examined in more detail. Significant spindle damage was seen at 4µg/ml and upwards to 10µg/ml.

In both experiments a dose related increase in chromosome dislocation was seen (Appendix, Plate 8). One multipolar division was observed in the low dose group of each experiment and in the vehicle control of the first experiment. These are thought to have arisen by chance and not be compound related.

At doses lower than 4µg/ml, DES disturbed the morphology of the spindle in a manner identical to that seen after treatment with colcemid. This suggests a common mechanism of action however DES also affected the cell morphology at 10µg/ml an appearance not noted with colcemid treatment. (Appendix, Plate 19).

Table 4.15 shows the mitotic profile of Diethylstiboestrol treated muntjac cells (experiment 1) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. µg/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	568	128	307	51	68	0	0	0	2	0	0	1	2	0	1	8	1.2
0.6	678	142	358	58	99	3	0	0	3	0	1	3	1	1	1	8	1.8
1.3	696	137	363	66	104	2	0	1	4	4	0	1	0	1	0	13	1.7
2.5	633	139	358	35	69	5	0	3	1	11	0	2	1	0	0	9	2.1
5	399	22	171	6	5	136	3	13	2	40	0	1	0	0	0	0	5.1
10	614	10	0	0	0	197	296	68	43	0	0	0	0	0	0	0	4.6

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

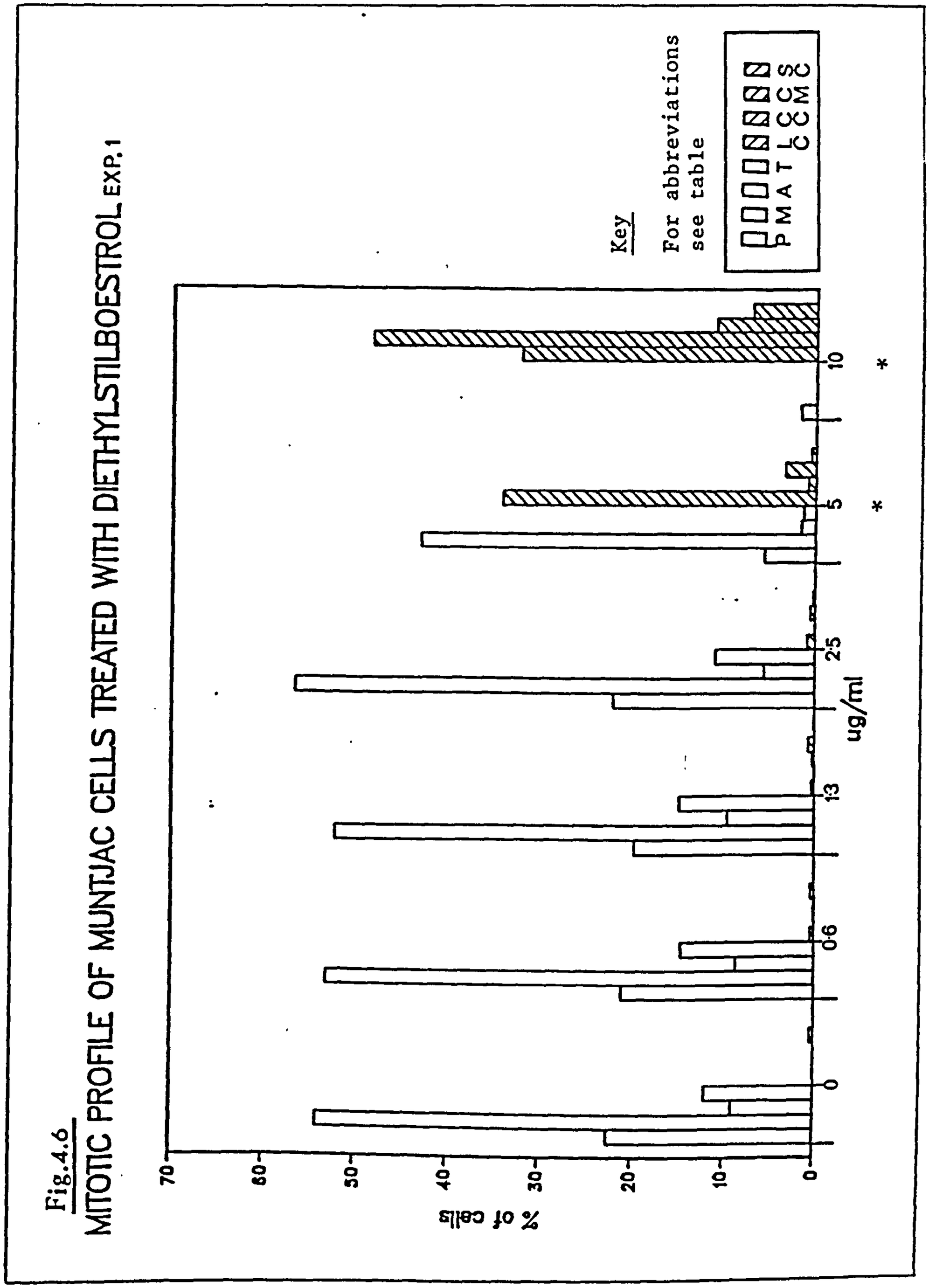


Fig.4.6
MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH DIETHYLSTILBOESTROL EXP.1
% of cells
70
60
50
40
30
20
10
0
0·6
13
25
5
10
ug/ml
Key
For abbreviations see table
PMATCCMC
* Difference is significant at p <0.01
*
*

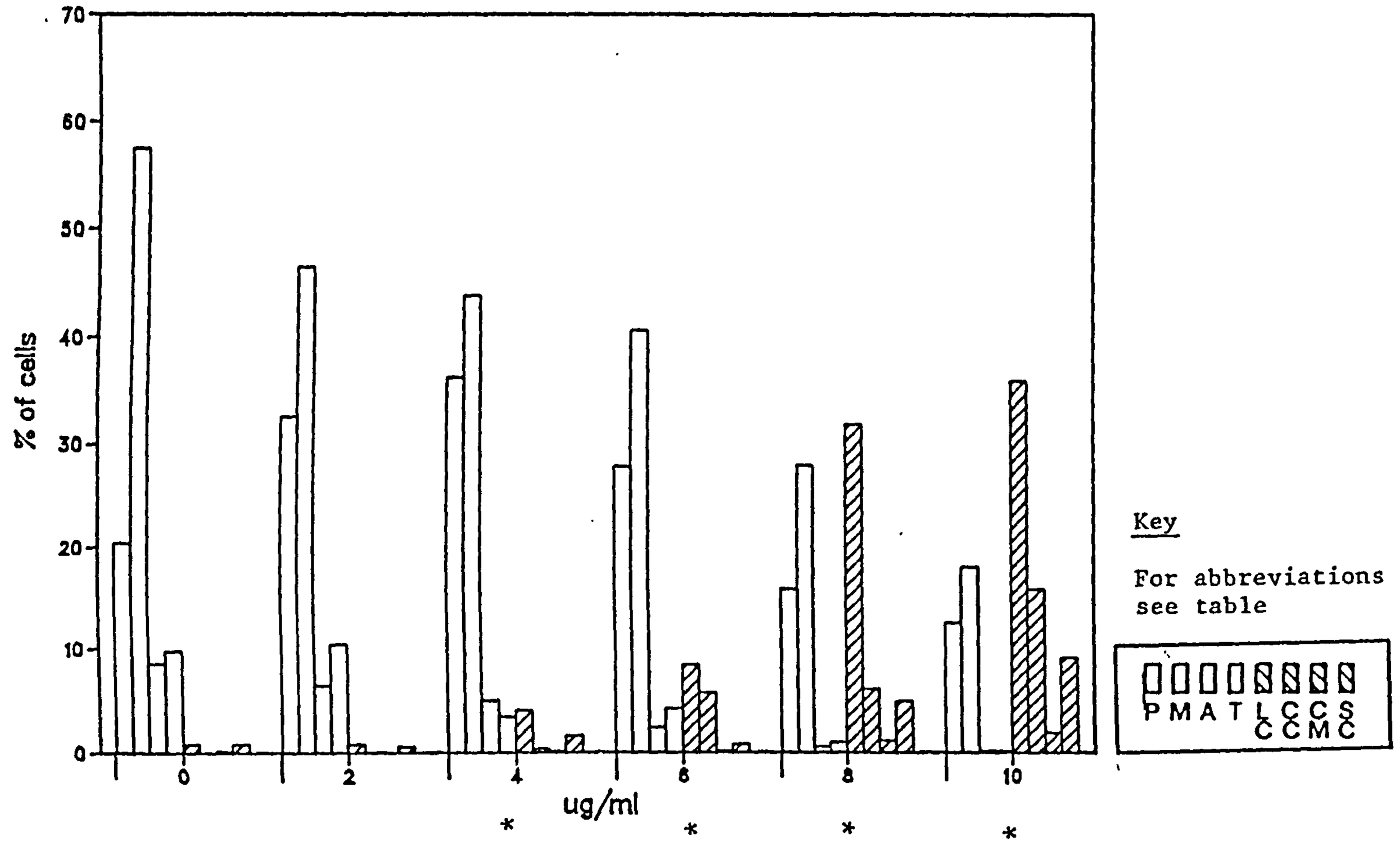
Table 4.16 shows the mitotic profile of Diethylstilboestrol treated muntjac cells (experiment 2) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. µg/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	262	49	140	27	33	4	0	1	1	0	2	1	0	0	0	4	2.1
	268	59	165	18	18	0	0	0	3	0	1	0	1	0	0	3	3.0
2	259	85	119	15	28	3	0	0	0	1	0	1	0	1	0	6	2.0
	261	85	122	18	26	1	0	0	3	0	2	2	0	0	1	1	2.4
4	250	85	107	11	17	8	1	0	6	10	1	1	0	0	0	3	1.8
	255	98	114	14	0	12	1	1	2	10	2	1	0	0	0	0	2.7
6	249	75	106	9	9	13	11	1	2	19	1	0	1	1	0	1	1.7
	275	70	106	3	13	32	19	0	2	23	0	0	4	0	0	3	2.3
8	240	41	70	0	0	72	23	4	11	15	0	0	2	0	0	2	3.2
	271	40	72	3	5	91	7	2	14	30	0	0	1	0	0	6	2.8
10	240	24	32	0	0	88	59	5	18	10	0	0	0	0	0	4	1.7
	242	36	55	1	1	85	17	4	26	14	0	0	0	0	0	3	1.0

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

Fig.4.7

MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH DIETHYLSTILBOESTROL EXP.2



* Difference is significant at p <0.01

Table 4.17
Effects of Diethylstilboestrol on cell division
Experiments 1 and 2

	Conc. µg/ml	No. of dividing cells scored	Spindle	% Metaphases with chromosome dislocation from the spindle (MDC) *1	% Anaphases and telophases with chromosome bridges and lagging *2	% Polyploid Metaphases *3	AT/M Ratio	MI $\bar{x}$
	0	568	+	0	0.5 (2.5)	1.4 (2.5)	0.388	1.2
	0.6	678	+	0	0.9 (3.7)	1.2 (2.2)	0.439	1.8
Exp 1	1.3	696	+	0.6 (1.1)	0.3 (1.2)	1.9 (3.4)	0.467	1.7
	2.5	633	+	1.7 (2.9)	0.5 (2.8)	1.4 (2.3)	0.290	2.1
	5	399	+	10 (19.0)	0.3 (8.3)	0	0.065	5.1
	10	614	-	0	0	0	0	4.6
	0	530	+	0	0.9 (5.0)	1.3 (2.2)	0.315	2.5
	2	520	+	0.2 (0.4)	1.2 (7.5)	1.4 (2.8)	0.361	2.2
Exp 2	4	505	+	4.0 (8.2)	1.0 (10.6)	0.6 (1.2)	0.192	2.3
	6	524	+	8.0 (16.3)	1.3 (17.1)	0.8 (1.6)	0.161	2.0
	8	511	+	8.8 (23.1)	0.6 (27.3)	1.6 (4.1)	0.058	3.0
	10	482	±	5.0 (20.3)	0	1.5 (5.9)	0.022	1.4

*1 Figure in brackets is % of MDC in total metaphase population only.
*2 Figure in brackets is % of cells with bridging and lagging in total anaphase and telophase population only.
*3 Figure in brackets is % of polyploid metaphases in total metaphase population only.

4.6 Nocodazole

Nocodazole was tested at doses between 2.5 and 40ng/ml (Tables 4.18 - 4.20, Figs. 4.8 - 4.9). Nocodazole induced a dose related metaphase arrest as indicated by the AT/M ratio and the mitotic index and significant spindle damage from 10-20ng/ml. Toxicity was reached at 40ng/ml in the first experiment and so the doses were lowered for the repeat experiment. This confirmed a significant dose related increase in chromosome dislocation. In both experiments there was inconclusive evidence for the induction of chromosome bridging and lagging. One tripolar metaphase was seen in the 2.5ng/ml dose group only and is thought to have arisen by chance.

Nocodazole affected the morphology of the spindle in a similar way to that induced by vinblastine ie. spindle shrinkage at 5ng/ml, followed by loss of shape and structure at 10 and 20ng/ml. This suggests a mode of action similar to vinblastine but slightly different from that of DES and colcemid. However nocodazole induced a loss of cell shape at 20ng/ml as seen previously after treatment with DES.

Nocodazole also appeared to affect chromosome morphology by inducing extreme condensation and stickiness at 20ng/ml. (Appendix, Plates 20, 21).

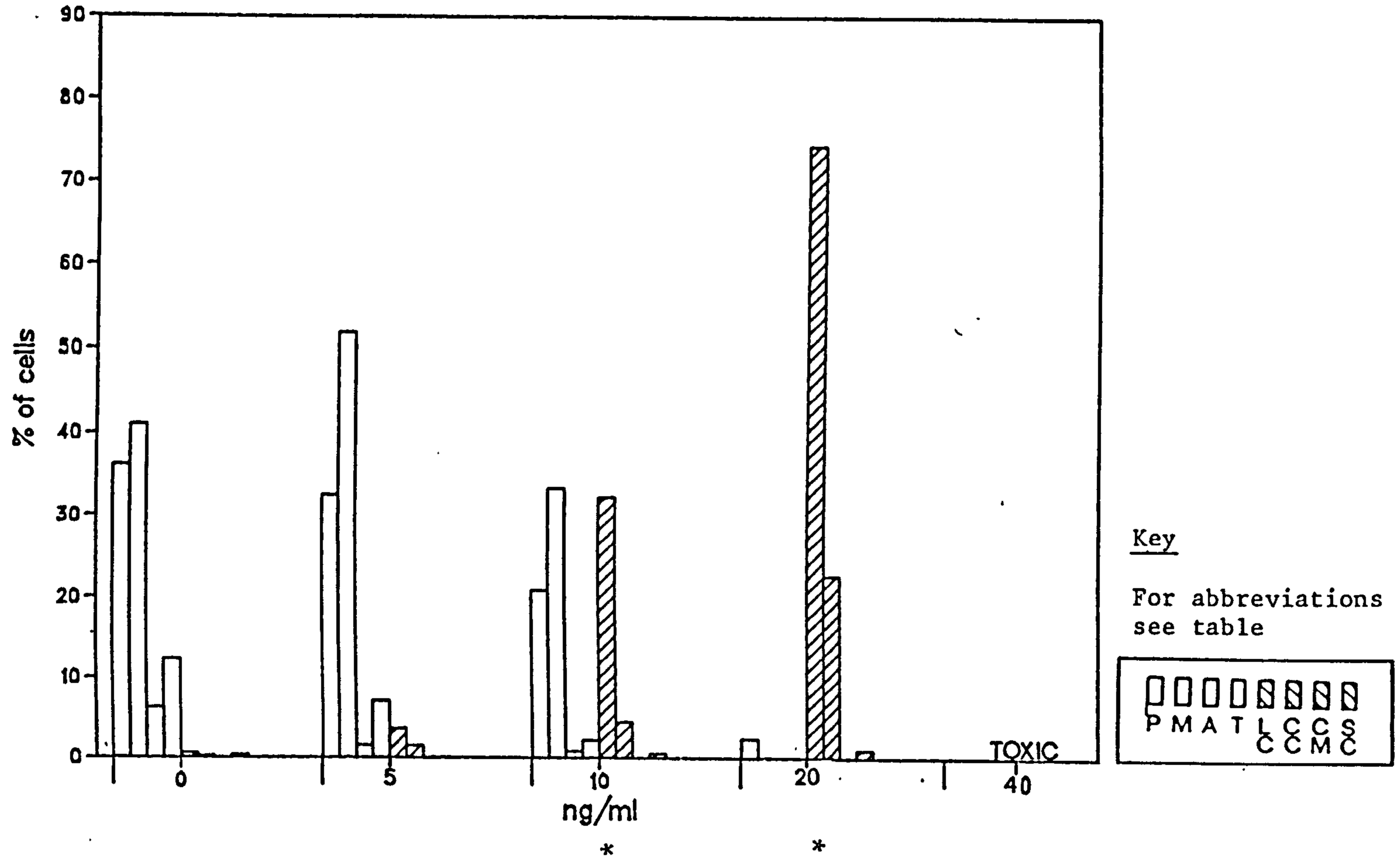
Table 4.18 shows the mitotic profile of Nocodazole treated muntjac cells (experiment 1) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. ng/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	256	94	103	15	32	0	1	0	2	0	1	2	0	2	0	4	2.8
	276	98	115	18	33	3	0	0	0	0	0	0	1	3	0	5	2.8
5	267	105	125	3	18	9	2	0	0	1	0	0	1	1	0	2	2.0
	262	67	149	5	19	10	6	0	0	3	0	0	0	0	0	3	2.1
10	265	58	61	3	6	98	18	0	1	17	0	0	1	0	0	2	3.1
	264	52	115	1	5	72	5	0	2	10	0	0	0	1	0	1	2.4
20	252	5	0	0	0	200	44	0	3	0	0	0	0	0	0	0	5.0
	390	10	0	0	0	269	108	0	3	0	0	0	0	0	0	0	4.7
40	0	Toxic															

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

Fig.4.8

MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH NOCODAZOLE EXP.1



* Difference is significant at p <0.01

Table 4.19 shows the mitotic profile of Nocodazole treated muntjac cells (experiment 2) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. ng/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE					ABNORMAL MITOSES					POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	260	4	155	30	66	0	0	0	1	0	0	0	1	3	0	0	1.3
	245	14	105	31	82	0	0	2	2	0	2	2	1	1	0	3	1.0
2.5	241	3	145	14	69	0	0	1	4	0	1	0	1	0	1	3	1.4
	252	30	135	21	48	0	1	0	7	0	1	2	0	7	0	0	1.6
5	236	12	150	21	41	0	1	0	10	0	0	0	0	1	0	0	1.1
	220	22	130	26	30	0	0	0	2	1	2	1	2	4	0	0	1.7
10	248	15	165	15	29	3	1	0	4	8	1	1	1	3	0	2	2.3
	349	25	235	15	27	15	8	0	8	6	1	1	0	3	0	5	2.3
20	239	10	170	1	8	17	20	4	3	5	0	0	0	0	0	1	3.3
	236	17	147	1	10	22	16	0	4	16	0	0	1	1	0	1	2.9

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

Fig.4.9 MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH NOCODAZOLE EXP.2

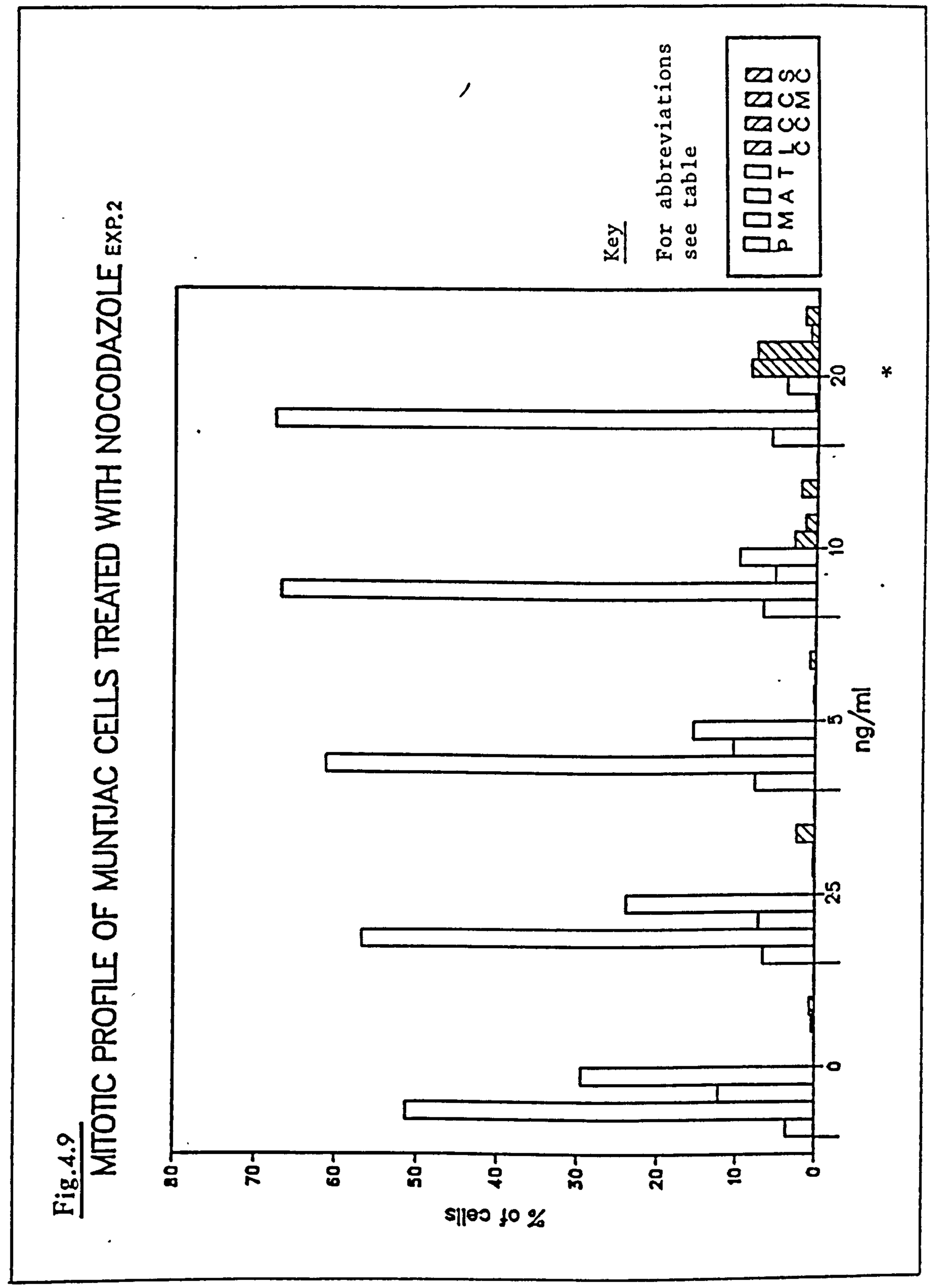


Table 4.20
Effects of Nocodazole on cell division
Experiments 1 and 2

	Conc. ng/ml	No. of dividing cells scored	Spindle	% Metaphases with chromosome dislocation from the spindle (MDC) *1	% Anaphases and telophases with chromosome bridges and lagging *2	% Polyploid Metaphases *3	AT/M Ratio	MI $\bar{x}$
	0	532	+	0	1.7 (8.4)	1.7 (4.0)	0.450	2.8
	5	529	+	0.8 (1.4)	0.4 (4.3)	1.0 (1.8)	0.164	2.1
Exp 1	10	528	+	5.1 (13.1)	0.4 (11.8)	0.6 (1.5)	0.085	2.8
	20	642	-	0	0	0	0	4.9
	40	TOXIC						
	0	405	+	0	2.0 (4.6)	0.6 (1.1)	0.804	1.2
	2.5	493	+	0	2.4 (7.3)	0.6 (1.1)	0.543	1.5
Exp 2	5	456	+	0.2 (0.4)	2.2 (7.8)	0	0.421	1.4
	10	597	+	2.3 (3.3)	1.8 (11.3)	1.2 (1.7)	0.215	2.3
	20	475	+	4.4 (6.2)	0.4 (9.1)	0.4 (0.6)	0.063	3.1

*1 Figure in brackets is % of MDC in total metaphase population only.
*2 Figure in brackets is % of cells with bridging and lagging in total anaphase and telophase population only.
*3 Figure in brackets is % of polyploid metaphases in total metaphase population only.

4.7 p-Fluorophenylalanine

pFPA was tested three times, twice with the standard protocol and once with the recovery protocol (Tables 4.21-4.31, Figs. 4.10 - 4.12). The dose range covered was 10-60µg/ml. pFPA induced a dose related mitotic arrest accompanied by a decrease in the AT/M ratio but without an increase in the mitotic index. There was a small but significant increase in aberrant division stages in the high (60µg/ml) dose only. These factors suggest toxicity of the compound rather than active spindle destruction. Inconclusive results were obtained for the induction of chromosome bridging and lagging (Appendix, Plate 9) or polyploidy.

During the 24 and 48 hour recovery periods the blocked cells recovered (as indicated by the rise, to control levels, in the AT/M ratio) however the treated cultures required approximately 24 hours to restore full mitotic activity. The most obvious effect of pFPA upon the cell division stages was a dose related occurrence of metaphases having a monopolar appearance (Appendix, Plate 22). They were similar to those seen after treatment with colcemid or DES although the effect was considerably more striking due to the increased number over control levels (approximately 40% of the total metaphase population at 50µg/ml against a control level of approximately 6%). (Tables 4.28, 4.29).

The recovery slides demonstrated that the compound induced 'monopolar' effect disappeared after removal of drug and appeared to have no ill effect on the subsequent division stages. This was indicated by the lack of aberrant cells in the recovering cell population.

No other compound related effects were noted except for one tripolar metaphase in the vehicle control and 40ng/ml dose groups which are thought to have arisen spontaneously.

4.7.1 Harlequin banding of cells treated with 50ug/ml pFPA (Table 4.30)

Harlequin banded cells did not appear in either the no recovery or 24 hour recovery cultures. There were 11.6% second division cells in the 48 hour recovery cultures and the remainder were first division cells. This demonstrated a very slow recovery rate of the cells following treatment.

This dividing cell population was comparable to the vehicle control cultures with no recovery as they contained 14.6% second division cells and 85.4% first division cells.

4.7.2 Metaphase analysis (table 4.31)

Metaphase analysis of these two sets of cultures was inconclusive for aneuploidy induction. The only point of note was the appearance of endoreduplication in the treated cell population, the frequency of which was significantly increased over the control level. (Appendix, Plate 23).

Table 4.21 shows the mitotic profile of p-Fluorophenylalanine treated muntjac cells (experiment 1) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. µg/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	259	16	131	41	51	1	0	0	6	0	2	3	4	1	1	2	0.9
	231	20	130	25	47	0	1	0	5	0	0	0	1	1	0	1	1.5
10	272	17	163	37	41	1	0	0	7	0	1	2	2	1	0	0	2.0
	235	12	156	27	32	1	0	1	3	1	0	0	1	1	0	0	0.8
20	246	33	149	27	28	2	2	0	0	1	1	1	1	0	0	1	0.9
	184	22	125	13	15	1	0	0	2	1	1	1	3	0	0	0	1.1
30	214	23	151	15	15	2	1	1	1	0	1	1	1	0	0	2	0.3
	227	22	152	21	15	1	0	0	9	0	4	0	2	0	0	1	0.8
40	147	9	107	16	14	0	0	0	0	0	0	0	0	0	0	1	0.9
	196	22	138	16	11	1	0	0	3	1	1	0	0	2	1	0	0.6
50	217	27	160	13	9	2	1	0	2	0	0	1	0	1	0	1	0.6
	136	10	104	8	3	2	3	1	4	0	0	0	0	0	0	1	0.8

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

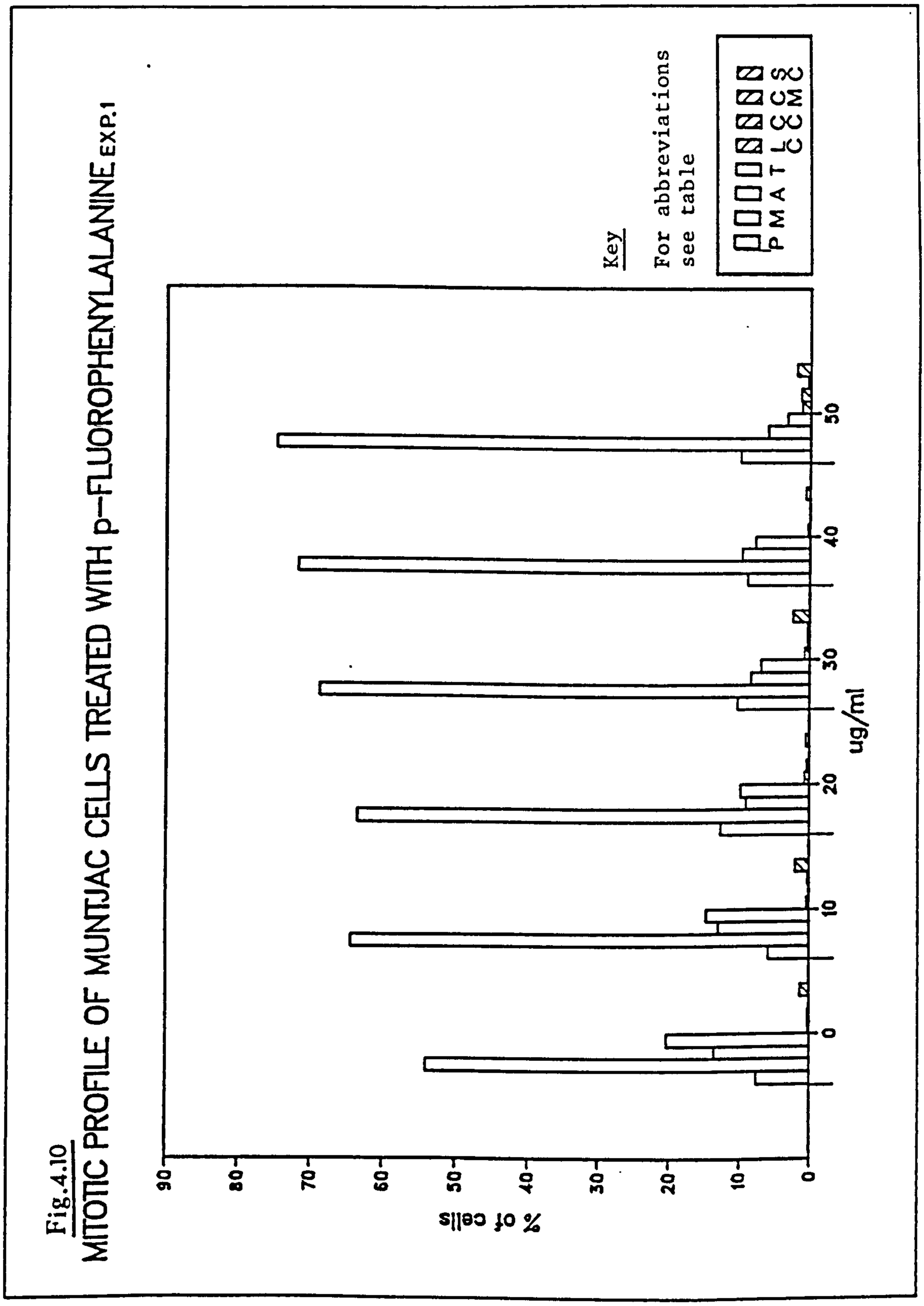

MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH p-FLUOROPHENYLALANINE EXP.1
Key
For abbreviations see table
PMATCCMC
% of cells
0
10
20
30
40
50
60
70
80
90
ug/ml
0
10
20
30
40
50

Fig.4.10

Table 4.22 shows the mitotic profile of p-Fluorophenylalanine treated muntjac cells (experiment 2) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. µg/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	250	29	113	31	58	0	0	1	8	1	3	0	3	2	0	1	1.2
	247	41	121	20	48	3	0	0	3	1	3	1	5	1	0	0	1.0
10	221	17	116	21	55	1	0	0	4	0	1	2	0	1	0	3	1.1
	247	32	129	20	42	2	0	0	11	2	2	1	4	0	0	2	0.6
20	228	30	132	21	35	1	0	0	3	0	0	0	3	1	0	2	1.1
	229	22	124	24	49	0	0	0	0	1	0	0	1	3	0	5	1.0
30	224	36	135	16	29	1	0	0	4	1	0	0	0	1	0	1	1.2
	222	36	127	18	34	1	0	0	4	1	0	0	1	0	0	0	1.2
40	241	31	152	23	16	3	0	0	9	1	1	1	1	0	0	3	1.4
	235	27	145	19	21	2	2	1	9	2	1	0	1	2	0	3	1.9
50	233	35	160	8	9	2	0	0	15	1	0	0	0	0	0	3	1.2
	230	22	169	9	10	1	0	0	7	1	0	1	3	0	0	7	0.6

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

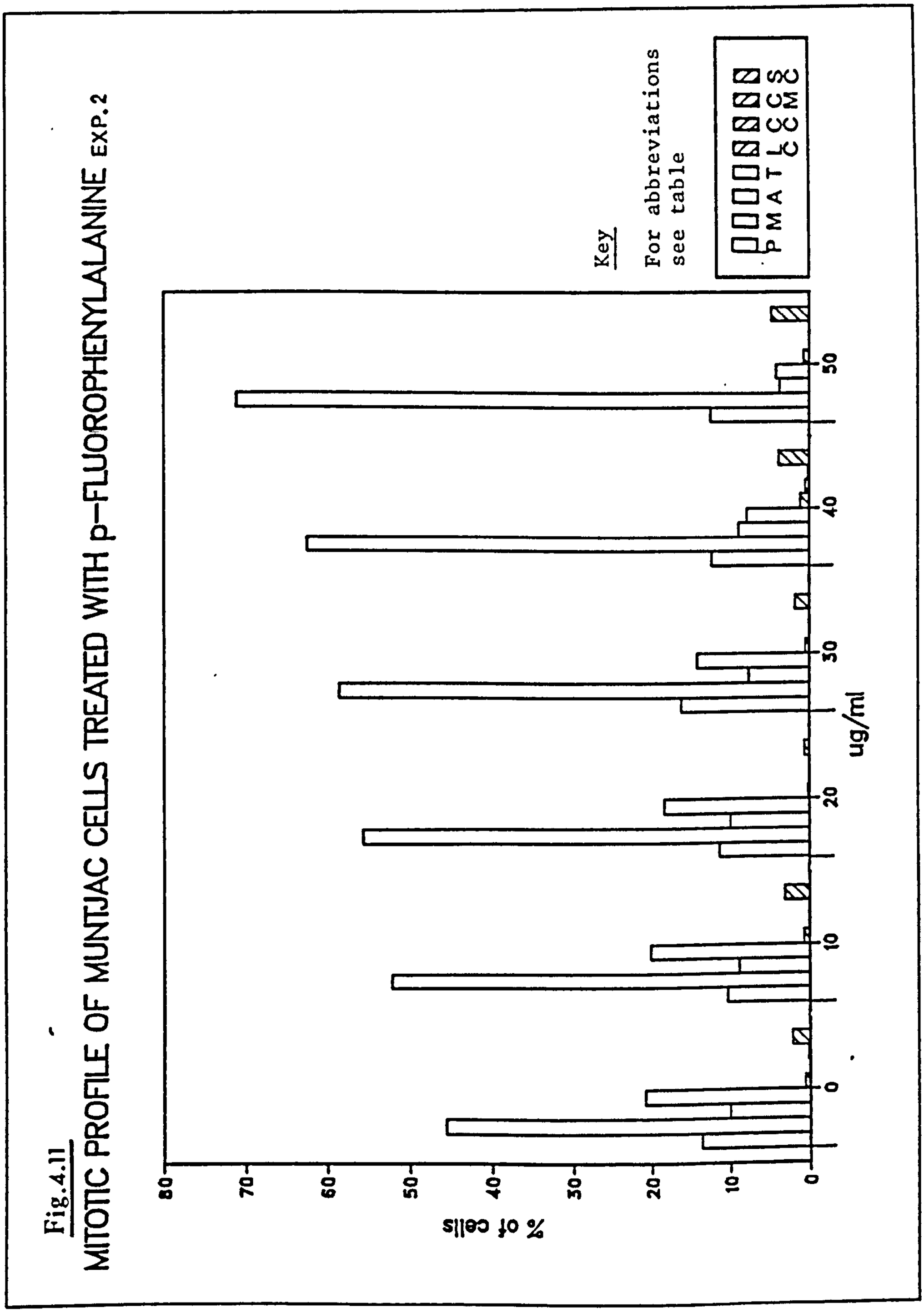


Fig.4.11
MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH p-FLUOROPHENYLALANINE EXP.2
Key
For abbreviations see table
PMATLCCMC
% of cells
0
10
20
30
40
50
60
70
80
ug/ml
0
10
20
30
40
50

Table 4.23

Effects of p-Fluorophenylalanine on cell division

Experiments 1 and 2

	Conc. µg/ml	No. of dividing cells scored	Spindle	% Metaphases with chromosome dislocation from the spindle (MDC) *1	% Anaphases and telophases with chromosome bridges and lagging *2	% Polyploid Metaphases *3	AT/M Ratio	MI $\bar{x}$
	0	490	+	0	2.5 (7.3)	0.6 (1.1)	0.628	1.2
	10	507	+	0.2 (0.3)	1.6 (5.5)	0	0.430	1.4
Exp 1	20	430	+	0.5 (0.7)	1.9 (8.8)	0.2 (0.4)	0.303	1.0
	30	441	+	0	2.0 (12.0)	0.7 (1.0)	0.218	0.6
	40	343	+	0.3 (0.4)	0.9 (5.0)	0.3 (0.4)	0.233	0.8
	50	353	+	0	0.6 (5.7)	0.6 (0.8)	0.125	0.7
	0	497	+	0.4 (0.8)	3.6 (10.3)	0.2 (0.4)	0.671	1.1
	10	468	+	0.4 (0.8)	2.4 (7.4)	1.1 (2.0)	0.563	0.9
Exp 2	20	457	+	0.2 (0.4)	1.8 (5.8)	1.5 (2.7)	0.504	1.1
	30	446	+	0.5 (0.8)	0.5 (2.0)	0.2 (0.4)	0.370	1.2
	40	476	+	0.6 (1.1)	1.5 (8.1)	1.3 (2.0)	0.266	1.7
	50	463	+	0.4 (0.6)	0.9 (10.0)	2.2 (2.9)	0.110	0.9

*1 Figure in brackets is % of MDC in total metaphase population only.
*2 Figure in brackets is % of cells with bridging and lagging in total anaphase and telophase population only.
*3 Figure in brackets is % of polyploid metaphases in total metaphase population only.

Table 4.24 shows the mitotic profile of p-Fluorophenylalanine treated muntjac cells (experiment 3), no recovery. All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. µg/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	462	104	228	49	68	4	0	0	4	0	2	0	0	1	0	2	3.3
	495	133	240	50	63	1	0	0	1	0	0	3	0	0	0	4	2.1
20	329	75	178	30	35	2	0	0	7	1	0	0	0	0	0	1	2.1
	421	91	217	40	54	5	0	0	4	2	0	1	1	1	1	4	1.8
40	392	48	287	24	9	7	0	0	8	1	1	2	0	0	0	5	1.2
	465	95	315	33	10	4	0	0	4	2	0	0	0	0	0	2	2.6
60	349	44	266	4	8	10	0	1	5	4	0	0	1	1	0	5	2.1
	242	23	189	8	0	10	0	2	5	1	0	1	0	0	0	3	1.0

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

Table 4.25 shows the mitotic profile of p-Fluorophenylalanine treated muntjac cells (experiment 3) after a 24 hour recovery period. All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. µg/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	426	100	205	62	52	1	0	0	1	0	1	1	0	0	0	3	2.7
	368	95	186	34	45	3	0	0	1	1	0	2	0	0	0	1	2.2
20	437	92	218	44	69	8	0	0	0	1	0	1	0	4	0	0	1.7
	394	101	183	34	64	5	0	0	0	0	1	0	1	1	0	4	0.7
40	385	98	192	43	44	0	0	0	3	1	0	1	0	1	0	2	0.7
	243	64	104	34	31	2	0	1	3	0	0	0	0	1	0	3	0.5
60	248	57	132	23	28	0	0	0	4	0	0	0	0	2	0	2	0.4
	275	68	133	31	21	2	0	0	14	2	0	0	0	0	1	3	0.2

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

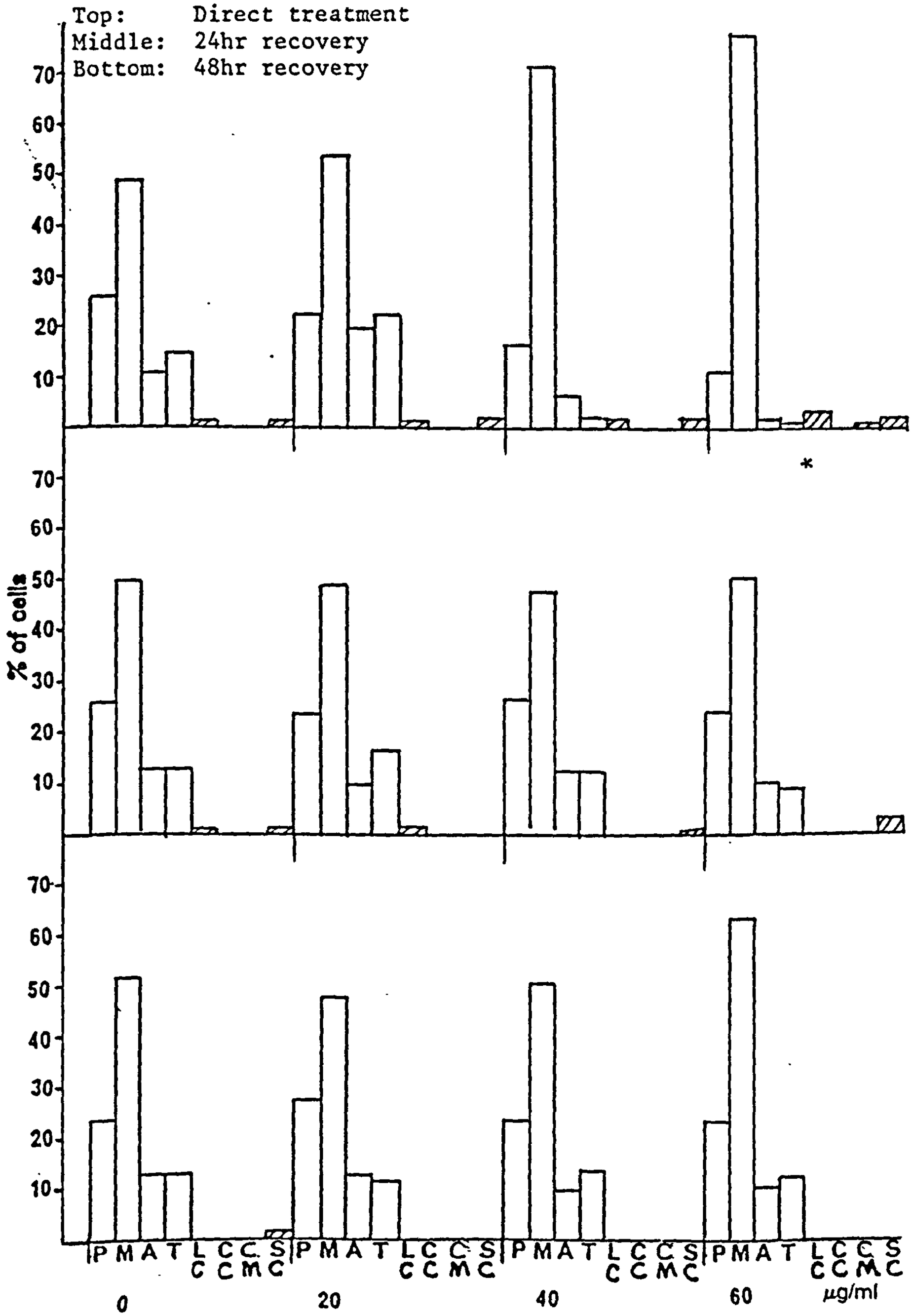
Table 4.26 shows the mitotic profile of p-Fluorophenylalanine treated muntjac cells (experiment 3) after a 48 hour recovery period. All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. µg/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	437	109	211	56	51	1	0	0	0	0	2	1	0	0	0	6	0.9
	390	84	209	44	44	0	0	0	4	1	2	0	0	0	0	2	1.8
20	445	123	210	57	49	0	0	0	1	1	0	0	1	0	0	3	1.9
	467	121	223	57	56	2	0	0	1	2	0	1	0	1	0	3	1.2
40	542	122	287	52	75	0	0	0	0	1	0	0	1	2	0	2	2.5
	401	97	197	37	55	0	0	0	1	3	3	3	0	1	1	3	2.2
60	473	92	262	47	60	0	0	0	0	1	1	1	3	0	0	6	2.7
	388	103	193	42	46	0	0	0	1	0	0	2	1	0	0	0	3.5

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

Fig.4.12

MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH p-FLUOROPHENYLALANINE EXP.3



* Difference is significant at p <0.01

Table 4.27

Effects of p-Fluorophenylalanine on cell division with recovery

Experiment 3

	Conc. µg/ml	No. of dividing cells scored	Spindle	% Metaphases with chromosome dislocation from the spindle (MDC) *1	% Anaphases and telophases with chromosome bridges and lagging *2	% Polyploid Metaphases *3	AT/M Ratio	MI $\bar{x}$
A	0	957	+	0	0.6(2.5)	0.6(1.3)	0.491	2.7
	20	750	+	0.4(0.7)	0.4(1.9)	0.7(1.3)	0.403	2.0
	40	857	+	0.4(0.5)	0.4(3.8)	0.8(1.1)	0.126	1.9
	60	591	+	0.9(1.1)	0.5(13.0)	1.4(1.7)	0.044	1.6
B	0	794	+	0.1(0.3)	0.5(2.0)	0.5(1.0)	0.496	2.5
	20	831	+	0.1(0.3)	1.0(3.7)	0.5(1.0)	0.399	1.2
	40	628	+	0.2(0.3)	0.5(1.9)	0.8(1.7)	0.514	0.6
	60	523	+	0.4(0.7)	0.4(1.9)	1.0(1.8)	0.389	0.3
C	0	827	+	0.1(0.2)	0.6(2.5)	1.1(1.9)	0.464	1.4
	20	912	+	0.3(0.7)	0.3(1.4)	0.7(1.4)	0.506	1.6
	40	943	+	0.4(0.8)	1.1(4.4)	0.5(1.0)	0.452	2.4
	60	861	+	0.1(0.2)	0.9(3.9)	0.7(1.3)	0.429	3.1

*1 Figure in brackets is % of MDC in total metaphase population only.
*2 Figure in brackets is % of cells with bridging and lagging in total anaphase and telophase population only.
*3 Figure in brackets is % of polyploid metaphases in total metaphase population only.

A No recovery
B 24 hour recovery
C 48 hour recovery

Table 4.28

p-Fluorophenylalanine.% of Monopolar metaphases in total metaphase population

Test Concentration µg/ml	Experiment I	Experiment II
Vehicle Control	3.1	8.9
10	5.7	9.3
20	13.2	8.3
30	12.8	13.5
40	28.7	24.4
50	32.5	44.8

Table 4.29

Treatment and Recovery p-Fluorophenylalanine

% of monopolar metaphases
in total metaphase population

Concentration µg/ml	% No recovery	24 hour recovery	48 hour recovery
0	1.0	1.5	2.6
20	11.1	2.7	5.0
40	19.1	1.4	2.3
60	15.6	2.7	4.2

Table 4.30

Number of cell cycles as indicated
by harlequin banding of muntjac fibroblasts
after treatment with p-Fluorophenylalanine (50µg/ml) and recovery

Treatment	Mitosis	No. of cells (Duplicate cultures)		%
		A	B	
Vehicle control	M1	107	116	85.4
no recovery	M2	18	20	14.6
	M3	0	0	0
Vehicle control	M1	36	44	36.4
24hr recovery	M2	75	59	60.1
	M3	0	6	2.9
Vehicle control	M1	10	10	14.7
48hr recovery	M2	38	28	48.5
	M3	23	27	36.8
pFPA	M1	cultures		-
no recovery	M2	contaminated		
	M3			
pFPA	M1	No		-
24hr	M2	dividing		
recovery	M3	cells		
pFPA	M1	111	101	88.3
48hr	M2	14	14	11.6
recovery	M3	0	0	0

Table 4.31
Metaphase analysis of muntjac fibroblasts after treatment with p-Fluorophenylalanine (50µg/ml) and recovery

Treatment	No. of Chromosomes	No. of cells (Duplicate cultures)	%	$\bar{x}$ %
Vehicle Control no recovery (14.6% second division cells)	7	A. 137	94.5	94.5
		B. 150	94.4	
	8-13	A. 6	4.2	3.7
		B. 5	3.1	
	14	A. 2	1.4	2.0
		B. 4	2.5	
pFPA 48 hour recovery (11.6% second division cells)	7	A. 100	91.7	93.8
		B. 94	95.9	
	8-13	A. 6	5.4	4.3
		B. 3	3.1	
	14	A. 3	2.8	1.9
		B. 1	1.0	

Endoreduplications

Vehicle control = 0/304 metaphases
pFPA = 16/207 metaphases

4.8 Acenaphthene

Acenaphthene was tested in two experiments at doses between 10 and 90µg/ml (Tables 4.32-4.34, Figs. 4.13-4.14). At these doses acenaphthene formed a very fine homogenous suspension in the vehicle.

On each occasion no conclusive spindle damage was seen. In the first experiment the significant increase in scattered chromatin at 30 and 60µg/ml, accompanied by a decreasing mitotic index indicated cytotoxic events only. Negative results were obtained for polyploidy, chromosome dislocation and chromosome bridging and lagging.

The second experiment, covering a reduced dose range, showed a dose related mitotic arrest and cytotoxicity but also some spindle damage at 60 and 80µg/ml. This effect requires confirmation. No other specific effects were seen.

Table 4.32 shows the mitotic profile of Acenaphthene treated muntjac cells (experiment 1) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. µg/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	238	46	102	19	65	0	0	0	2	0	1	0	0	1	0	2	2.8
	255	40	113	15	69	1	0	0	9	0	0	3	3	1	0	1	3.0
10	247	37	121	30	49	1	1	0	1	1	0	0	2	2	0	2	2.5
	261	45	134	30	40	0	0	0	2	0	2	1	4	0	0	3	2.7
30	288	43	113	18	74	3	1	2	26	0	0	1	3	1	0	3	1.7
	307	32	135	29	63	2	1	1	37	0	1	0	4	1	0	1	0.7
60	333	7	30	5	13	5	4	19	250	0	0	0	0	0	0	0	0.5
	311	13	37	6	13	2	6	8	224	0	0	1	1	0	0	0	0.3
90	0	Toxic															
	0																

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

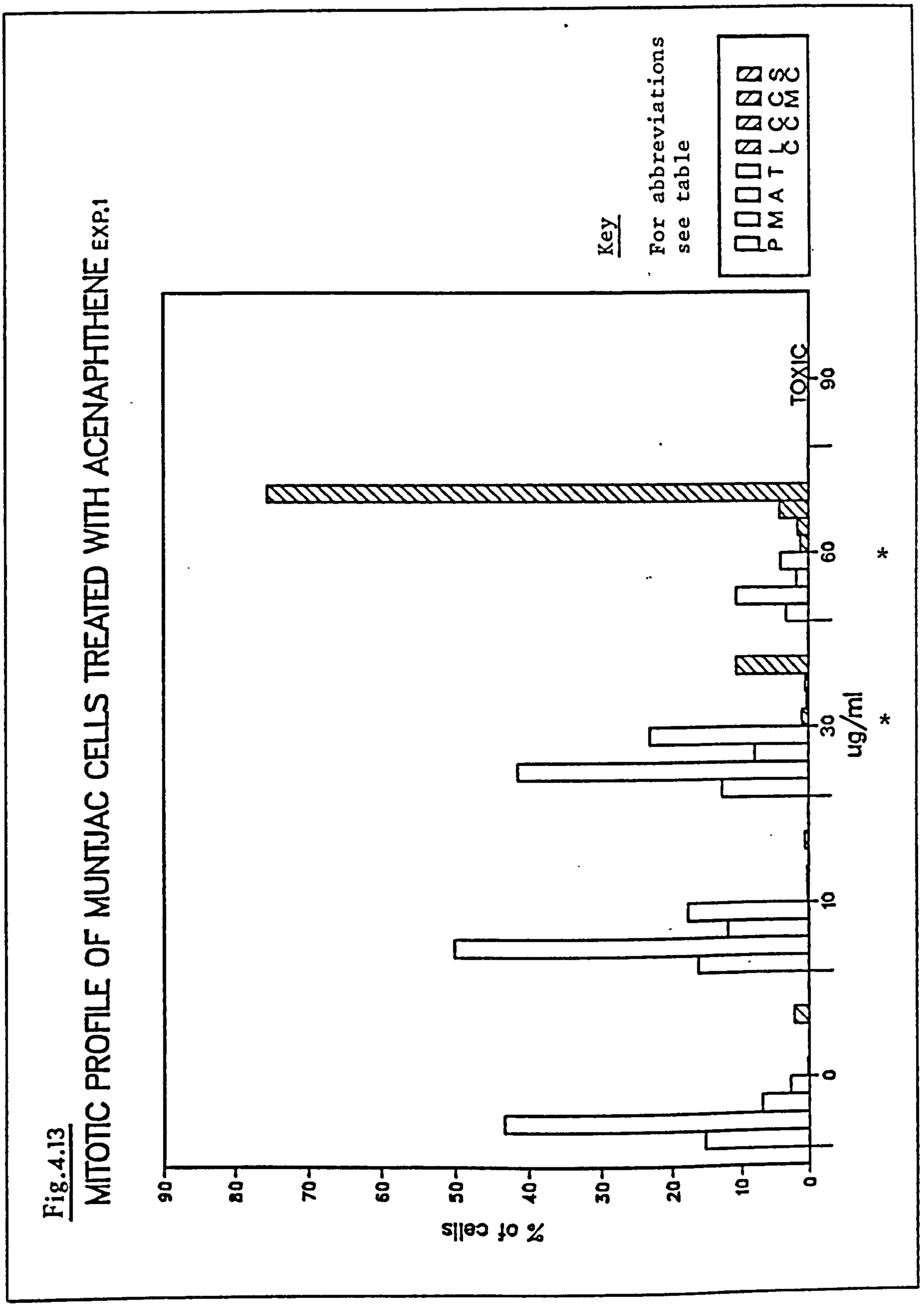

MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH ACENAPHTHENE EXP.1
Key
For abbreviations see table
PMATCCMC
TOXIC
90
60
30
10
0
ug/ml
*
* Difference is significant at p <0.01
90
80
70
60
50
40
30
20
10
0
% of cells

Fig.4.13

Table 4.33 shows the mitotic profile of Acenaphthene treated muntjac cells (experiment 2) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. µg/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	240	28	140	20	34	0	0	0	14	0	0	0	3	1	0	0	1.0
	223	28	120	24	47	0	0	0	3	1	0	0	0	0	0	0	0.9
20	237	21	135	24	46	0	0	0	8	0	1	1	0	1	0	0	1.4
	256	17	155	26	43	1	1	0	7	0	2	0	3	1	0	0	0.8
40	223	33	125	20	25	2	0	5	9	2	1	0	1	0	0	0	0.6
	223	31	115	22	37	1	0	4	8	0	3	0	0	1	0	1	0.3
60	224	19	105	18	26	2	0	20	31	0	2	0	1	0	0	0	0.8
	232	20	115	7	19	2	6	23	36	1	0	0	3	0	0	0	0.4
80	214	5	38	4	5	1	0	60	100	1	0	0	0	0	0	0	0
	220	14	96	11	11	2	0	40	36	2	4	0	2	0	0	2	0

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

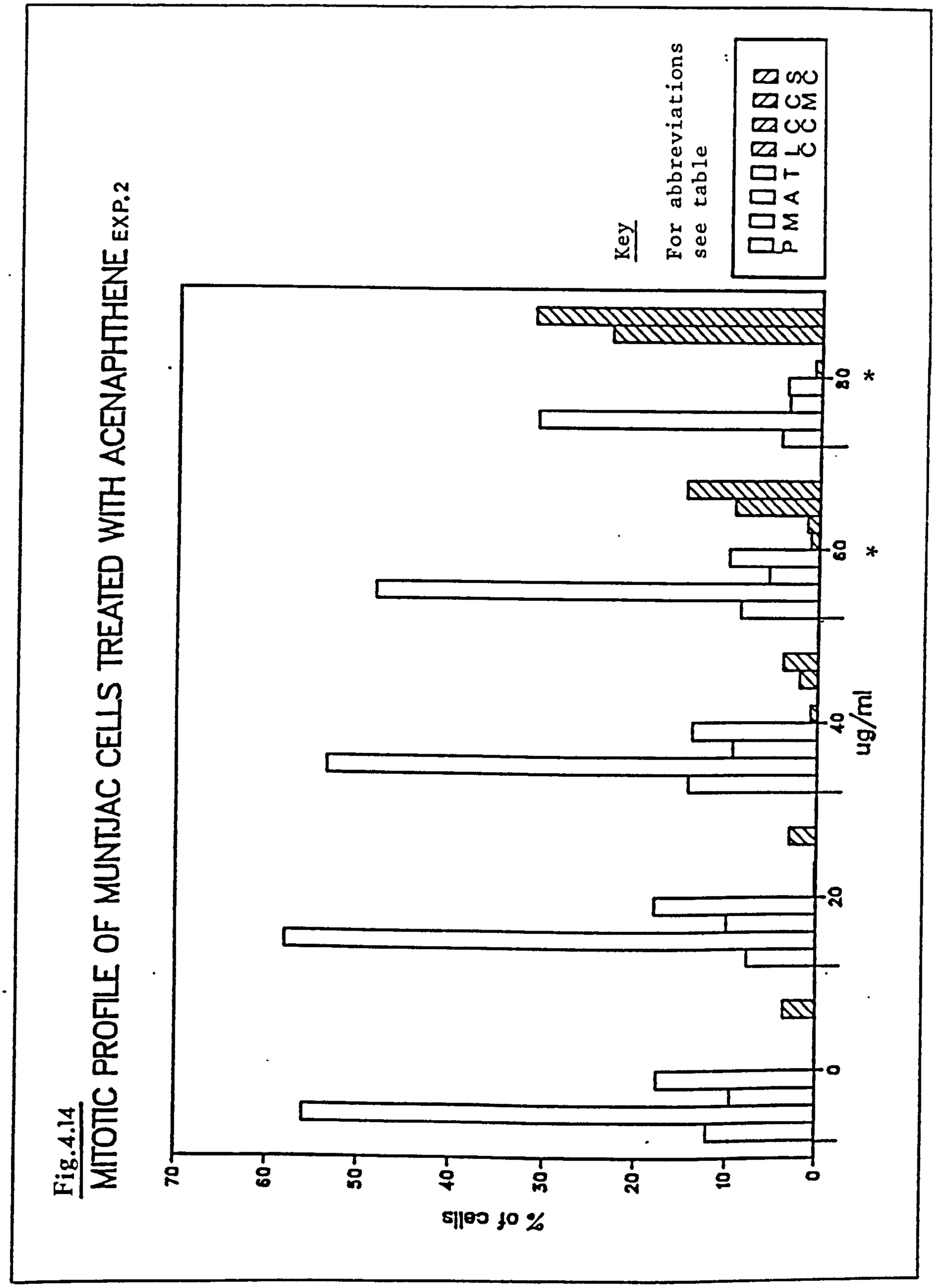


Fig.4.14
MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH ACENAPHTHENE EXP.2
% of cells
0
10
20
30
40
50
60
70
ug/ml
20
40
60
80
Key
For abbreviations
see table
PMATICS
* Difference is significant at p <0.01

Table 4.34

Effects of Acenaphthene on cell division

Experiments 1 and 2

	Conc. µg/ml	No. of dividing cells scored	Spindle	% Metaphases with chromosome dislocation from the spindle (MDC) *1	% Anaphases and telophases with chromosome bridges and lagging *2	% Polyploid Metaphases *3	AT/M Ratio	MI $\bar{x}$
Exp 1	0	493	+	0	1.8 (5.1)	0.6 (1.4)	0.780	2.9
	10	508	+	0.2 (0.4)	2.2 (6.9)	1.0 (1.9)	0.584	2.6
	30	595	+	0	1.9 (5.6)	0.7 (1.6)	0.741	1.2
	60	644	+	0	0.3 (5.1)	0	0.548	0.4
	90	TOXIC						
Exp 2	0	463	+	0.2 (0.4)	0.9 (3.1)	0	0.480	1.0
	20	493	+	0	1.8 (6.1)	0	0.479	1.1
	40	446	+	0.5 (0.8)	1.4 (5.5)	0.2 (0.4)	0.433	0.5
	60	456	+	0.2 (0.5)	1.3 (7.9)	0	0.319	0.6
	80	434	+	0.7 (2.2)	1.4 (16.2)	0.5 (1.4)	0.231	0

*1 Figure in brackets is % of MDC in total metaphase population only.
*2 Figure in brackets is % of cells with bridging and lagging in total anaphase and telophase population only.
*3 Figure in brackets is % of polyploid metaphases in total metaphase population only.

4.9 Hydroquinone

Hydroquinone was tested at a dose range between 3 and 18µg/ml (Tables 4.35-4.37 Figs. 4.15-4.16). Hydroquinone induced significant dose related spindle damage at doses very close to the toxic levels. Toxicity is indicated by the decrease in the mitotic index below the vehicle control level, and by an increase in scattered chromatin in the first experiment at 18µg/ml. Both experiments demonstrated a dose related metaphase block as illustrated by the decrease in the AT/M ratio. There was no increase in the mitotic index. By reducing the dose range in the second experiment hydroquinone was shown to have a very narrow band of activity inducing small but significant effects on the spindle. The shape of the spindle was affected but not the size resulting in loosely constructed, irregular or crescent shaped spindles (Appendix, Plate 24). Hydroquinone also induced extreme condensation of the chromosomes whilst they were still attached to the spindle fibres, this is often indicative of prolonged metaphase arrest although the spindle was not completely destroyed.

Table 4.35 shows the mitotic profile of Hydroquinone treated muntjac cells (experiment 1) All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. µg/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	474	93	170	64	115	10	0	5	18	0	1	2	4	2	0	0	1.2
3	439	100	180	46	85	12	1	1	11	0	2	0	1	0	0	0	1.5
9	466	100	183	59	69	21	2	5	14	0	4	1	4	2	0	2	2.3
12	539	72	283	35	41	17	8	22	52	3	0	0	2	2	0	2	0.5
18	86	9	32	0	3	2	5	9	25	1	0	0	0	0	0	0	0.2

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

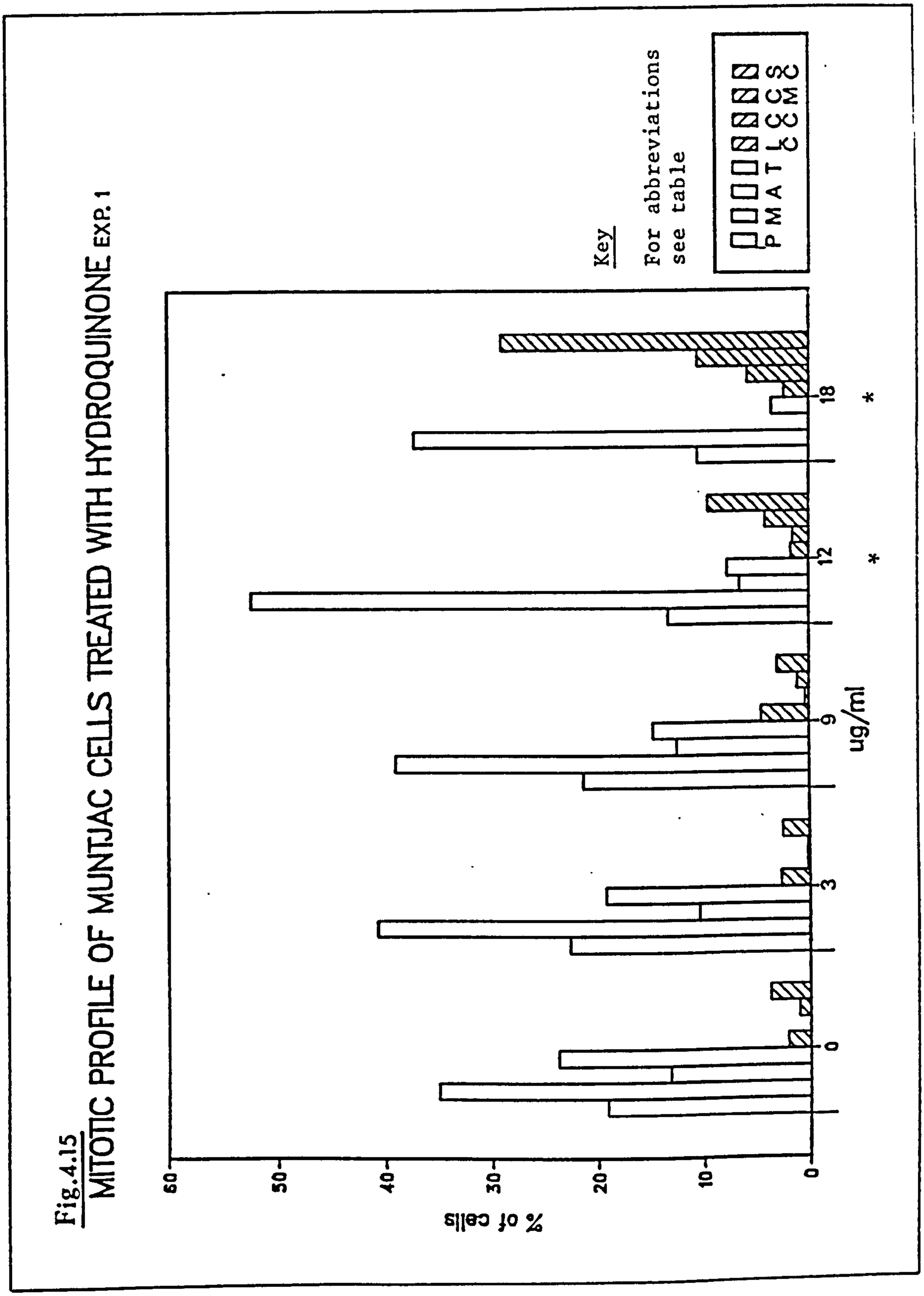


Fig.4.15
MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH HYDROQUINONE EXP.1
% of cells
60
50
40
30
20
10
0
0
3
6
12
18
ug/ml
Key
For abbreviations see table
PMATICCS
* Difference is significant at p <0.01
*
*

Table 4.36 shows the mitotic profile of Hydroquinone treated muntjac cells (experiment 2). All data are actual cell numbers except for the Mitotic Index (MI) which is expressed as a percentage.

TEST CONC. µg/ml	NUMBER OF CELLS SCORED	NORMAL MITOSIS				SPINDLE DAMAGE				ABNORMAL MITOSES						POLY. METS.	MI
		P	M	A	T	LC	CC	CM	SC	MDC	AFL	AB	TFL	TB	MP		
0	236	8	124	24	65	2	0	0	7	0	1	0	3	1	0	1	2.5
	215	14	128	20	46	2	0	0	0	0	3	1	0	1	0	0	2.8
10	268	64	130	22	33	9	0	0	1	0	1	2	2	2	0	2	2.8
	261	36	140	13	50	4	0	1	9	0	2	0	5	1	0	0	2.5
12	226	29	127	16	35	5	0	0	8	2	1	0	0	3	0	0	1.5
	227	38	130	16	24	2	2	3	6	0	3	0	1	1	0	1	1.7
14	262	28	160	4	16	22	2	0	21	4	2	0	1	1	0	1	1.6
	246	38	140	12	23	10	3	1	13	1	1	1	1	1	0	1	1.6
16	282	21	185	5	3	19	16	7	19	2	0	0	1	0	0	4	2.0
	283	21	165	1	10	18	15	16	34	3	0	0	0	0	0	0	1.4

P = Prophase, M = Metaphase, A = Anaphase, T = Telophase, LC = Loose chromosomes, CC = Chromosome Clumps, CM = Chromatin Mass, SC = Scattered Chromatin, MDC = Metaphase Dislocating Chromosome, AFL = Anaphase Fragment or laggard, AB = Anaphase Bridge, TFL = Telophase Fragment or laggard, TB = Telophase Bridge, MP = Multipolar division, POLY. MET. = Polyploid Metaphases.

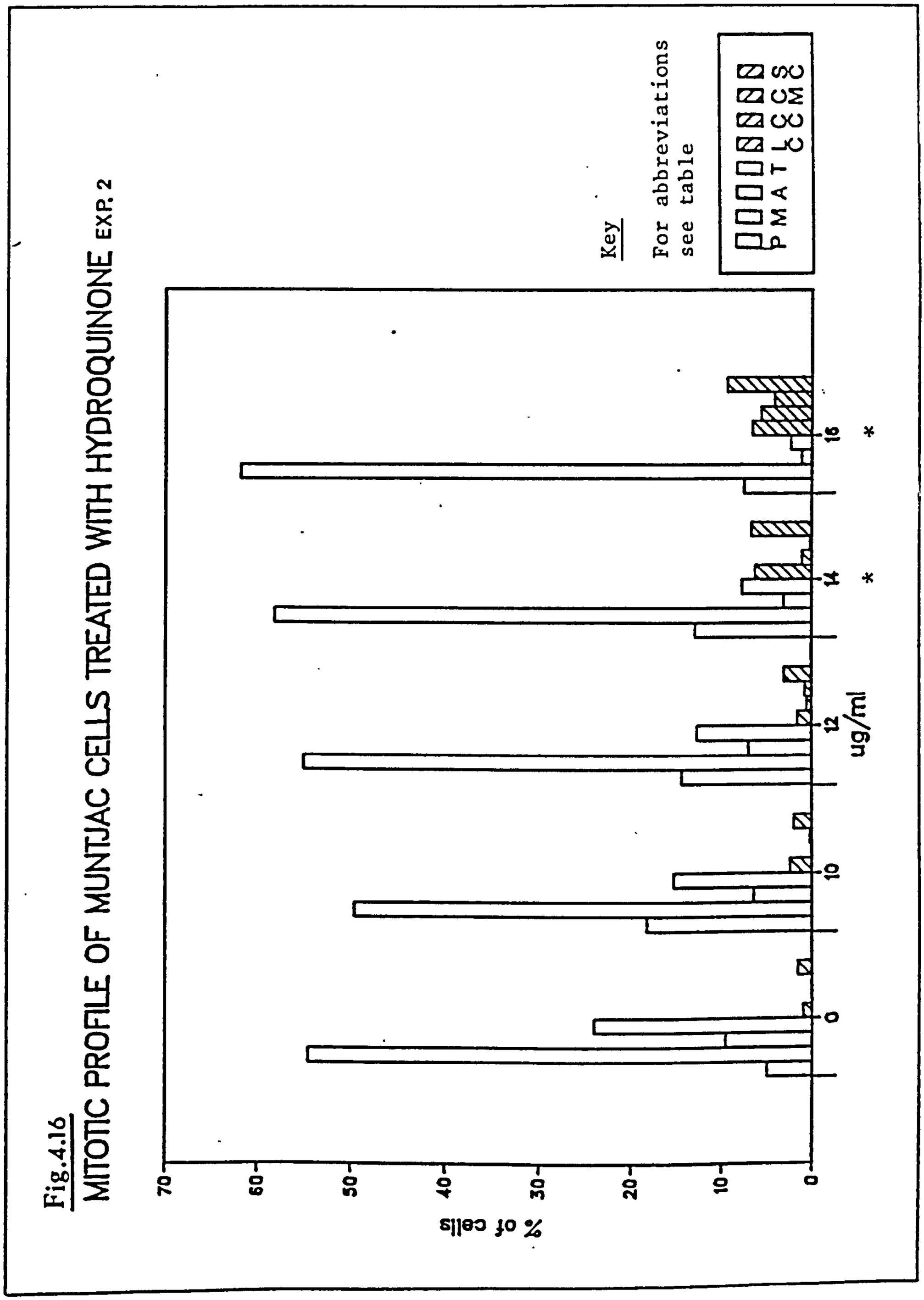


Fig.4.16
MITOTIC PROFILE OF MUNTJAC CELLS TREATED WITH HYDROQUINONE EXP.2
% of cells
70
60
50
40
30
20
10
0
0
10
12
14
16
ug/ml
Key
For abbreviations see table
PMATCCMC
* Difference is significant at p <0.01
*
*

Table 4.37
Effects of Hydroquinone on cell division
Experiments 1 and 2

	Conc. µg/ml	No. of dividing cells scored	Spindle	% Metaphases with chromosome dislocation from the spindle (MDC) *1	% Anaphases and telophases with chromosome bridges and lagging *2	% Polyploid Metaphases *3	AT/M Ratio	MI $\bar{x}$
Exp 1	0	474	+	0	1.9 (4.8)	0	1.052	1.2
	3	439	+	0 (0.4)	0.7 (6.9)	0 (1.9)	0.728	1.5
	9	466	+	0	2.4 (7.9)	0.4 (1.1)	0.700	2.3
	12	539	+	0.6 (1.0)	0.7 (5.0)	0.4 (0.7)	0.269	0.5
	18	86	+	1.2 (3.1)	0	0	0.094	0.2
Exp 2	0	452	+	0	2.2 (6.1)	0.2 (0.4)	0.615	2.7
	10	529	+	0	2.8 (11.3)	0.4 (0.7)	0.437	2.7
	12	543	+	0.4 (0.8)	1.7 (9.0)	0.2 (0.4)	0.354	1.6
	14	508	+	1.0 (1.6)	1.6 (12.7)	0.4 (0.7)	0.184	1.6
	16	565	+	0.9 (1.4)	0.2 (5.0)	0.7 (1.1)	0.055	1.7

*1 Figure in brackets is % of MDC in total metaphase population only.
*2 Figure in brackets is % of cells with bridging and lagging in total anaphase and telophase population only.
*3 Figure in brackets is % of polyploid metaphases in total metaphase population only.

5. DISCUSSION

5.1 The muntjac cells.

These cells proved to be very suitable for use in the assays under investigation. They were reliable and easy to culture and recovered well after frozen storage. Provided that the cells were grown and used within a limited number of subcultures (7-8) the background frequency of division aberrations and variation in chromosome number could be maintained at a low level. The small number of chromosomes and their characteristic morphology simplified the metaphase analysis procedure providing an accurate measure of spindle dysfunction.

5.1.1 The cytotoxicity assay

Toxic effects could be easily identified by disruption of the normal cell and culture morphology and inhibition of cell growth. The cells grew well in the Linbro plates although they were often more sensitive to drug treatment than the cells grown in the flaskettes. This may have been due to better cell attachment to the glass of the flaskette than to the plastic of the Linbro plate, even tissue culture treated plastic culture vessels can show batch and supplier variation with regard to cell adhesion (Freshney, 1983). The cytotoxicity assay gave a good indication of the compound toxicity towards the muntjac cells, however it was usually necessary to include in the spindle assay a concentration of the test compound which was apparently cytotoxic according to the preliminary assay. This ensured that for at least one test concentration, the drug could be seen to be entering the cells and having an effect.

The sensitivity of the cytotoxicity assay may have been improved by repeating the experiment using a narrower dose concentration around the suspected toxic dose. However with compounds that were toxic at very low (ng) concentrations ie. colcemid, nocodazole and vinblastine, the preparation of accurate concentrations over such a low range was extremely difficult. Therefore, it was considered that the single test results were sufficient to allow the determination of a suitable dose range providing that the limitations of the cytotoxicity assay were fully realised.

5.1.2 The spindle assay

The large cells and chromosomes were easy to stain and could be examined under low magnification. The individual division stages, both normal and aberrant, were rapidly identified since the mitotic elements were clearly differentially stained. Unless the compound was very toxic the cells grew well in the flaskettes and there were always sufficient mitotic cells for examination per slide. The enclosed culture environment within the flaskette provided very safe conditions for treating the cells with hazardous chemicals and also protected the cells from microbial contamination.

The muntjac doubling time of approximately 20 hours was very useful in the design of the assay procedures. Treatment with the test chemical for 24 hours ensured that the cells were

exposed for at least one cell cycle therefore allowing full expression of the compound related effects. This treatment period also fitted easily into a normal working routine and allowed each assay to be completed within one week.

5.1.3 The recovery protocol

This protocol was designed firstly to see whether the muntjac cells were capable of recovery following treatment with a mitotic inhibitor and secondly, whether aneuploid cells could be detected in the recovery cell population. Recovery was confirmed by the presence of harlequin stained cells in the slide preparations, however the demonstration of aneuploidy induction following treatment, although promising, was inconclusive due to insufficient data. Growing the cells in roller cultures produced many cells for analysis and harvesting by the "mitotic shake off" technique (Carrano et al, 1976) was very successful since the majority of the cells in the slide preparations were in mitosis. Careful preparation of the metaphase spreads enabled the individual chromosomes to be counted whilst still enclosed by the cytoplasmic membrane. Danford (1984) used a modified hypotonic solution to achieve similar results in mammalian cells in vitro. Thus the muntjac cells are well suited to both the spindle staining assay and for use in a recovery protocol where metaphase analysis is required.

5.2 Comparison with other cell lines

The spindle staining assay has been readily applied to a variety of cell lines since the introduction of the technique in 1982 by Parry et al (Table 5.1). However a stable and reasonably low background frequency of spindle dysfunction and aneuploidy are important criteria when choosing a cell line for use in this assay. Both Kirsch-Volders (1986) and Parry (1988) encountered instability problems when using primary cultures of mammalian cells and the levels of aneuploidy increased with increasing passages, thus rendering the cell lines unsuitable for use. Alternatively continuous or established cell lines with more stable frequencies of spindle dysfunction could be considered, however these are often characterised by divergence from the donor euploid karyotype. Therefore a diploid cell line such as the muntjac fibroblasts when used at a low passage number compared favourably with the other cell lines that have been used in the spindle assay.

When the spindle staining assays using different cell lines are compared, (Table 5.1 A.) it can be seen that the experimental methodology varies only slightly from that originally suggested by Parry et al in 1982. For example the treatment time is between 1 and 2 x the doubling time of the cell line used and the concentration of the acetic acid in the staining solution varies between 10 and 15%. The concentration of acetic acid affects the staining intensity of Brilliant Blue R, and the

CELLS USED*1	DOUBLING TIME h	TREATMENT TIME h	% ACETIC ACID	COLCEMID POSITIVE µg/ml	MI NO. CELLS SCORED/ DOSE POINT	SPINDLE ASSAY NO. REPLICATES /DOSE POINT	SPINDLE ASSAY NO. CELLS /REPLICATE
HSBP	-	24	10	0.3	1500+	3	50+
HSBP CH1-L DON	20 20 12	24	12	0.1	3000	3	100
DON	12	14	15	0.04	3000	3	400-500
VB	21	21	10	-	4000	4+	50
FLOW (2002) VB	22 21	22 21	10	-	2000	4+	25+
PK	24-28	39	15	0.04	2000	3	100
MOUSE EMBRYO FIBROBLASTS - WISH		24 24	12	4.0	2000	4	50
MUNTJAC FIBROBLASTS	20	24	10	0.01	2000	2	250+

Table 5.1 A) Comparison of spindle staining assays using different cell lines; Methodology.
(for references see table 5.1 B) *1 For explanation of abbreviations see page 152.

Cells Used*1	Mitotic Profile	MDC	Chromosome Bridging + Lagging	Multi/Mono Polar Spinde	Polyploid Meta-phases	Spindle Morpho-logy	Cytoplasmic Cleavage	AT/M Ratio	Multi-nucle-ation	Reference
HSBP	✓		✓	✓	✓	✓	✓			Parry et al (1982)
HSBP CH1-L DON	✓	✓	✓	✓		✓		✓		Parry et al (1985)
DON	✓			✓			✓			Lafi et al (1986)
VB	✓	✓	✓			✓				Nijs and Kirsch-Volders (1986)
FLOW (2002) VB	✓	✓	✓							Kirch-Volders (1986)
PK	✓	✓	✓	✓	✓	✓	✓	✓	✓	Somers (1986)
MOUSE EMBRYO FIBROBLASTS WISH	✓									De Oliveria and Machado-Santelli(1987)
MUNTJAC FIBROBLASTS	✓	✓	✓	✓	✓	✓	✓	✓		Present Study

Table 5.1 B) Comparison of spindle staining assays using different cell lines; End points discussed.

*1 For explanation of abbreviations see page 152.

Table 5.1 cont.
Explanation of abbreviations

1. H S B P = Primary human fibroblasts
2. CH1-L = Chinese hamster liver cells
3. DON = Chinese hamster lung cells
4. VB = Primary human fibroblasts
5. FLOW 2002 = Primary human fibroblasts
6. PK = Poteroo kidney cells
7. WISH = Human amnion cells

variation in use may reflect cell line differences or just individual preferences. Colcemid appears to be the positive control treatment of choice in this assay although the concentration varies considerably between laboratories, ranging from 0.04 - 4.0µg/ml. Even at the lowest concentration, colcemid would be toxic to the muntjac fibroblasts after 24 hours treatment. This variation indicates possible differences in the sensitivity of different cell lines to colcemid or variation in activity between batches of colcemid. A suitable concentration of colcemid for use as a positive control in the muntjac spindle assay would be between 0.01 and 0.02µg/ml. Variation in the sensitivities of different cell lines to colcemid has been reported by Somers (1986). This investigator compared the results obtained with colcemid in the poteroo PK line with those from the primary cell lines CH1-L and HSBP and the established cell line DON and concluded that the PK line showed the greatest resistance to the effects of colcemid treatment. It may be possible that the muntjac fibroblasts are more sensitive to the effects of other mitotic poisons [in a way similar to colcemid] than other cell lines and may therefore be more suitable for use in the spindle staining assay. A possible reason for the increased sensitivity may be due to the very low chromosome number which allows good visualisation of the mitotic spindle and any defects which may otherwise be obscured by overcrowding with chromosomes.

When the scoring procedures are compared between assays more subtle differences can be discerned (Table 5.1 A). The number of cells scored per slide for estimation of the mitotic index remains fairly constant at 1000, and 3 or 4 replicates per dose are usually included. However the number of cells per replicate, and therefore the total number of cells per treatment, which are examined for division aberrations varies considerably between different lines and published methods (ie. between 100 and 1500 cells). In the muntjac assay each treatment was carried out in duplicate; however approximately 500 cells were examined per dose point which is the second highest sample size from amongst the systems investigated. This scoring procedure was considered adequate throughout this study since the historical control data indicated a low level of spindle dysfunction when the passage number was restricted (<8 passages). It is not possible to comment on the adequacy of the number of cells examined in the other systems without knowing the individual historical control data.

Table 5.1 B. illustrates the number of end points discussed in the various spindle staining assays. All contain the classification of normal and aberrant division stages as introduced by Parry et al in 1982. Apart from these results a variety of other effects are discussed depending on the size of the report, the compounds tested and the authors preferences. The relative merits of these end points will be discussed together with the results of the muntjac spindle assays (summarised in Table 5.2) and comparison with other published data.

Compound	Significant spindle damage	Chromosome dislocation from spindle	Chromosome bridging and lagging	Multipolar divisions	Poly. Mets.	Dec. AT/M	Inc. MI	ALTERED MORPHOLOGY OF: Spindle *	Chromosomes *	Cytoplasm *
Colcemid	+ [10ng]	I	–	–	–	+ [5ng]	+ [10ng]	+ [5ng]	–	–
Vinblastine	+ [1ng]	I	I	–	I	+ [2.5ng]	+ [10ng]	+ [2.5ng]	–	–
DES	+ [4µg]	+ [4µg]	–	–	–	+ [5µg]	I	+ [2µg]	–	+ [10µg]
Nocodazole	+ [5ng]	+ [10ng]	I	–	–	+ [5ng]	+ [20ng]	+ [5ng]	+ [20ng]	+ [20ng]
pFPA	–	–	I	–	I	+ [40µg]	–	+ [20ng]	–	–
Acenaphthene	–	–	–	–	–	I	–	–	–	–
Hydroquinone	+ [12µg]	I	I	–	–	+ [12µg]	–	+ [12µg]	+ [14µg]	–

*Observed effects only.

Table 5.2 Summary of compound induced effects seen in muntjac spindle assay.

+ positive
– negative
I inconclusive
[] lowest effective dose

Compound	Chromosome bridging and lagging	Multipolar divisions	Polyploidy	Hyperdiploidy	Endoreduplication *
Colcemid	+ [20ng]	+ [20ng]	-	I	I
pFPA	I	-	-	I	+

+ Positive
- Negative
I Inconclusive
[] Lowest effective dose

* Observed effect only

Table 5.3 Summary of compound induced effects seen in the muntjac recovery protocol.

The results for the recovery examination are further summarised in Table 5.3.

5.3 The results

5.3.1 The mitotic profile

The comparison of the normal division stages with the aberrant cells devoid of a spindle illustrates one of the ways in which a compound can disturb mitosis. The increase or decrease in individual division phases, when compared to control levels can indicate specific phase related effects. For example, Somers (1986) reported a significant decrease in prophase stages in poteroo (PK) cells after treatment with vincristine (another vinca-alkaloid, related to vinblastine). It was suggested that this may be due to vincristine affecting the cells during interphase and leading to cell death and lysis before division. The effect was not seen in the muntjac cells after treatment with vinblastine. This may be due to the differences in activity between the two compounds which, although structurally very similar, have been reported to have different anti-tumour properties (Degraeve, 1978) or to variation in the sensitivities of the two cell lines. Within the present study, the most obvious effects on the normal division stages occurred when the compound induced a metaphase block. A dose related increase in metaphases was seen after treatment with colcemid, nocodazole, pFPA and hydroquinone. For the compounds active over a narrow concentration range such as vinblastine and DES it was difficult to identify a dose response for metaphase blockage since the spindles were destroyed so rapidly.

Nocodazole, whilst being a potent spindle inhibitor was active over a larger dose range and therefore produced a 'typical' mitotic profile illustrating metaphase arrest. This was also observed with pFPA, however this was the only compound to induce mitotic arrest without an associated increase in chromosome loss and aberrant cells. This suggests that pFPA is not a spindle poison like colcemid or vinblastine, but induces its effects via an entirely different mechanism eg. by incorporation into division related proteins at G2 (Sisken and Wilkes, 1967). Metaphase arrest is usually associated with a decrease in anaphase and telophase cells. If this effect is not immediately obvious from the mitotic profile a dose response can usually be detected by examining the AT/M ratio. Each of the compounds tested, except acenaphthene, caused a reduction in this ratio. The AT/M ratio decreases from the level present within the vehicle control until at zero the metaphase block is total or all the normal metaphases are destroyed. The AT/M ratio proved to be a very useful tool for identifying mitotic arrest. The vehicle control data for this value showed some variation between experiments however this was not a problem providing that adequate concurrent control data was used .

An increase in the MI can also indicate mitotic arrest. It can be used as rapid test for such, however it must be remembered than an increase in the number of cells in mitosis may be due alternatively to a compound related stimulation in cell growth. This effect has been reported by Somers (1986) after treatment of poteroo cells with epidermal growth factor (EGF) which is also a tumour promoter.

A decrease in the MI is often taken as an indication of compound toxicity especially if dose related (Scott et al, 1983). Such effects were observed in this study after treatment with pFPA, acenaphthene and hydroquinone. When compounds are tested at toxic levels the results must be analysed very carefully since it has been reported (Parry et al, 1985) that spindle damage can also result from disruption to the normal protein metabolism and may not reflect a direct effect of the compound itself on the division apparatus. pFPA and acenaphthene did not induce significant spindle damage at doses where the mitotic index was reduced, however hydroquinone demonstrated both spindle damage and toxicity in the first experiment but spindle damage only in the repeat experiment. This suggests that hydroquinone may be a spindle poison in this cell line, but at near to or at toxic doses only. This confirms the results of Parmentier and Dustin (1953) who found that hydroquinone rapidly caused mitotic anomalies in intestinal cells in vivo but which were soon masked by pycnonecrotic nuclear distruction. A decrease in the MI may also be due to a compound preventing cells from entering mitosis. This should be considered when no other toxic effects are evident.

Spindle damage is evident when the proportion of normal cell division stages decreases in relation to an increase in abnormal (- spindle) cell types. The original classification of four different aberrant phenotypes (Parry, 1982) suggested different mechanisms or effects of spindle damage. However,

conclusions drawn in this study and reports from other workers eg. Somers (1986) and Parry (1988) tend to simplify the phenotypes into 2 catagories ie. potentially viable and non viable cell types. None of the cell types contain a spindle structure and it is therefore the condition of the chromosomes that is being examined. Since cells containing loose chromosomes and chromosome clusters have been seen to recover after removal of the compound, either by forming a new spindle and recommencing mitosis or by regressing back to an interphase state, it is thought that these phenotypes represent potentially viable cells. In contrast the cells containing a single chromatin mass or scattered chromatin are assumed to be non viable due to the severity of the damage to the normal chromosome morphology. For this reason, an increase in the latter two phenotypes alone can also be considered to be an indicator of toxicity and not necessarily spindle damage. Such a situation was found here with acenaphthene for which the increase in chromatin masses and scattered chromatin was matched by a reduction in mitotic index.

For the potent spindle poisons examined in the muntjac assay ie. colcemid, vinblastine, DES and nocodazole, the loose chromosome phenotype was always the first to appear and usually remained as the largest proportion of all the aberrant phenotypes throughout the dose range examined. As the concentration of compound increased so did the proportion of the other three aberrant phenotypes with chromosome clusters appearing next and chromatin masses and scattered chromatin

appearing last. The conclusions drawn from these observations were, that following spindle attack by a compound, the severity of the damage was indicated by the type of aberrant cells produced and this would influence the viability of the cells. However, on occasions non viable phenotypes could be induced by acute toxicity alone.

5.3.2 Dislocation of one or more chromosomes from an otherwise normal metaphase spindle (MDC)

This effect appeared to increase in frequency in several of the experiments but the effect was not always reproducible. The effect was usually seen at about the same time as the appearance of aberrant cells with the loose chromosome or chromosome clusters phenotype. For this reason it is considered to represent one of the early effects of a spindle damaging agent but may also be due to effects on the kinetochores or centromeres of individual chromosomes thereby loosening their attachment to the spindle fibres. The effect was seen with nocodazole but was most marked after treatment of the muntjac cells with DES. Since electron microscopy has revealed that DES can affect the kinetochores and induce unusual inter-kinetochore microtubule associations (Brinkley, 1985) it is possible that this compound may act by two different mechanisms, one affecting the spindle tubulin and one affecting the kinetochores/kinetochore tubulin.

An increase in MDC was seen in the second experiment with

vinblastine when the effective dose range had been reduced considerably. Vinblastine has not been reported to affect the kinetochores/centromeres of chromosomes, however similar results for the induction of MDC have been reported by Somers (1986) after the treatment with Vendoline, a monomeric vinca-alkaloid.

5.3.3 Chromosome lagging and bridging

Whole chromosomes lagging behind at anaphase will most certainly result in a chromosomal aneuploidy for one of the daughter cells. The chromosome may not have migrated to the spindle pole because of dislocation from the metaphase spindle or because of an error in the mechanism which moves the chromosomes to the spindle mid plane before anaphase separation. The latter is a mechanism suggested by Parmentier and Dustin (1953) to explain the effects of hydroquinone on mouse intestinal glands treated *in vivo*. Unless the chromosome is readily identifiable as being whole, it is possible that the laggard may be acentric and would therefore have no point of attachment to the mitotic spindle. An acentric chromosome probably arises following a clastogenic event which may or may not be associated with an aneugenic event. In this case partial, rather than whole chromosome aneuploidy may be induced in the daughter cells (colcemid, vinblastine and DES have all been found to be clastogenic in mammalian cells *in vivo* (Satya-Prakash *et al*, 1986 ; Shelby, 1988). Clastogenic events can also induce chromosome bridges at anaphase. The bridge is

formed by the breaking and rejoining of chromosomes or chromatids so that they contain more than one centromere which go separate ways at anaphase and form a chromatin bridge between the two. The bridge will eventually break and partial aneuploidy will probably be induced due to maldistribution of chromatin segments. Chromosome bridges can also be caused by excessive stickiness between the chromosomes resulting in malsegregation at anaphase. Hsu and Satya-Prakash (1985) have reported that the muntjac cell line is prone to the appearance of "pseudochromatin bridges" at anaphase. This is due to the ends of the sister chromatids of a no. 1 chromosome being temporarily held together by a remnant of a nucleolus thus creating the impression of a chromatin bridge (Appendix, Plate 25). This effect was not a problem in this assay providing that adequate concurrent control data was available.

The results for several of the compounds tested eg. vinblastine, nocodazole, hydroquinone and pFPA suggested that they may be inducing bridging or lagging. This effect has been reported for hydroquinone and pFPA by Parmentier and Dustin (1948) and Biesell and Jacquez (1954) respectively. The cell numbers examined here were too low for the results to be conclusive and probably this accounts for the apparently negative results obtained with DES, which has been reported to induce lagging and bridging in mammalian cells <u>in vitro</u> by other workers (Danford and Parry, 1982; Parry <u>et al</u>, 1982). When the results for DES were examined as a proportion of the

anaphase/telophase population only, small increases in bridging and lagging were seen. Recovery experiments may demonstrate an increase in anaphase/telophase lagging chromosomes or fragments following the recovery of previously blocked metaphases containing dislocated chromosomes.

An increase in chromosome lagging and bridging was seen with colcemid treatment but only after recovery. This was not surprising since colcemid has been reported to elevate the levels of chromosomal breakage (mainly the chromatid type) in recovering mouse bone marrow cells. (Satya-Prakash et al, 1986).

5.3.4 Multipolar divisions

These represent a potential source of aneuploidy. They are thought to arise following damage to the centrioles at replication or by malfunction of the spindle resulting in random chromosome dislocation and migration to a number (3-5) of poles (Lafi et al, 1986). This will result in more than two cells being produced at telophase resulting in maldistribution of the chromosomes.

The spontaneous frequency for multipolar divisions in the muntjac spindle assay was very low (<0.08% total cell population) and none of the compounds tested with the standard protocol appeared to induce an increase in their frequency and

so the reported multipolar induction by vinblastine (Degraeve, 1978) in mammalian cells in vitro could not be confirmed. However after recovery from colcemid treatment a small increase in multipolar division was seen (0.45%). This confirms the reported effects of colcemid by Hsu et al (1983) and Hsu and Satya—Prakash (1985) who also noted increases in multipolar divisions in recovery cell populations. This effect is not unexpected since colcemid is known to affect centriole separation and migration (Dustin, 1984).

5.3.5 Polyploid metaphases

These are cells in which the chromosome complement has at least doubled. Polyploidy can occur following prolonged arrest when the cells either regress to an interphase state or proceed to cytokinesis without separation and migration of the duplicated chromosomes. These situations will probably result in either cells with giant nuclei or multinucleation. Large polyploid metaphases may then be seen at the next mitotic division. Since the diploid number of chromosomes for the muntjac cell line was only 7 it was often possible to detect a doubling in the chromosome number in the spindle assay especially after metaphase arrest. However none of the compounds tested with the standard protocol induced reproducible, conclusive polyploid effects. This is probably due to the cell population examined being inappropriate for this type of investigation. The failure of the recovery spindle assay to detect any

increase in polyploidy after colcemid treatment was disappointing since this is a recognised effect of this compound (Sawada and Ishidate, 1978). This failure suggests that the recovery protocol may require further refinement if the induction of polyploidy is to be examined eg. longer recovery times.

5.3.6 Observed effects

One of the most frequent effects induced by the compounds tested in the muntjac spindle assay and previously reported as an effect of DES in mammalian cells in vitro (Parry et al, 1982, and Tucker and Barrett, 1986) related to the apparent size of the spindle. Small darkly staining spindles were seen after treatment with colcemid, vinblastine, DES and nocodazole and monopolar-like metaphases were induced in a dose related manner with pFPA.

Large and irregular spindles were also seen especially after treatment with colcemid, DES and hydroquinone which appeared similar to those reported by Tucker and Barrett (1986) induced by DES. The interpretation of such effects is difficult since it is known that the spindle has a differing sensitivities to Ca^{2+} and Mg^{2+} (Wissinger et al, 1981). These chemicals are added to the fixative solution in specific concentrations to prevent artifactual shrinkage or elongation of the spindle. Therefore careful comparisons must be made between the treated

cells and the concurrent vehicle control cells before alteration to spindle size can be regarded as a compound related effect. The alteration to spindle size often appeared at doses below those which induced significant spindle distruction as indicated by an increase in the loose chromosome or chromosome cluster phenotypes. Since this occurred with both colcemid and DES, which are known potent spindle inhibitors, this observation may serve as an early warning of potential spindle poison effects. The reproducible dose related monopolar effect seen after treatment with pFPA may represent either extreme spindle shrinkage or effects to the centrioles preventing migration and resulting in a single pole surrounded by the chromosomes. Since the effect was seen to be reversible and the recovering cell populations did not appear to suffer from an increased frequency of multipolar divisions [one of the possible effects resulting from the recovery of monopolar metaphases (Lafi et al, 1986)] it is thought that the former mechanism is the most likely.

The loss of normal spindle shape induced by hydroquinone may represent a selective effect on the growth of individual tubulin fibres. It is possible that the abnormal crescent shaped spindles were formed by spindle fibres being shorter on

one side of the structure and longer on the other causing the apparatus to curve between the poles. This theory requires further investigation and consideration.

Extreme chromosome condensation is a recognised effect of prolonged metaphase arrest, however it was only noticeable in the muntjac spindle assay after treatment with nocodazole and hydroquinone. Since metaphase arrest was induced by all of the compounds tested except for acenaphthene it is possible that nocodazole and hydroquinone have targets (other than tubulin) within the DNA of the chromosomes. Hydroquinone has been reported as inducing short, thick chromosomes in mammalian cells <u>in vivo</u> (Parmentier and Dustin, 1948).

The morphology of the cytoplasm was affected by both DES and nocodazole resulting in loss of cell shape. Cytoplasmic budding producing gourd shaped cells induced by DES has also been reported by Sawada and Ishidate (1978). This suggests that the compound can destroy both cytoskeletal and spindle tubulin.

Abnormal cytoplasmic cleavage was seen occasionally in the spindle assay after treatment with vinblastine. The aberrations included cytokinesis without chromosome separation and migration and cytokinesis without decondensation of the

metaphase chromosomes. These cell types were not seen after DES treatment although this is a reported effect for this compound in cultured human fibroblasts (Danford and Parry, 1982). Similar effects have also been reported by Lafi et al (1986) after treatment of Chinese hamster (DON) cells with Michler's ketone and Somers (1986) after treatment of poteroo kidney (PK) cells with vincristine.

5.3.7 Metaphase analysis

Metaphase analysis of the recovery cell populations following treatment with colcemid or pFPA revealed no evidence for polyploidy induction and inconclusive results for hyperdiploidy. pFPA, although reported to induce metaphase arrest (Biesele and Jacquez, 1954) is usually negative for aneuploidy induction in mammalian test systems, however it is reported as a potent aneugen in fungal systems (Bond and Chandley, 1983). Therefore a negative result with muntjac cells was not unexpected, however a more conclusive indication of aneugenic activity was anticipated for colcemid treated cells. Colcemid-induced aneuploidy and polyploidy in recovering cells has been reported by Tsutsui et al (1984); Satya-Prakash et al (1984) and Hsu and Satya-Prakash (1985) using Syrian hamster embryo cells and Chinese hamster cells in culture. The negative/inconclusive results obtained in this experiment are probably due to inadequate study design and the limited number of cells examined in both the vehicle control and treated groups. Since it appears that at least some of the

aneuploid cells can exist within the population it is hoped that the repetition of similar experiments involving more dose points and treatment times may provide more conclusive results.

A most interesting observation to come out of the metaphase analysis was the suggestion of induction of endoreduplication by pFPA and possibly by colcemid. Endoreduplication occurs when the chromosomes replicate at least twice between mitoses instead of once as in normal mitosis, and this results in a form of polyploidization. Endoreduplication leads at first to exact karyotype doubling. If there is no discord between chromosome quadruplication and centriole duplication in the endoreduplication cell cycle, such polyploids resume normal mitosis and usually reproduce more slowly than diploids. However, this can be followed readily by mitotic nondisjunction and consequent selection of better-adapted hypotetraploid variant cells (Sutou and Tokuyama, 1974). Endoreduplication probably occurs occasionally in all tissues and has been observed in tumour tissues (Therman, 1986). Aneuploid stem lines derived from endoreduplication are often more malignant than the original stem line they have outgrown (Sutou and Tokuyama, 1974).

The induction of endoreduplication by pFPA may be the initiating event for aneuploid cell production in mammalian cells and requires further investigation.

The results demonstrate that a variety of effects can be detected using the muntjac spindle assay and recovery protocol. It is possible that the different effects reflect different mechanisms of action for the chemicals examined. The most obvious target is the mitotic spindle and although damage to other mitotic elements can be indicated, the information gained, regarding mechanism(s) of action is limited.

Thus the muntjac spindle assay has been shown to be capable of detecting chemically induced effects to the mitotic spindle resulting in malformation or malfunction. It cannot provide definite answers but the results provide valuable insight into the possible mechanisms of action and by comparison with known aneugens can indicate the aneugenic potential of an unknown compound.

The recovery protocol using muntjac cells has the advantage of simplicity of metaphase analysis since hyperdiploidy is readily recognisable. However it must be remembered that the loss or gain of a muntjac chromosome represents a very sizable proportion of the genome. As such it is a potentially more damaging event than in a cell line eg. human where the variation in chromosome number may be better tolerated. This may account for the low number of aneuploid cells observed after treatment with the aneugen colcemid and suggests that the use of an alternative cell line should be considered in the future.

6. CONCLUSIONS

The muntjac cells compare favourably with other cell lines used in spindle staining assays and have the advantages of being a relatively stable diploid cell line with a low number of very large chromosomes. The cytotoxicity and recovery protocols require further refinement and development to increase sensitivity and accuracy of the results. The muntjac spindle assay provides a great deal of information but this is not always interpretable and therefore the number and complexicity of the end points should be carefully considered when specific effects are being analysed. However, the results obtained with the known aneugens colcemid, vinblastine and DES compared well with other published data and gave insight to the possible mechanisms of action and aneugenic potential of the other compounds tested. Nocodazole was shown to be a potent spindle inhibitor similar to colcemid in action and effect and therefore a potential aneugen. Hydroquinone induced mitotic arrest and significant spindle destruction but, unlike colcemid, this effect was associated with general toxicity. pFPA induced mitotic arrest but no significant spindle damage and the recovery results suggested that it may have an aneugenic potential via the induction of endoreduplication or polyploidy. Acenaphthene appeared to have no effect on mitosis up to toxic levels, however this compound is a polycyclic aromatic hydrocarbon (PAH) and the majority of chemicals of this class are indirectly acting agents. This means that they

require metabolic activation with mixed-function oxygenases in order to form the genetically active molecule. Mixed function oxygenases are located mainly on the surface of cytoplasmic membranes and active preparations (ie. an S9 mix) are easily made by homogenising tissues rich in these enzymes (eg. the liver). At the present time activation systems such as S9-mix have not been used in in vitro assays for spindle poisons and therefore compounds such as acenaphthene may go undetected. Therefore the incorporation of an external metabolising system into the muntjac spindle assay and additional validation is regarded as essential future work.

With the growing realization of the impact of aneuploidy on human health, demand for test systems such as the muntjac spindle assay is increasing. The recent proposed revisions of the guidelines for mutagenicity testing included investigations for aneuploidy and it is anticipated that this may soon become a regulatory requirement in certain cases. It is hoped that such action may prevent the occurrence of another genetic disaster similar to that induced by DES and generally decrease the number of aneugenic agents in the environment.

In conclusion, the muntjac spindle assay can be used as a relatively quick and sensitive primary screening assay for potential aneugens especially those which act via the mitotic spindle. The test system, following refinement, should be able to fill the gap not covered by existing mutation and clastogen assays. However further validation is required and confirmation of positive results in short term in vivo tests such as the micronucleus assay (Schmid, 1975) will be necessary.

ACKNOWLEDGEMENTS

My thanks go to my supervisors Dr D Gatehouse and Dr B Harold for their help, encouragement and advice during and in the completion of my research. To Dr DJ Tweats for his reading and helpful comments on the manuscript, to Miss G Amphlett for the statistical analysis of the results and to the staff of the photographic department for the development and printing of the plates contained herewithin.

I would also like to express my gratitude to many other friends and colleagues who have listened, advised and kept me going throughout the course of this study. My special thanks go to Miss K Varley for her patience and skill in typing this manuscript and to Glaxo Group Research Ltd. for allowing me to embark on this research.

APPENDIX

Plate Index

Plate number	Subject	Chemical treatment
1	Normal prophase (P)	-
2	Normal metaphase (M)	-
3	Normal anaphase (A) early/late	-
4	Normal telophase (T)	-
5	Loose chromosomes (LC)	Vinblastine 30ng/ml
6	Chromosome clumps (CC)	Vinblastine 30ng/ml
7	Chromatin mass (CM) and scattered chromatin (SC)	Vinblastine 30ng/ml
8	Chromosome dislocation from metaphase spindle (MDC)	DES 8µg/ml
9	Lagging chromosomes and bridging	pFPA 10µg/ml
10	Multipolar metaphase	Colchicine 20ng/ml
11	Polyploid metaphase (Poly. met) and normal metaphase	Hydroquinone 12µg/ml
12	Multipolar telophase	Colchicine 20ng/ml, 48 hour recovery

Plate number	Subject	Chemical treatment
13	Aneuploid metaphase chromsomes	Colchicine 20ng/ml, 48 hour recovery
14	Aberrant metaphase chromosomes	Colchicine 20ng/ml, 48 hour recovery
15	Star metaphase	Vinblastine 10ng/ml
16	Irregular spindle	Vinblastine 5ng/ml
17	Cytokinesis without chromosome decondensation	Vinblastine 30ng/ml
18	Cytokinesis without chromosome separation	Vinblastine 1ng/ml
19	Loss of cytoplasmic struture	DES 10µg/ml
20	Extreme chromosome condensation	Nocodazole 20ng/ml
21	Sticky chromosomes	Nocodazole 20ng/ml
22	Monopolar metaphase	pFPA 50µg/ml
23	Endoreduplication	pFPA 50µg/ml
24	Crescent spindle	Hydroquinone 16µg/ml
25	Pseudo chromosome bridge	Nocodazole 10ng/ml

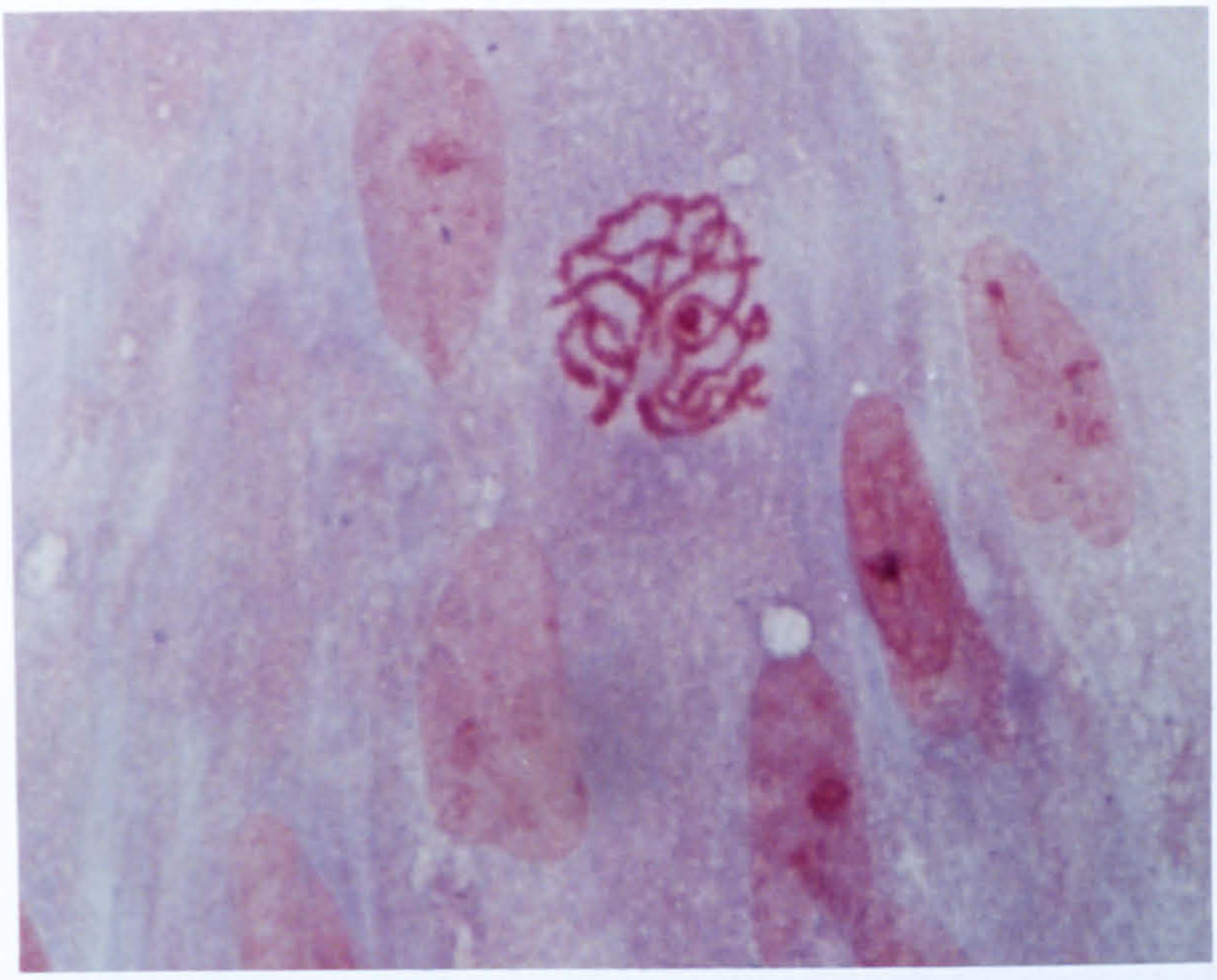


Plate 1 Normal prophase (P)

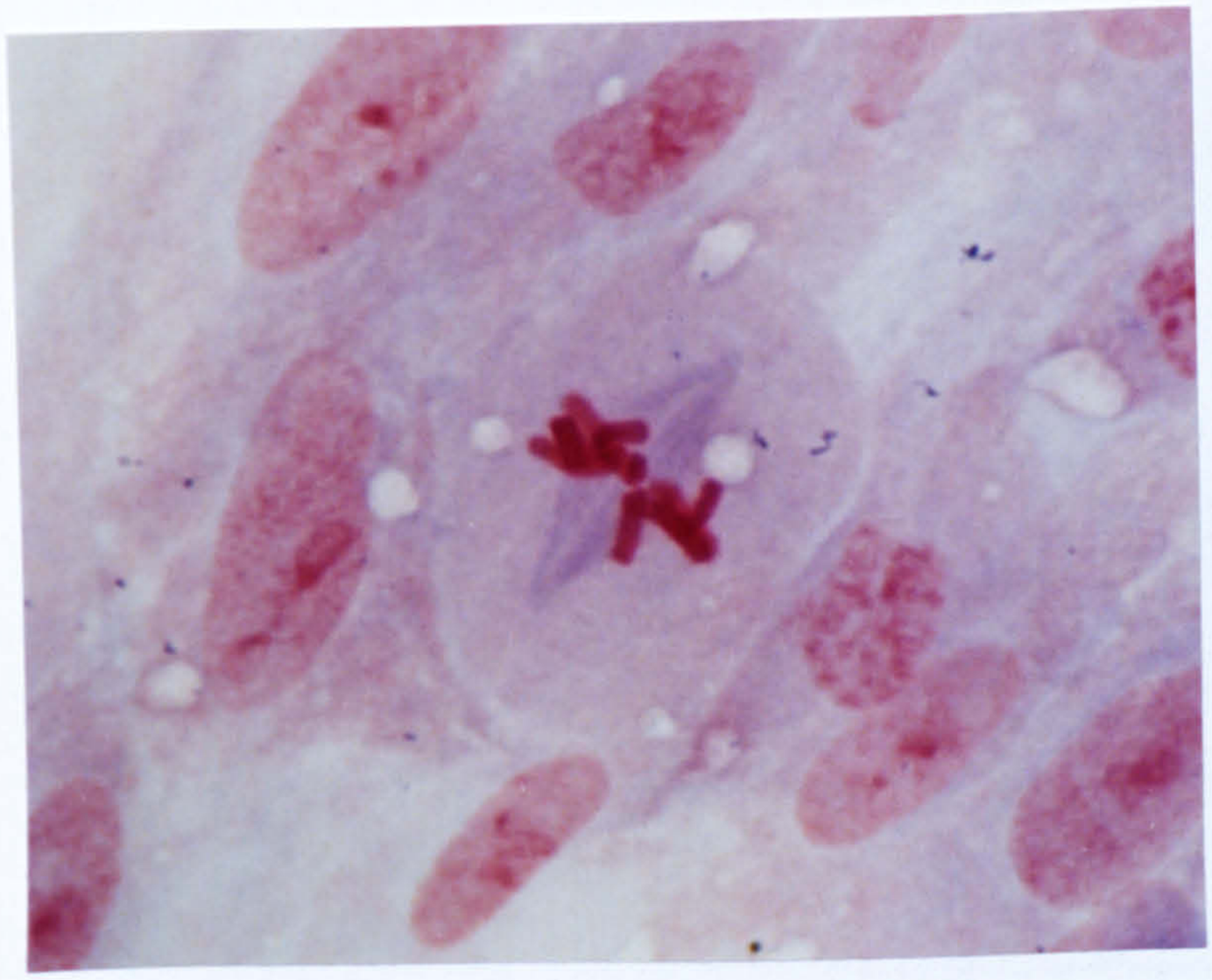


Plate 2 Normal metaphase (M)

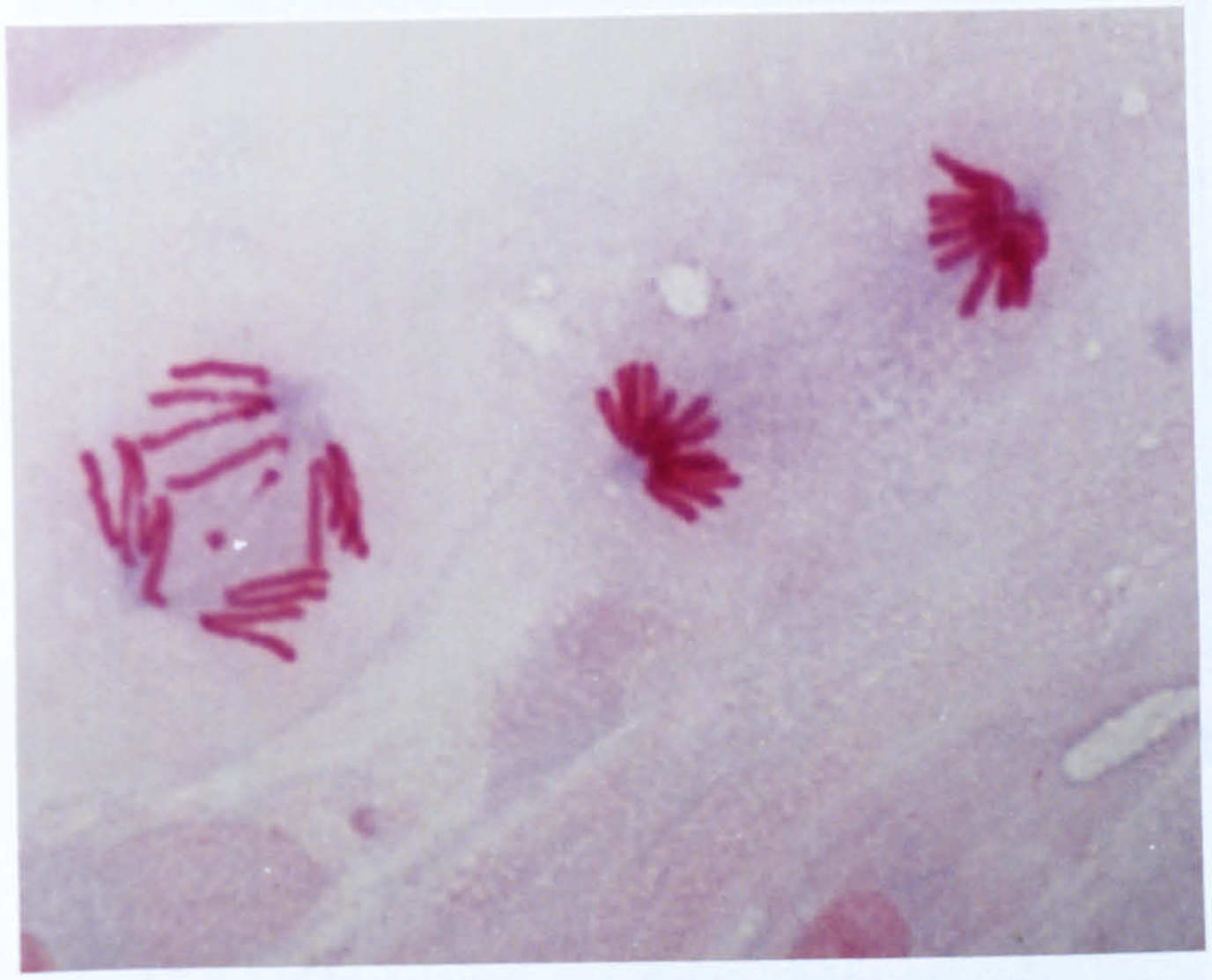


Plate 3 Normal anaphase (A) early/late

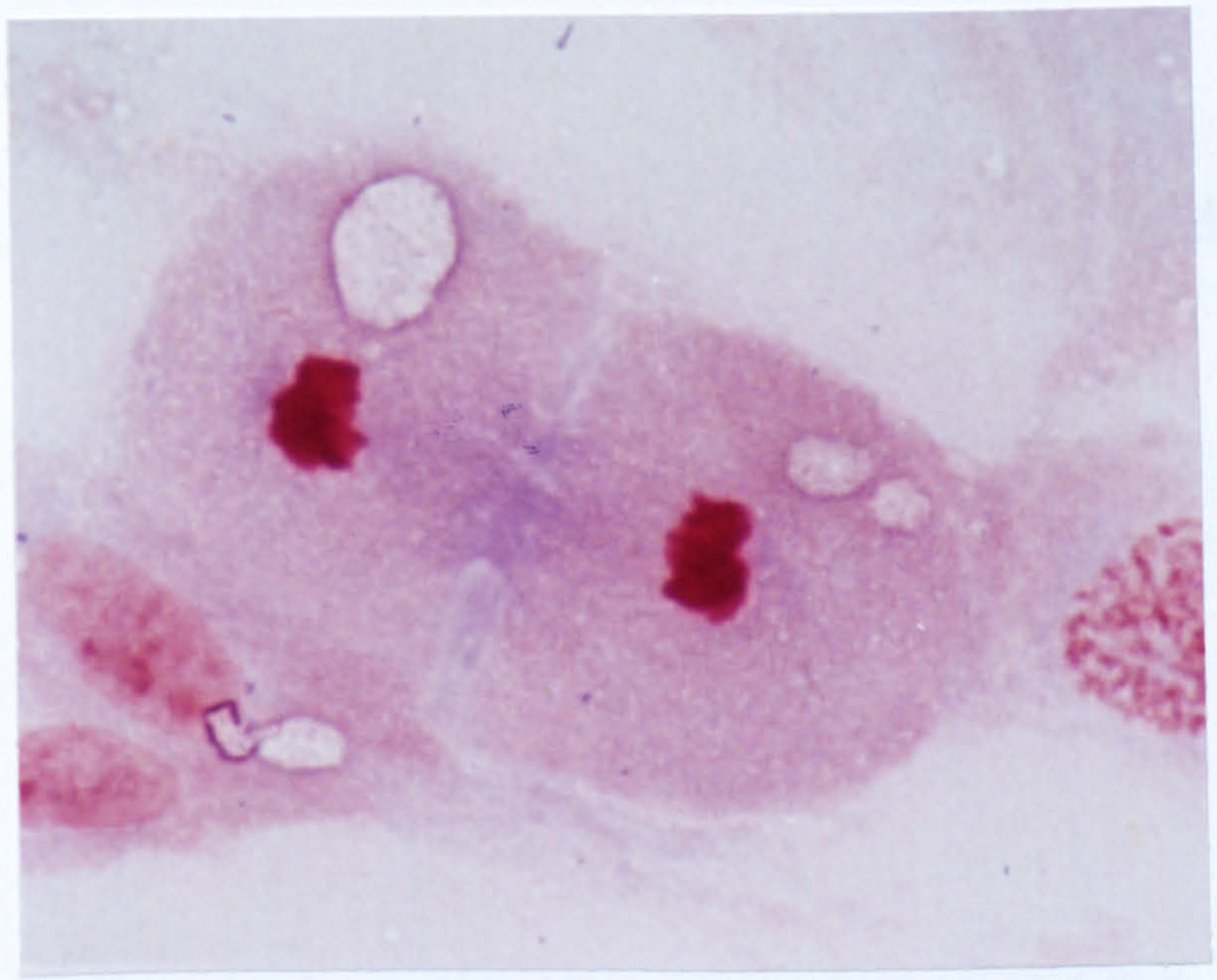


Plate 4 Normal telophase (T)

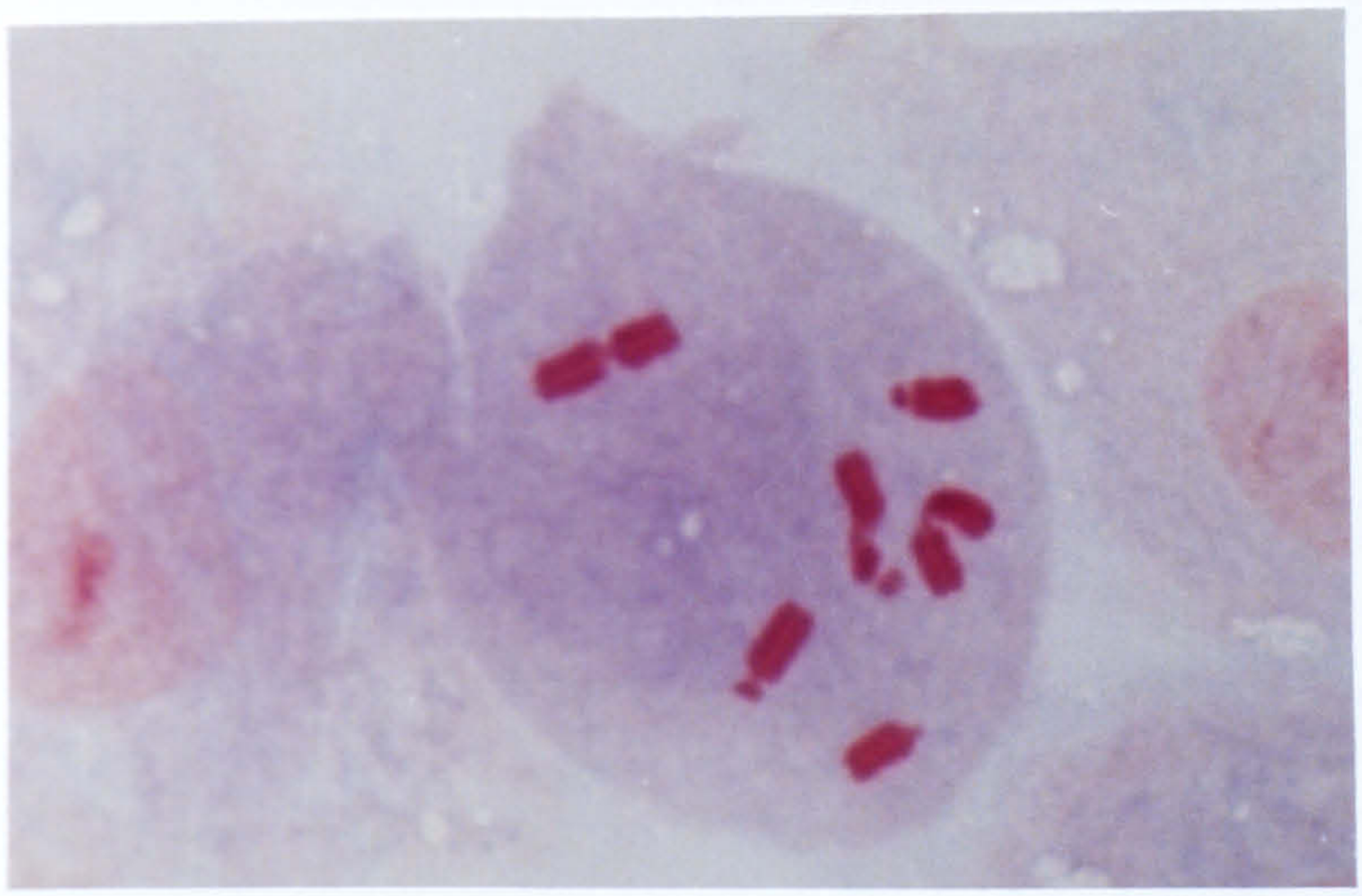


Plate 5 Loose chromosomes (LC) (Vinblastine 30ng/ml)

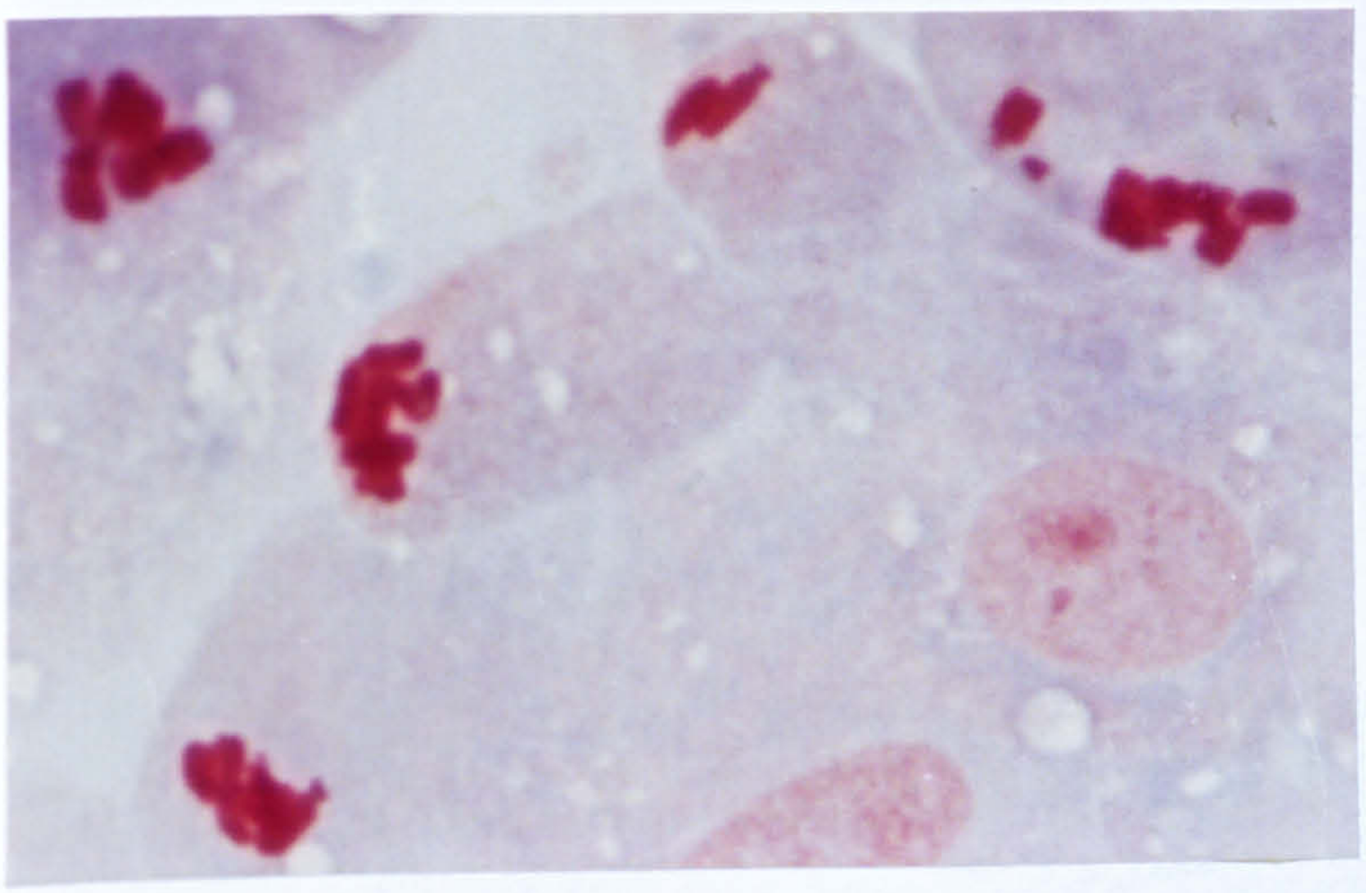


Plate 6 Chromosome clumps (CC) (Vinblastine 30ng/ml)

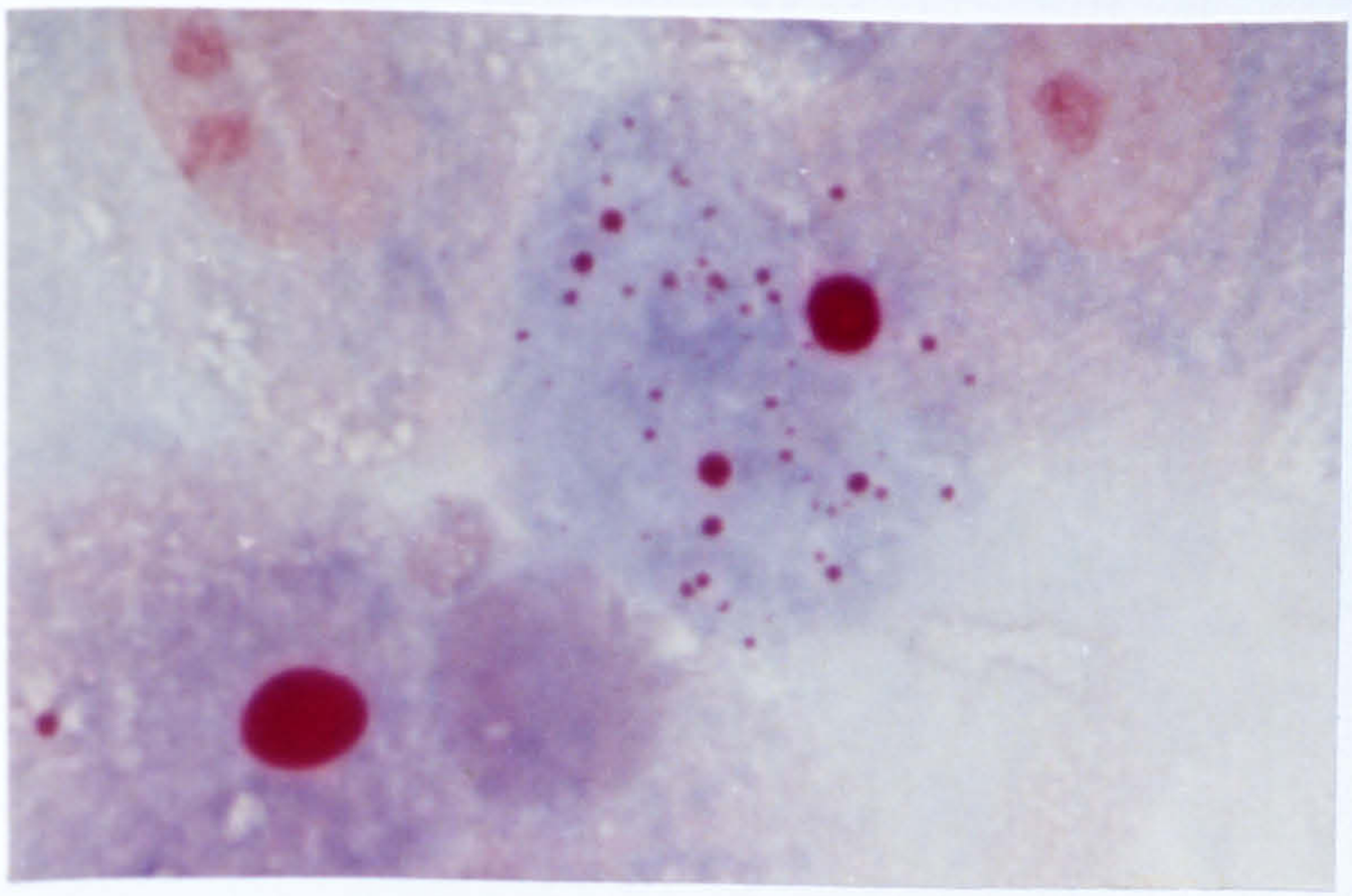


Plate 7 Chromatin mass (CM) and scattered chromatin (SC) (Vinblastine 30ng/ml)

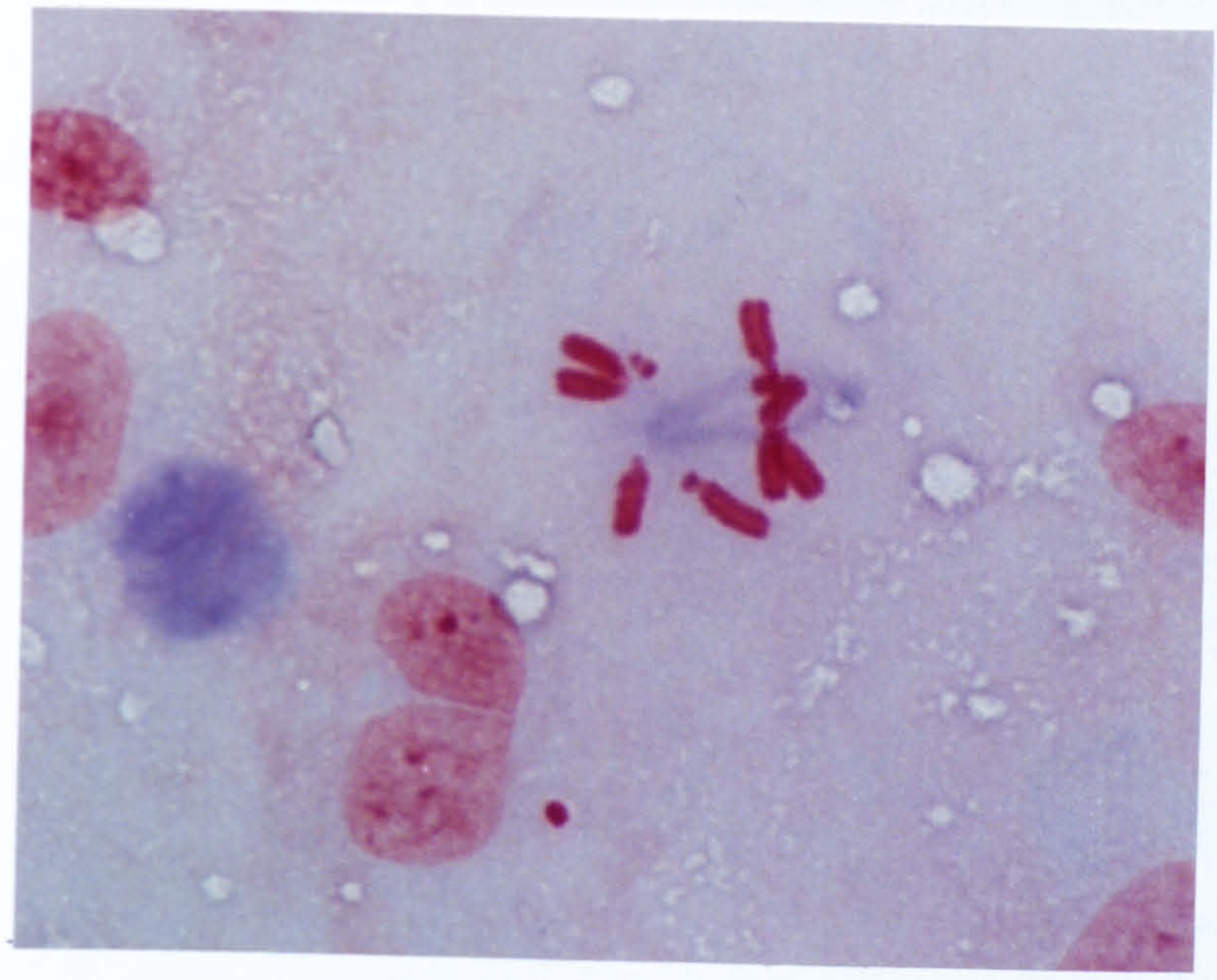


Plate 8 Chromosome dislocation from metaphase spindle (MDC) (DES 8µg/ml)

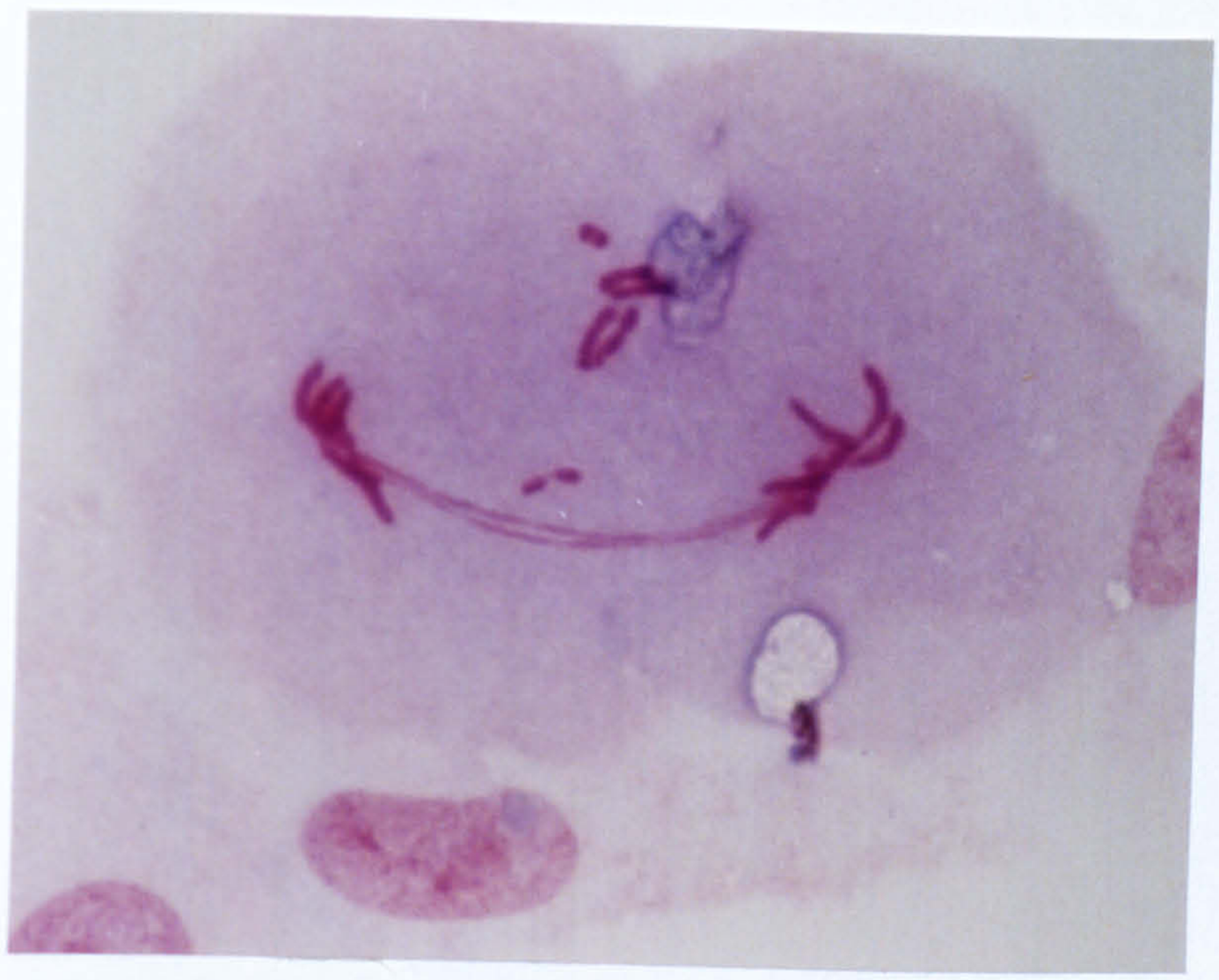


Plate 9 Lagging chromosomes and bridging (pFPA 10µg/ml)

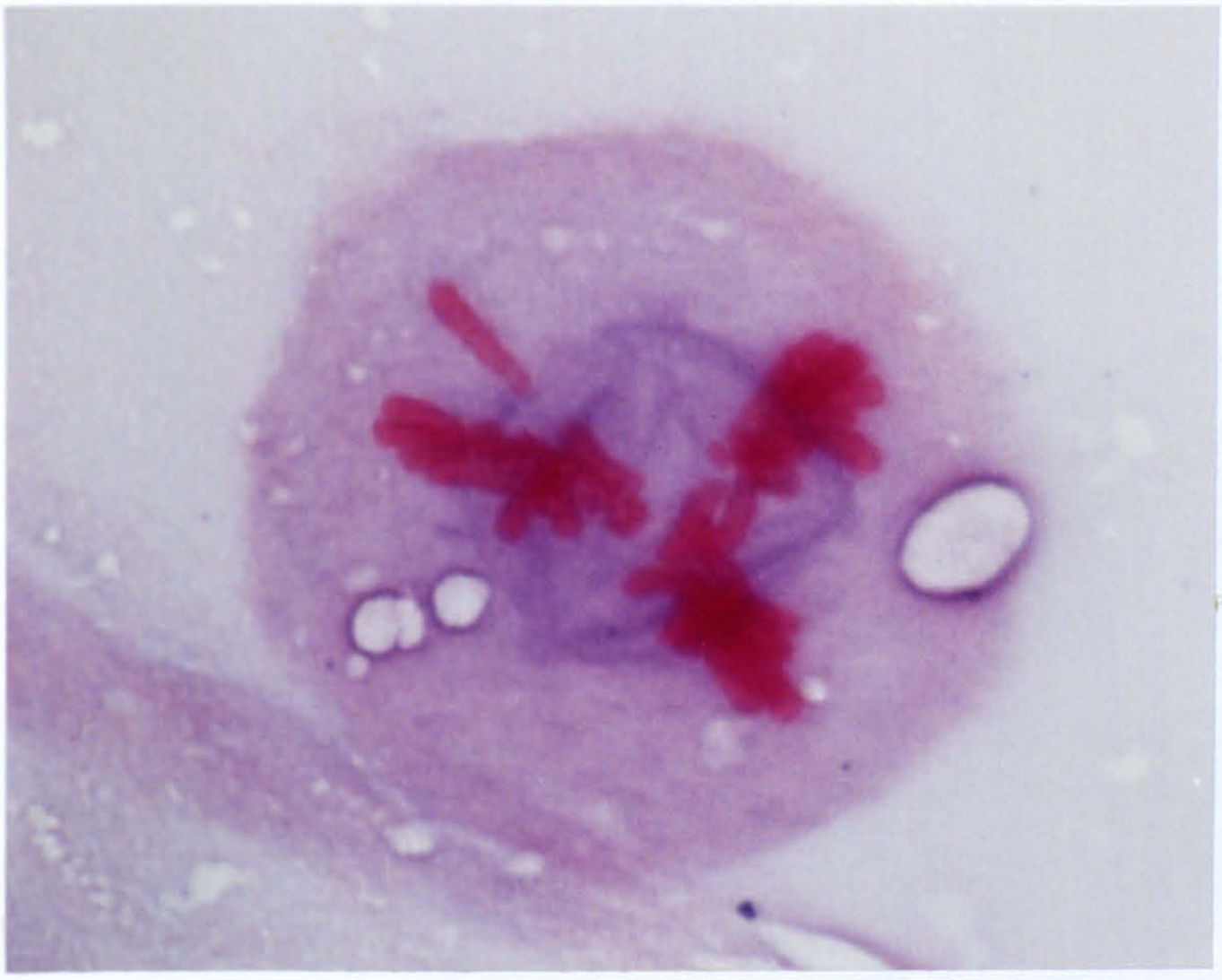


Plate 10 Multipolar metaphase (Colchicine 20ng/ml)

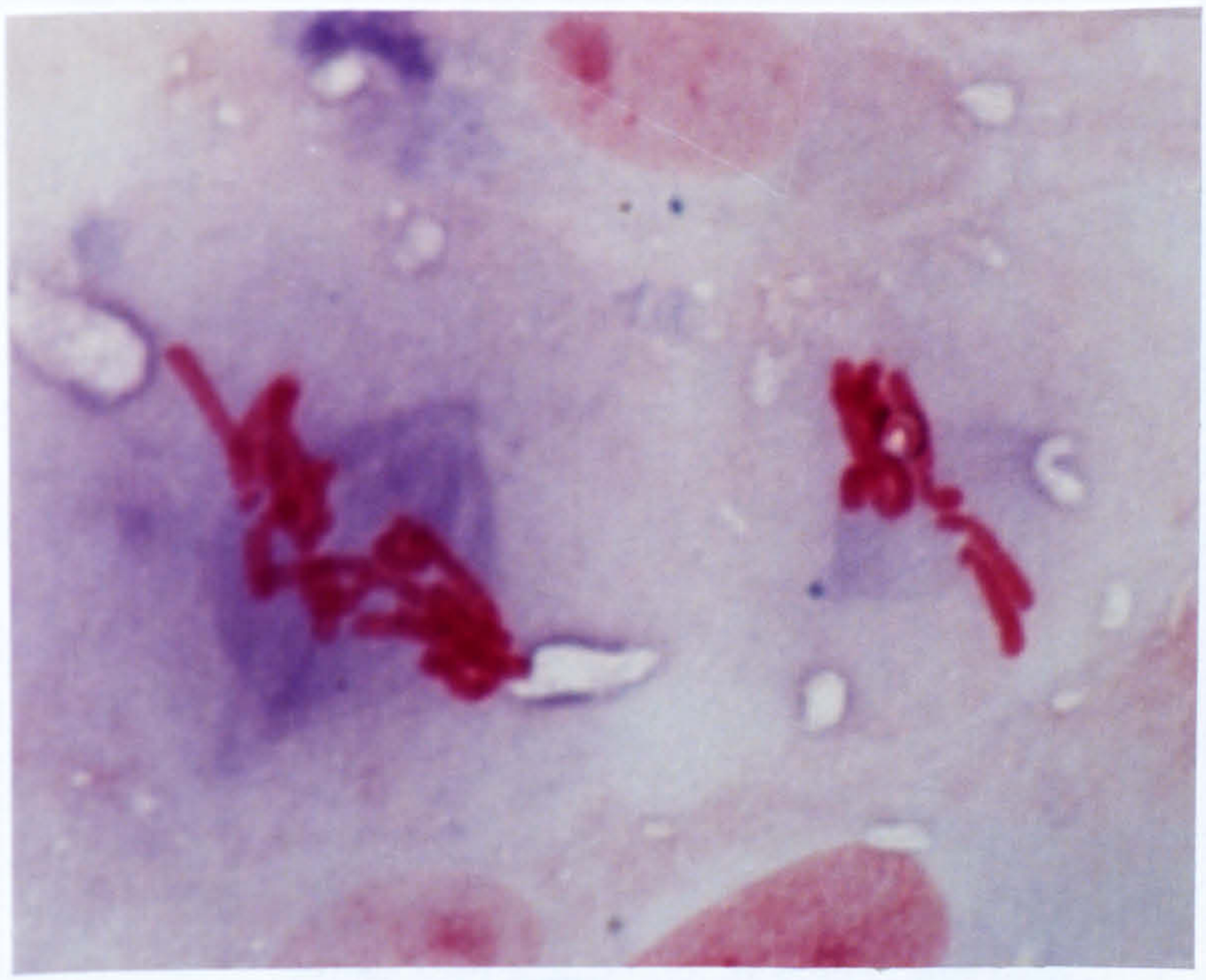


Plate 11 Polyploid metaphase (Poly. met)
and normal metaphase (Hydroquinone 12µg/ml)

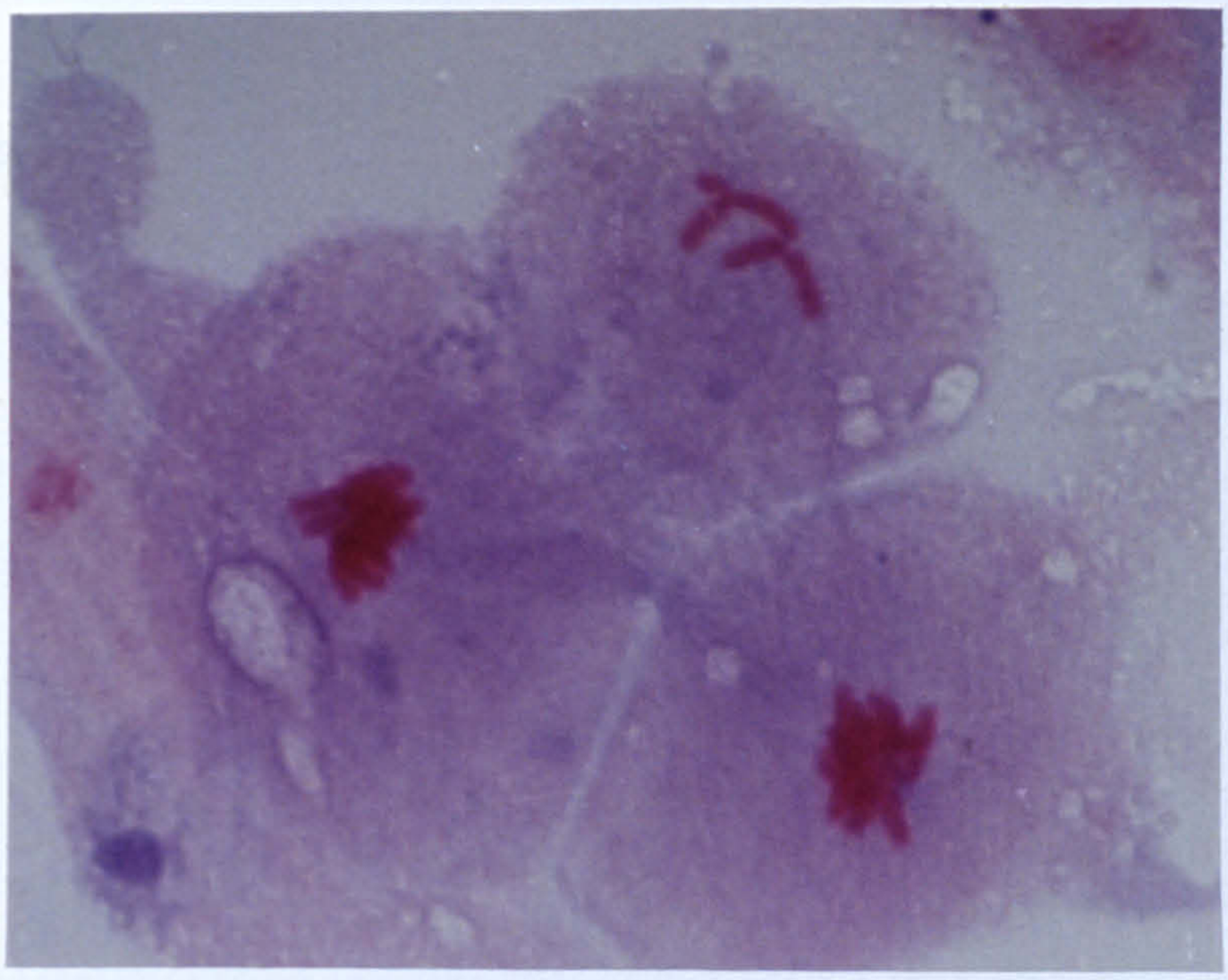


Plate 12 Multipolar telophase (Colchicine 20ng/ml, 48 hour recovery)

Plate 13 Aneuploid metaphase chromsomes (Colchicine 20ng/ml, 48 hour recovery)

Plate 14 Aberrant metaphase chromosomes (Colchicine 20ng/ml, 48 hour recovery)

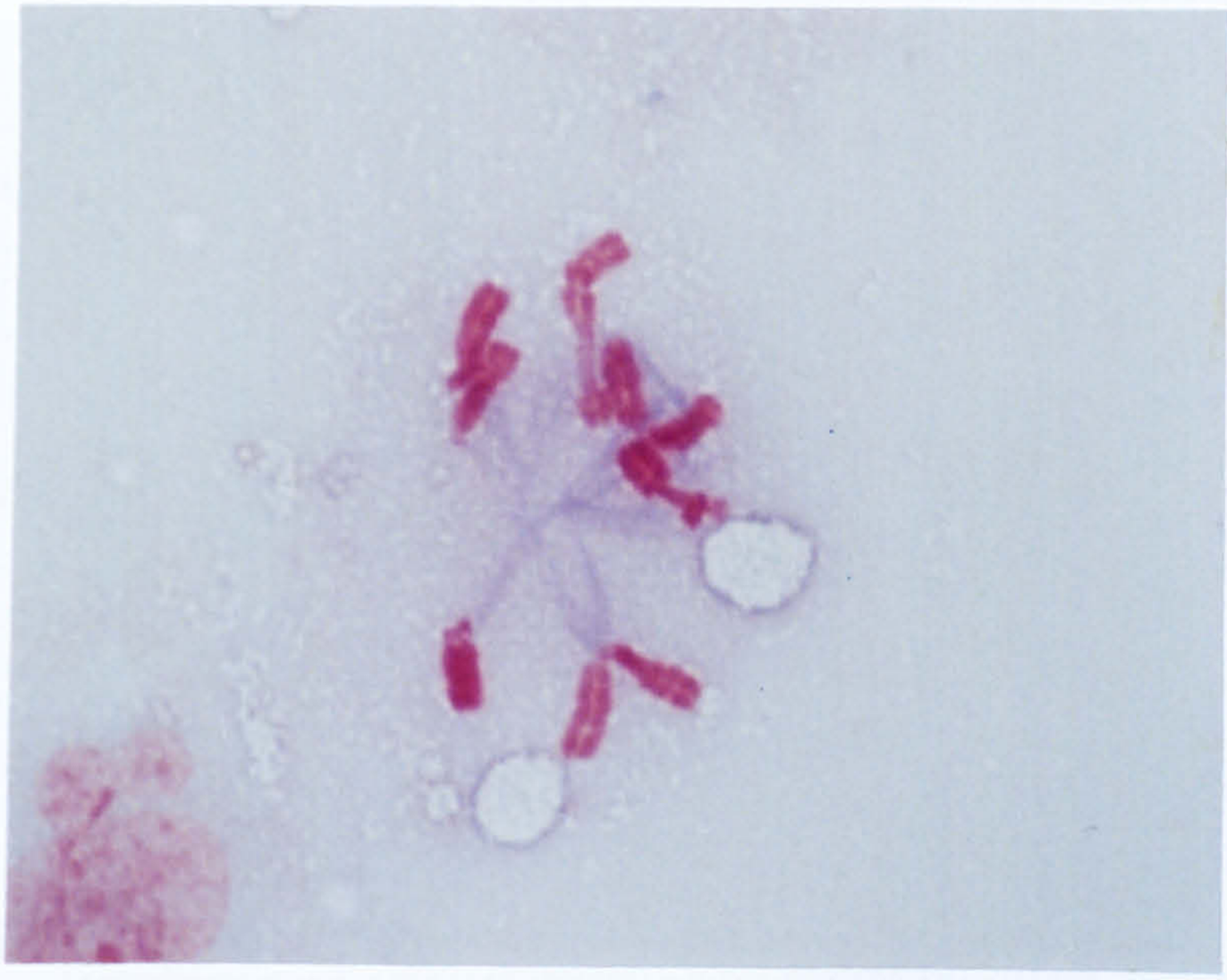


Plate 15 Star metaphase (Vinblastine 10ng/ml)

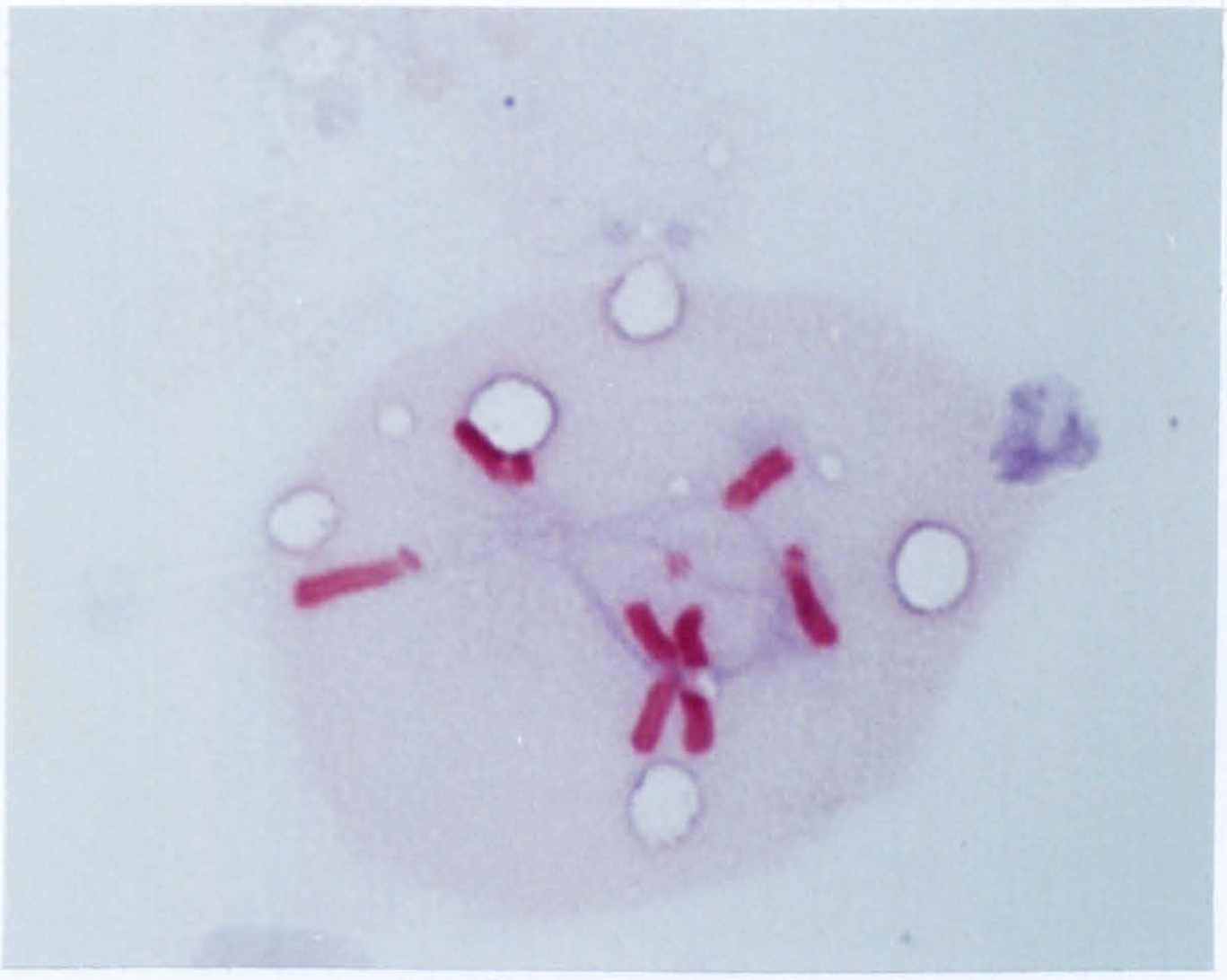


Plate 16 Irregular spindle (Vinblastine 5ng/ml)

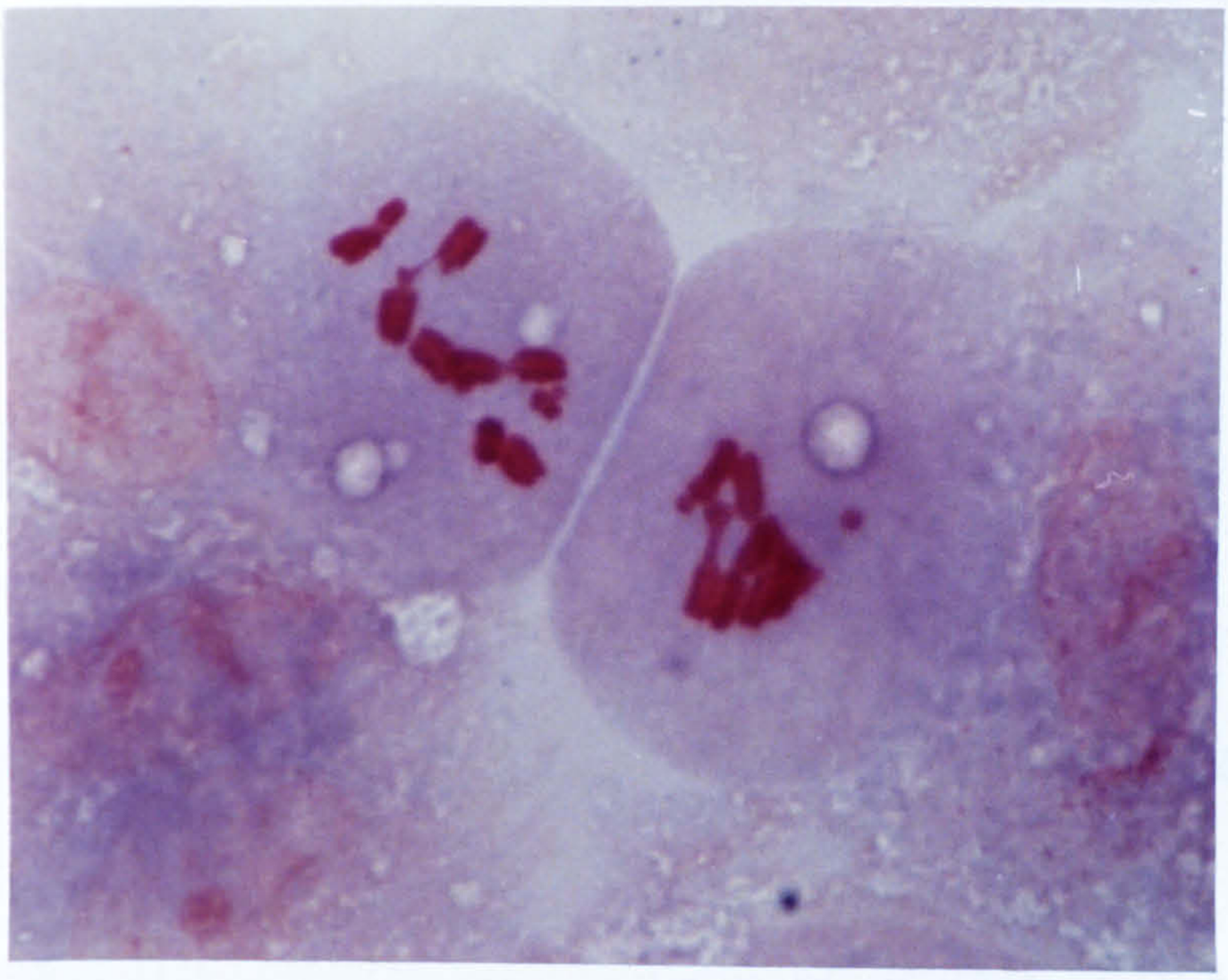


Plate 17 Cytokinesis without chromosome decondensation (Vinblastine 30ng/ml)

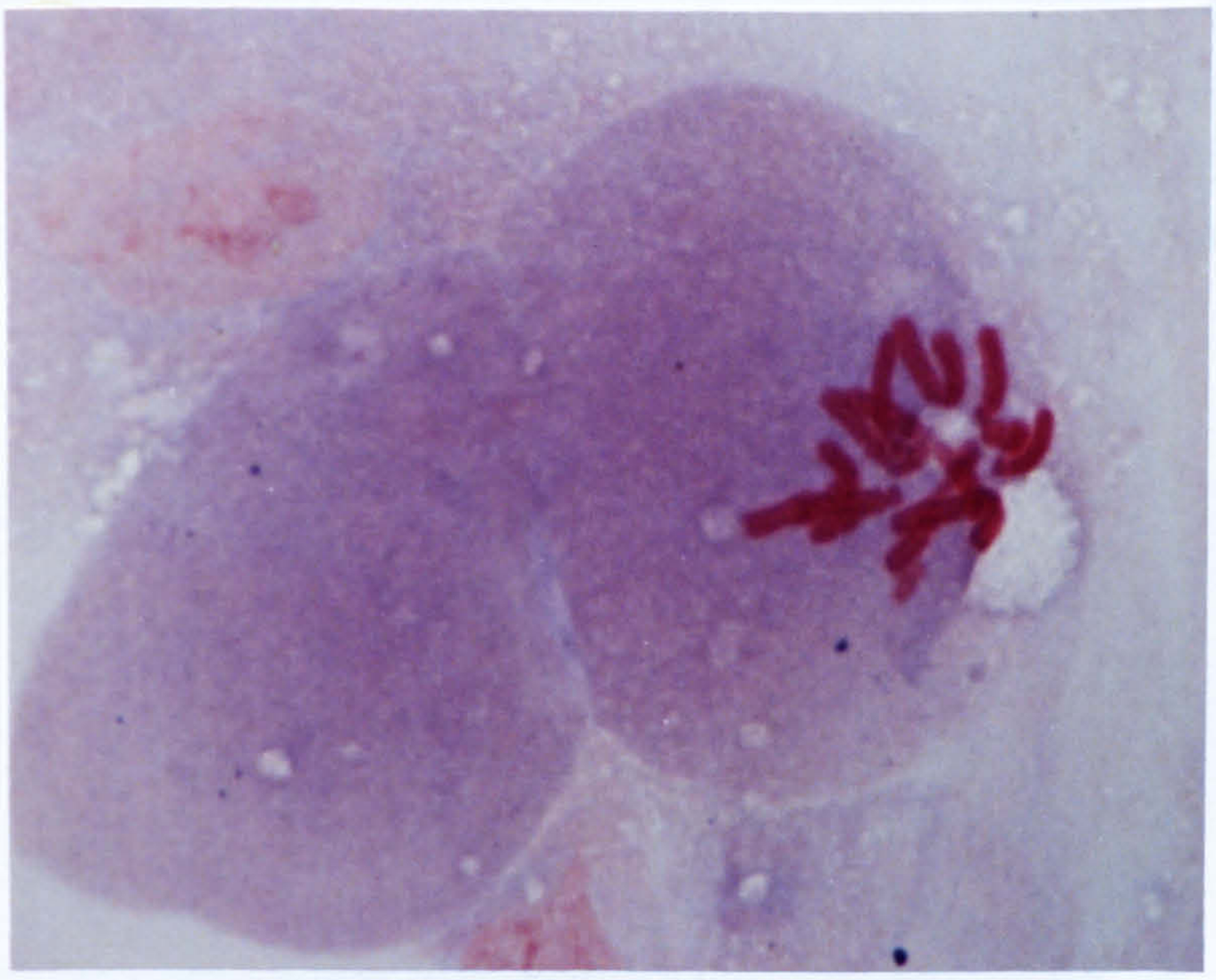


Plate 18 Cytokinesis without chromosome separation (Vinblastine 1ng/ml)

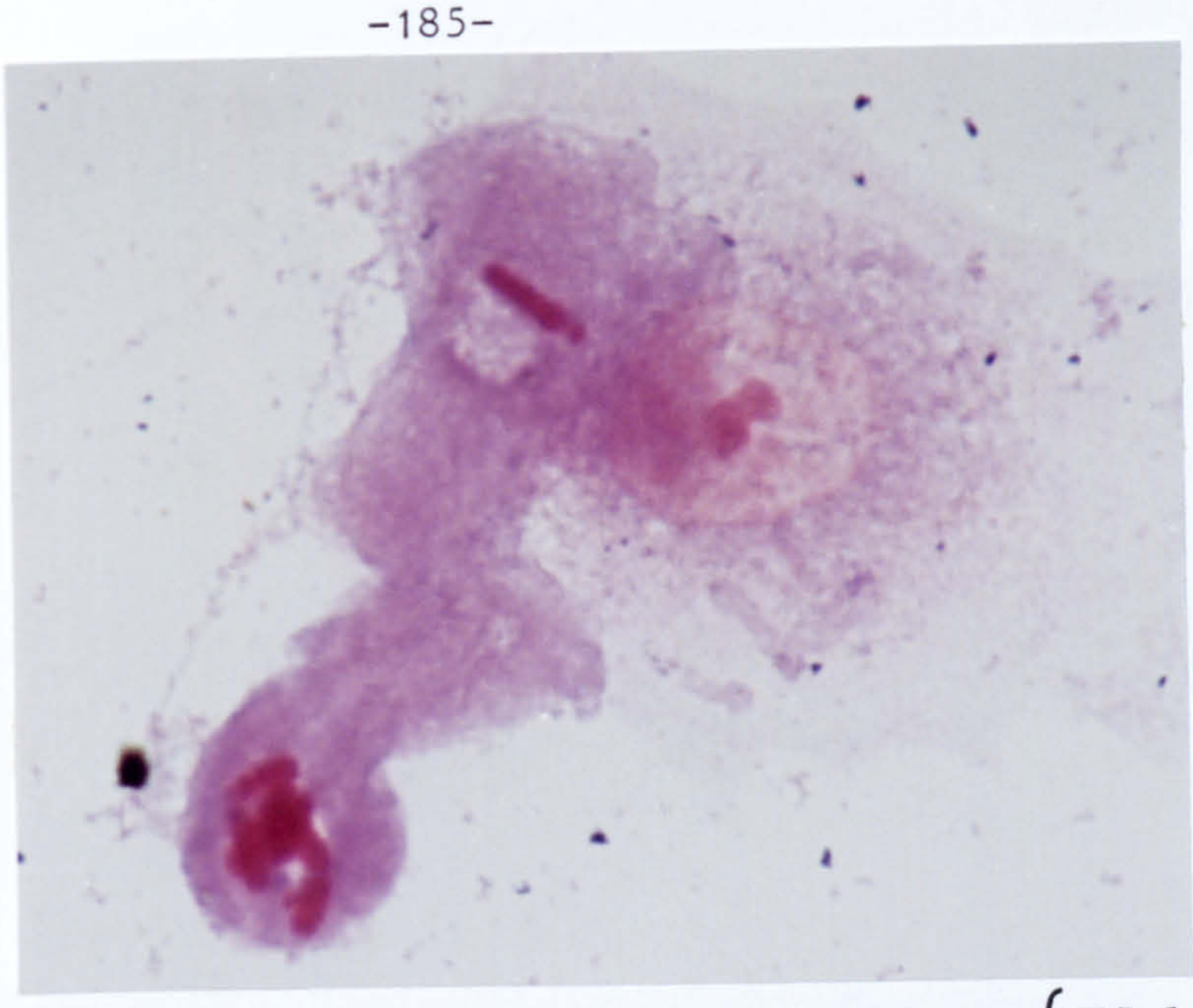


Plate 19 Loss of cytoplasmic struture (DES 10μg/ml)

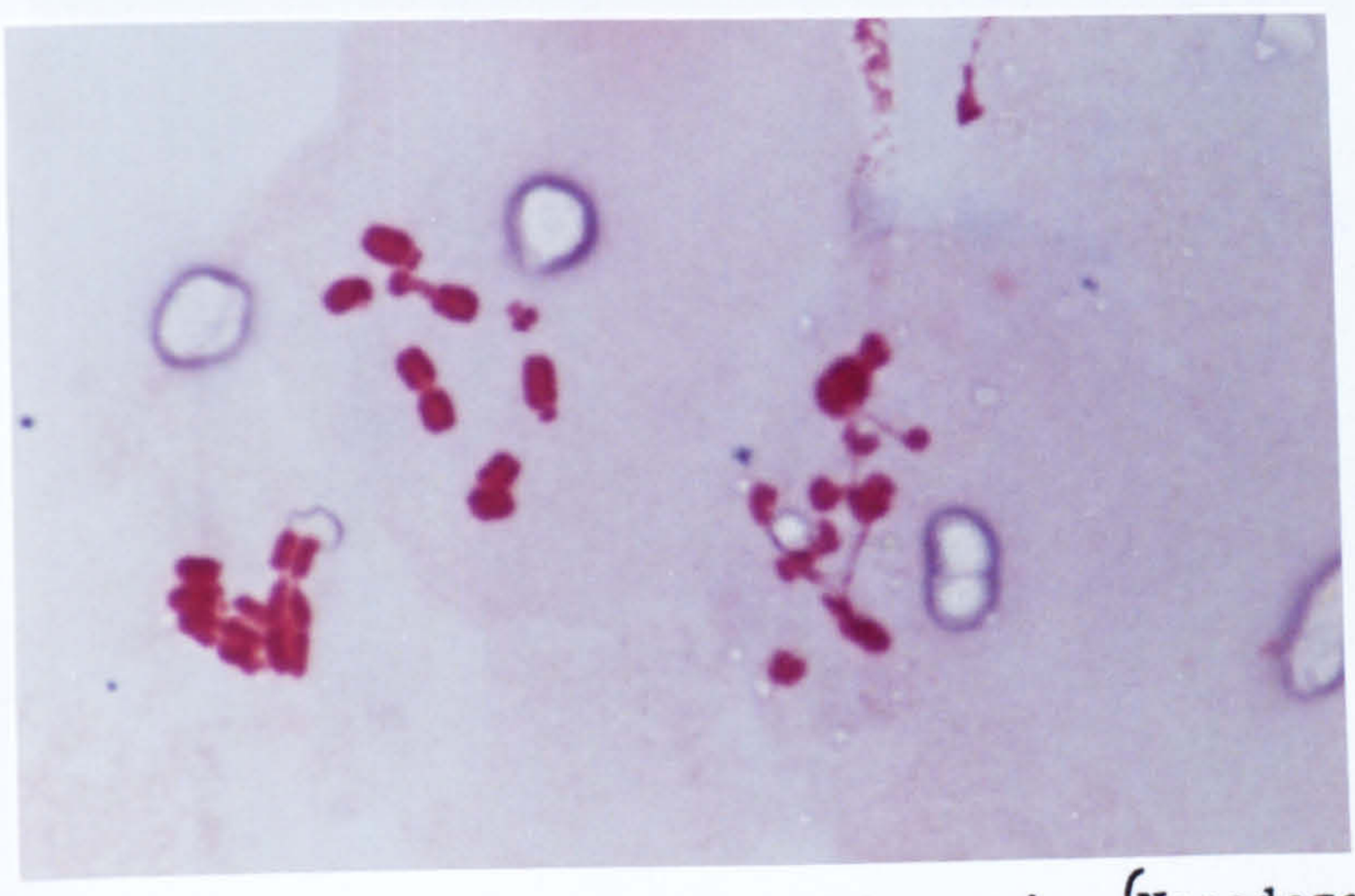


Plate 20 Extreme chromosome condensation (Nocodazole 20ng/ml)

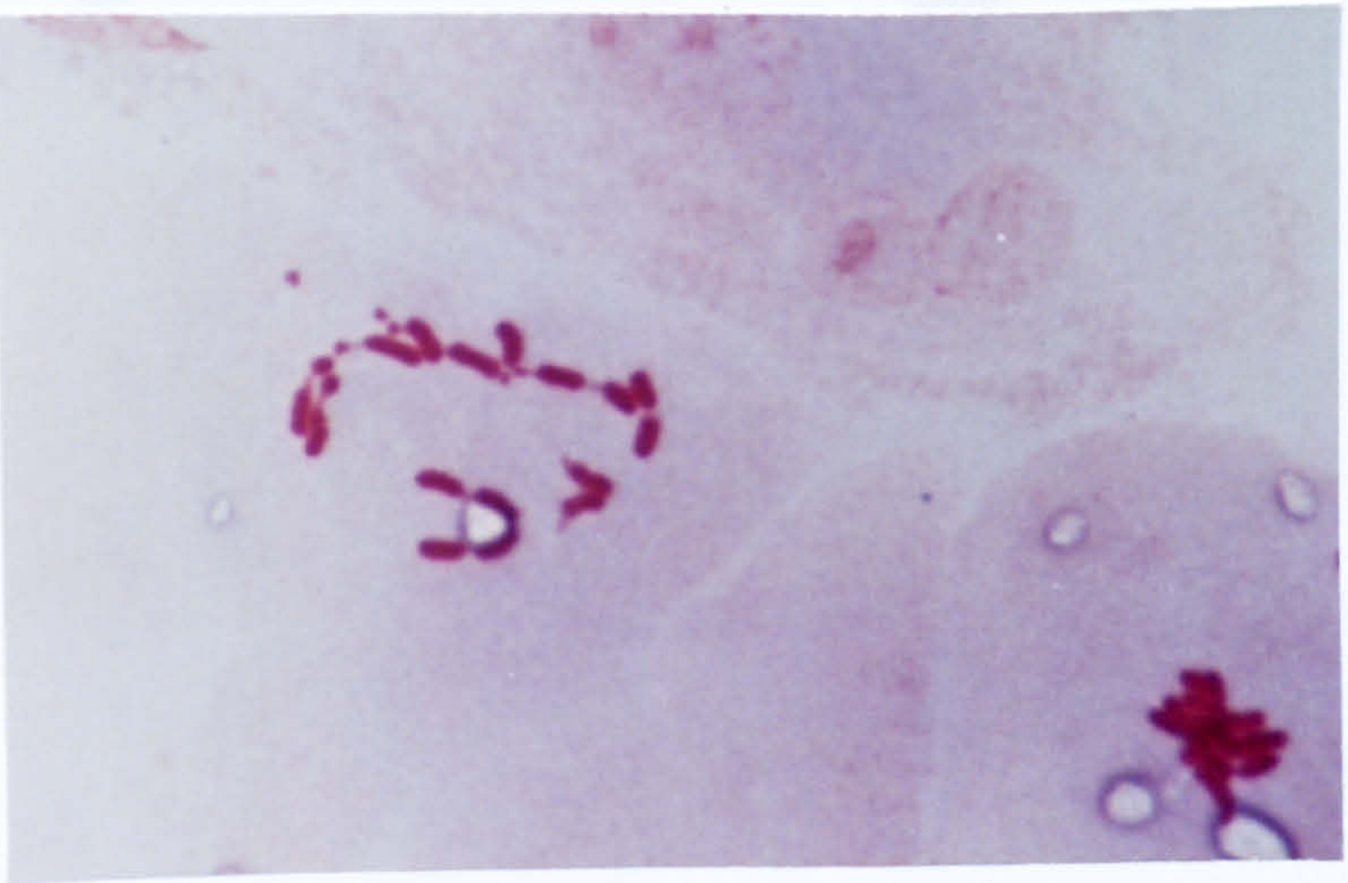


Plate 21 Sticky chromosomes (Nocodazole 20ng/ml)

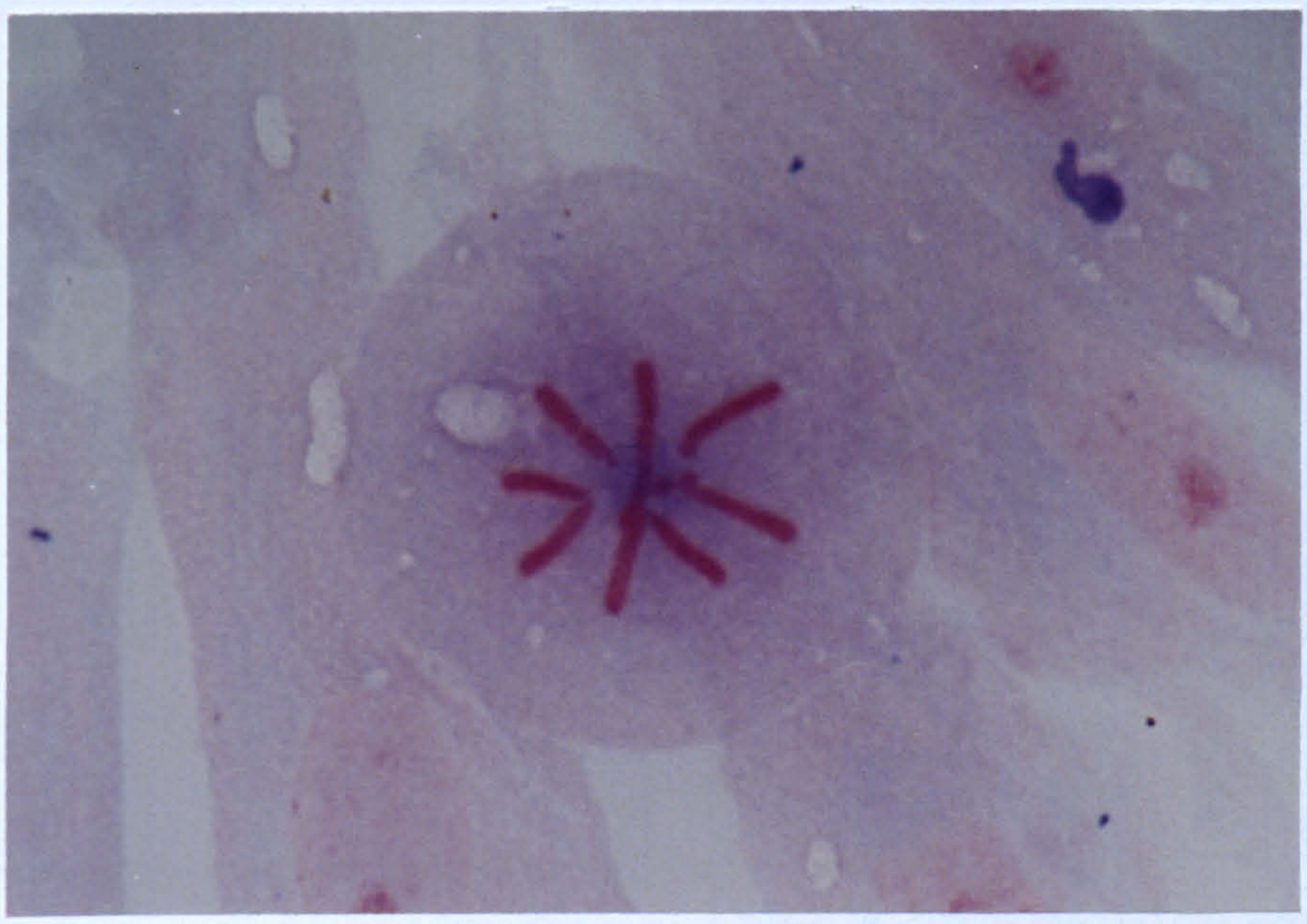


Plate 22 Monopolar metaphase (pFPA 50µg/ml)

Plate 23 Endoreduplication (pFPA 50µg/ml)

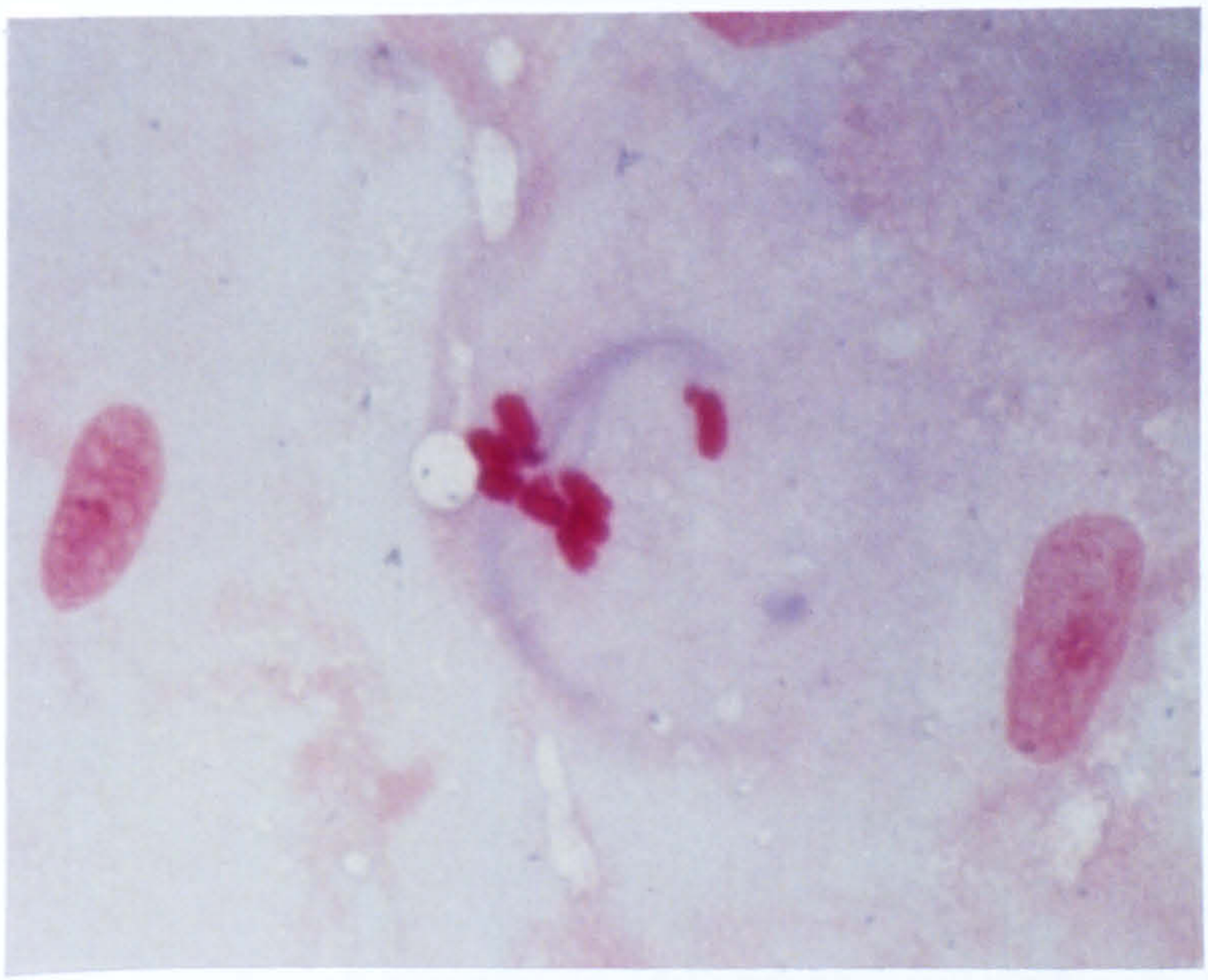


Plate 24 Crescent spindle (Hydroquinone 16μg/ml)

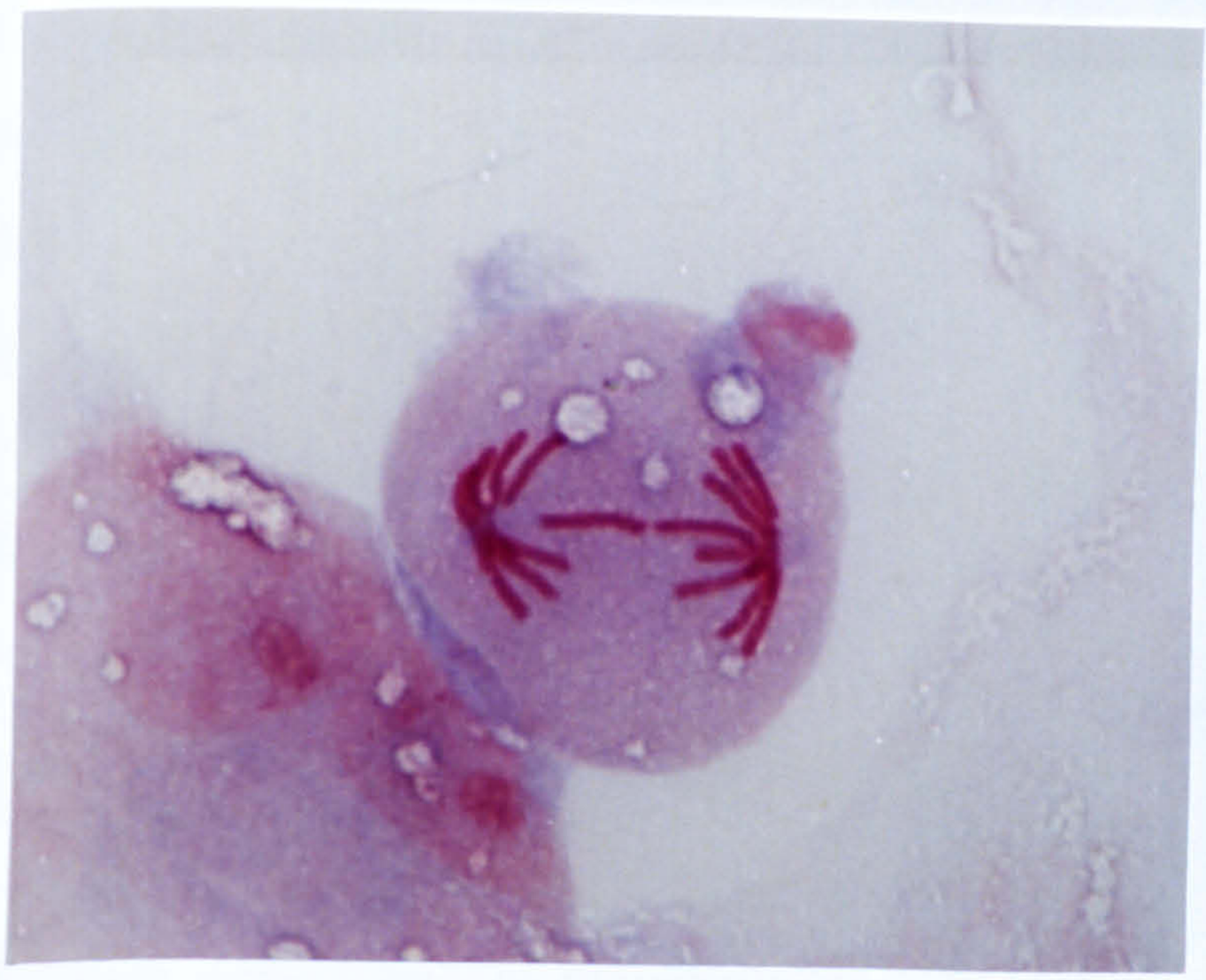


Plate 25 Pseudo chromosome bridge (Nocodazole 10ng/ml)

REFERENCES

ALBANESE, R. (1988). The clastogenicity of modulators of pH and osmotic pressure in cultured human lymphocytes. Mutagenesis. In press.

ALLEN, J.W., LIANG, J.C., CARRANO, A.V. and PRESTON, R.J. (1986). Review of literature on chemical-induced aneuploidy in mammalian male germ cells. Mutation Research, 167, 123-137.

ASHBY, J. and BRAITHWAITE, I. (1987). A rat liver micronucleus assay employing 4AAF as mitogen. Absract from the European Environmental Mutagen Society meeting, Zurich, July 19-23, p. 90.

BARLOGIE, B., RABER, M.N., SCHUMANN, J., JOHNSON, T.S., DREWINKO, B., SWARTZENDRUBER, D.E., GOHDE, W., ANDREEFF, M and FREIREICH, E.J (1983). Flow cytometry in clinical cancer research. Cancer Research, 43, 3982-3997.

BIESELE, J.J. and JACQUEZ, J.A. (1954). Mitotic effects of certain amino acid analogs in tissue culture. Annals New York Academy of Sciences, 58, 1276-1287.

BOND, D.J. (1986). Mechanisms of aneuploid induction. In, Proceedings of the XVIth Annual Meeting of the European Environmental Mutagen Society, 25-30th August, Brussels, Belgium. 34-39. Leonard and Kirsch-Volders.

BOND, D.J. and CHANDLEY, A.C. (1983). Aneuploidy. Oxford Monographs on Medical Genetics no. 11. Oxford, New York, Toronto. Oxford University Press.

BRINKLEY, B.R., TOUSSON, A. and VALDIVIA, M. (1985). The kinetochore of mammalian chromosomes : Structure and function in normal mitosis and aneuploidy. In Aneuploidy, Etiology and Mechanisms. 243-278. Dellarco, Voytek and Hollaender. New York and London, Plenum Press.

BRINKLEY, B.R., VALDIVIA, M.M., TOUSSON, A. and BRENNER, S.L. (1984). Compound kinetochores of the Indian Muntjac. Evolution by linear fusion of unit kinetochores. Chromosoma (Berl.), 91, 1-11.

BUITENDIJK, S. (1984). DES : The time bomb drug. In, Drugs in pregnancy and delivery. Report on the thirteenth European Symposium on Clinical Pharmacological Evaluation in Drug Control. 53-60. World Health Organisation Regional Office for Europe, Copenhagen.

CARRANO, A,V., GRAY, J,W., MOORE II, D,H., MINKLER, J.L., MAYALL, B.H., VAN DILLA, M.A. and MENDELSOHN, M.L. (1976). Purification of the chromosomes of the Indian Muntjac by flow sorting. The Journal of Histochemistry and Cytochemistry, 24, no. 1 348-354.

CONTI, C.J., ALDAZ, C.M., O'CONNELL, J., KLEIN-SZANTO, A.J.P. and SLAGA, T.J. (1986). Aneuploidy, an early event in mouse skin tumour development. Carcinogenesis, 7, no. 11, 1845-1848.

DANFORD, N. (1984). Measurement of levels of aneuploidy in mammalian cells using a modified hypotonic treatment. Mutation Research, 139, 127-132.

DANFORD, N., BALFE, P. (1987). Methods of analysis of data on mitotic aneuploidy. Mutagenesis, 2, no. 5, 391-396.

DANFORD, N. and PARRY, J.M. (1982). Abnormal cell division in cultured human fibroblasts after exposure to diethylstilboestrol. Mutation Research, 103, 379-383.

DEAN, B.J. and DANFORD, N. (1984). Assays for the detection of chemically induced chromosome damage in cultured mammalian cells. In, Mutagenicity Testing, a Practical Approach. 187-232. S. Venitt and J.M. Parry, Oxford, Washington DC. IRL Press.

De BRABANDER. (1986). Dynamics of cell division and maintenance of euploidy, presented at the XVI. annual meeting of the European Environmental Mutagen Society. 25-30th August Brussels, Belgium.

DEGRAEVE, N. (1978). Genetic and related effects of Vinca rosea alkaloids. Mutation Research, 55, 31-42.

DELLARCO, V.L., MAVOURNIN, K.H. and TICE, R.R. (1985). Aneuploidy and health risk assessment : Current status and future directions. Environmental Mutagenesis, 7, 405-424.

De OLIVEIRA, A.R. and MACHADO-SANTELLI, G.M. (1987). Diphenylhydantoin and mitotic spindle abnormalities in cultured mouse and human cells. Mutation Research, 187, 91-97.

DUNNETT, C.W. (1955). A multiple comparison procedure for comparing several treatments with a control. Journal of the American Statistical Association, 50, 1096-1121.

DUSTIN, P. (1980). Microtubules. Scientific American, 243, (2), 58-68.

DUSTIN, P. (1984). Microtubules (2nd Edition) Springer-verlag, Berlin, Heidelberg, New York, Tokyo.

DYSON, R.D. (1978). Cell Biology : A molecular approach. Second edition, Chapter 11, 510-517. Allyn and Bacon Inc. Boston, London, Sydney, Toronto.

EHRHARDT, A.A. (1987). Behaviour patterns of women exposed to prenatal DES. Abstract from the 15th International Summer School of Brain Research. In, Neurochemistry of Functional Neuroteratology : permanent effects of chemicals on the developing brain. Amsterdam, August 31st-September 4th, p. 37.

EVANS, H.J. (1985). Neoplasia and Cytogenetic Abnormalities. In, Aneuploidy, Etiology and Mechanisms. 165-178. Dellarco, Voytek and Hollaender. New York and London. Plenum Press.

FOUREMAN, P.A. (1986). Detection of Aneuploidy in Drosophila. Chemical Mutagens : Principles and Methods for their detection. Vol. 10, 183-213. de Serres, FJ., New York and London. Plenum Press.

FRESHNEY, R.I. (1983). Culture of Animal Cells. A manual of basic technique. Alan R. Liss, Inc., New York.

GALLOWAY, S.M., BEAN, C.L., ARMSTRONG, M.A., DEASY, D., KRAYNAK, A. and BRADLEY, M.O. (1985). False positive in vitro chromosome aberration tests with non-mutagens at high concentrations and osmolalities. Abstracts of the sixteenth annual meeting of the Environmental Mutagen Society, February 25th-March 1st. Environmental Mutagenesis, 7, Supplement 3, 48-49.

GALLOWAY, S.M. and IVETT, J.L. (1986). Chemically induced aneuploidy in mammalian cells in culture. Mutation Research, 167, 89-105.

GAULDEN, M.E., PROCTOR, B.L. and WEBER, N.B. (1985). Varying responses of centromeres to Benzene, Formaldehyde and Mitomycin C, some of which can lead to aneuploidy. Abstracts of the sixteenth annual meeting of the Environmental Mutagen Society. Febuary 25th-March 1st, Nevada USA. Environmental Mutagenesis, 7, Supplement 3, p. 13.

GRATZER, W. (1987). Protean proteins perceived. Nature, 328, 669-670.

GRIFFITHS, A.J.F. (1982). Short-term tests for chemicals that promote aneuploidy. In Chemical Mutagens; Principles and Methods for their Detection Vol. 7, Chapter 5, 189-210. de Serres, FJ., New York and London. Plenum Press.

GRIFFITHS, A.J.F., BROCKMAN, H.E., DeMARINI, D.M. and de SERRES, F.J. (1986). The efficacy of Neurospora in detecting agents that cause aneuploidy. Mutation Research, 167, nos. 1/2, 35-45.

GRIFFITHS, A.J.F. and DELANGE, A.M. (1977). p-Fluorophenylalanine increases meiotic non-disjunction in a neurospora test system. Mutation Research, 46, 345-354.

HERBST, A.L., ULFELDER, H. and POSKANZER, D.C. (1971). Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumour appearance in young women. New England Journal of Medicine, 284, no. 16, 878-881.

HOOK, E.B. (1985). The impact of Aneuploidy Upon Public Health : Mortality and morbidity associated with human chromosome abnormalities. In Aneuploidy, Etiology and Mechanisms. 7-33. Dellarco, Voytek and Hollaender. New York and London. Plenum Press.

HSU, T.C., LIANG, J.C. and SATYA-PRAKASH, K.L. (1986). Cytogenetic assays for mitotic poisons using somatic animal cells. In Chemical mutagens: principles and methods for their detection. Vol. 10, 155-181. de Serres, F.J., New York and London. Plenum Press.

HSU, T.C. and SATYA-PRAKASH, K.L. (1985). Aneuploidy induction by mitotic arrestants in animal cell systems : possible mechanisms. In, Aneuploidy, Etiology and Mechanisms, 279-289. Dellarco, Voytek and Hollaender. New York and London. Plenum Press.

HSU, T.C., SHIRLEY, L.R. and TAKANARI, H. (1983). Cytogenetic Assays for mitotic poisons : The diploid Chinese hamster cell system. Anticancer Research, 3, 155-160.

HUTTNER, K.H. and RUDDLE, F.H. (1976). Study of Mitomycin C-induced chromosomal exchange. Chromosomea (Berl.), 56, 1-13.

JENSSEN, D. and RAMEL, C. (1980). The micronucleus test as part of a short term mutagenicity test program for the prediction of carcinogenicity evaluated by 143 agents tested. Mutation Research, 75, 191-202.

KAFER, E., SCOTT, B.R. and KAPPAS, A. (1986). Systems and results of tests for chemical induction of mitotic malsegregation and aneuploidy in Aspergillus nidulans. Mutation Research, 167, nos. 1/2, 9-34.

KIRSCH-VOLDERS, M. (1986). Differential staining of chromosomes and spindle cannot be used as an assay to determine the effects of cancer promoters on primary cultures of human fibroblasts. Mutation Research, 171, 177-183.

KOSTOFF, D. (1938). Irregular mitosis and meiosis induced by acenaphthene. Nature, 141, 1144-1145.

KRAHN, D.F. (1983). Chinese hamster cell mutagenesis: A comparison of the CHO and V79 systems. Annals New York Academy of Sciencies, 407, 231-238.

LAFI, A., PARRY, J.M. and PARRY, E.M. (1986). The effect of Michler's ketone on cell division, chromosome number and structure in cultured Chinese hamster cells. Mutagenesis, 1, no. 1, 17-20.

LEVAN, A. and OSTERGREN, G. (1943). The mechanism of c-mitotic action. Observations on the naphthalene series. Hereditas, 29, 381-443.

LIANG, J.C. and BRINKLEY, B.R. (1985). Chemical probes and possible targets for the induction of aneuploidy. In, Aneuploidy, Etiology and Mechanisms. 491-505. Dellarco, Voytek and Hollaender. New York and London Plenum Press.

LIANG, J.C., HSU, T.C. and HENRY, J.E. (1983). Cytogenetic assays for mitotic poisons : The grasshopper embryo system for volatile liquids. Mutation Research, 113, 467-479.

MAILHES, J.B., PRESTON, R.J. and LAVAPPA, K.S. (1986). Mammalian in vivo assays for aneuploidy in female germ cells. Mutation Research, 167, no. 1/2 139-148.

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

McINTOSH, J.R., HEPLER, P.K. and VAN WIE, D.G. (1969). Model for mitosis. Nature, 224, 659-663.

MELNICK, S., COLE, P., ANERSON, D. and HERBST, A. (1987). Rates and risks of diethylstilboestrol-related clear-cell adenocarcinoma of the vagina and cervix. The New England Journal of Medicine, 316, 514-516.

MITCHISON, T. and KIRSCHNER, M. (1984). Dynamic instability of microtubule growth. Nature, 312, 237-242.

NIJS, M. and KIRSCH-VOLDERS, M. (1986). Induction of spindle inhibition and abnormal mitotic figures by Cr(II), Cr(III) and Cr(VI) ions. Mutagenesis, 1, no. 4, 247-252.

NOWAK, C. (1987). Studies on the ability of hypotonic solutions to induce chromosomal aberrations in V79 cells. Teratogenesis, Carcinogenesis and Mutagenesis, 7, 515-525.

ONFELT, A. (1986). Mechanistic aspects of chemical induction of spindle disturbance and abnormal chromosome numbers. Mutation Research, 168, 249-300.

OSHIMURA, M. and BARRETT, J.C. (1986). Chemically Induced Aneuploidy in Mammalian Cells : Mechanisms and biological significance in cancer. Environmental Mutagenesis, 8, 129-159.

PARDUE, M.L. and HSU, T.C. (1975). Locations of 18S and 28S Ribosomal genes on the chromosomes of the Indian Muntjac. The Journal of Cell Biology, 64, 251-254.

PARMENTIER, R. and DUSTIN, P. jun. (1948). Early effects of hydroquinone on mitosis. Nature, 161, 527-528.

PARMENTIER, R. and DUSTIN, P. jun. (1953). On the mechanism of the mitotic abnormalities induced by hydroquinone in animal tissues. Revue Belge. Path. Med. exp., 23, 20-30.

PARRY, J.M. (1988). Studies upon the genetic effects of environmental chemicals: the coordinated research programme of the European Economic Community. Mutagenesis, 3, no. 2, 105-136.

PARRY, J.M., DANFORD, N. and PARRY, E.M. (1984). In vitro techniques for the detection of chemicals capable of inducing mitotic chromosome aneuploidy. ATLA, 11, 117-28.

PARRY, E.M., DANFORD, N. and PARRY, J.M. (1982). Differential staining of chromosomes and spindle and its use as an assay for determining the effect of diethylstilboestrol on cultured mammalian cells. Mutation Research, 105, 243-252.

PARRY, E.M., SHARP, D.C. and PARRY, J.M. (1985). The observation of mitotic division aberrations in mammalian cells exposed to chemical and radiation treatments. Mutation Research, 150, 369-381.

PATHAK, S. and HSU, T.C. (1977). Monitoring the effects of chemical mutagens with mammalian meiotic cells. Mammalian Chromosomes Newsletter, 18, p. 42.

PATTERSON, R.M. and PETRICCIANI, J.C. (1973). A comparison of prophase and metaphase G-bands in the muntjac. The Journal of Heredity, 64, 80-82.

PAUL, J. (1975). Cell and tissue culture. Fifth edition. p.30. Churchill Livingstone. Edinburgh, London and New York.

PAWELETZ, N. and SCHROETER, D. (1986). On the fine structure of the mitotic apparatus of mammalian cells. In, Genetic Toxicology of Environmental Chemicals, Part A, Basic principles and Mechanisms of action. 307-314 Ramel, Lambert, Magnusson. New York. Alan R Liss, inc.

PERRY, P. and WOLFE, S. (1974). New Giemsa method for the differential staining of sister chromatids. Nature, 251, 156-158.

RABINOVITCH, P.S., MARTIN, G.M. and HOEHN, H. (1982). Interphase Flow-cytogenetics : Correlation of DNA fluorescence to aneuploidy in human flbroblast cultures. Human Genetics, 61, 246-249.

RAINALDI, G., FLORI, L., COLELLA, C.M., MARIANI, T., PIRAS, A., SIMI, S. and SIMILI, M. (1987). Analysis by BrUdR-labelling technique of induced aneuploidy in mammalian cells in culture. Mutation Research, 177, 255-260.

RESNICK, M.A., MAYER, V.W. and ZIMMERMANN, F.K. (1986). The detection of chemically induced aneuploidy in Saccharomyces cerevisiae : an assesment of mitotic and meiotic systems. Mutation Research, 167, nos. 1/2, 47-60.

SANDHU, S.S., VIG, B.K. and CONSTANTIN, M.J. (1986). Detection of chemically induced aneuploidy with plant test systems. Mutation Research, 167, nos. 1/2, 61-69.

SATYA-PRAKASH, K.L., HSU, T.C. and WHEELER, W.J. (1984). Metaphase arrest, anaphase recovery and aneuploidy induction in cultured Chinese hamster cells following exposure to Mitotic arrestants. Anticancer Research, 4, 351-356.

SATYA-PRAKASH, K.L., LIANG, J.C., HSU, T.C. and JOHNSTON D.A. (1986). Chromosome abberrations in mouse bone marrow cells following treatment in vivo with vinblastine and colcemid. Environmental Mutagenesis, 8, 273-282.

SAWADA, M. and ISHIDATE, M. Jr. (1978). Colchicine-like effect of diethylstilboestrol (DES) on mammalian cells in vitro. Mutation Research, 57, 175-182.

SCOTT, D., DANFORD, N., DEAN, B., KIRKLAND, D. and RICHARDSON, C. (1983). In vitro chromosome aberration assays. Report of the United Kingdom Environmental Mutagen Society sub-committee on guidelines for mutagenicity testing. Part 1, Chapter 3, 41-64. B.J. Dean. UKEMS.

SCHMID, M. (1975). The Micronucleus Test. Mutation Research, 31, 9-15.

SHELBY, M.D. (1988). The genetic toxicity of human carcinogens and its implications. Mutation Research, 204, 3-15.

SHIMADA, T., and INGALLS, T.H. (1975). Chromosome mutations and pH disturbances. Archives of Environmental Health, 30, 196-200.

SISKEN, J.E., and WILKES, E. (1967). The time of synthesis and the conservation of mitosis-related proteins in cultured human amnion cells. The Journal of Cell Biology, 34, 97-110.

SOMERS, A. (1986). An investigation into the mechanisms of aneuploidy induction in cultured mammalian cells. PhD. thesis, University College of Swansea, Wales.

SUTOU, S. and TOYUYAMA, F. (1974). Induction of endoreduplication in cultured mammalian cells by some chemical mutagens. Cancer Research, 34, 2615-2623.

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

TENCHINI, M.L., MOTTURA, A., VELICOGNA, M., PESSINA, M., RAINALDI, G., and DeCARLI, L. (1983). Double Y as an indicator in a test of mitotic non-disjunction in cultured human lymphocytes. Mutation Research, 121, 139-146.

TUCKER, R.W., and BARRETT, J.C. (1986). Decreased numbers of spindle and cytoplasmic microtubules in hamster embryo cells treated with a carcinogen, diethylstilboestrol. Cancer Research, 46, 2088-2095.

THERMAN, E. (1986). Human chromosomes. Structure, behaviour effects. Second Edition, Chapter 12. 110-118. New York, Berlin, Heidelberg, Tokyo. Springer-Verlag.

TSUTSUI, T., MAIZUMI, H., McLACHLAN, J.A. and BARRETT, J.C. (1983). Aneuploidy induction and cell transformation by diethylstilboestrol: a possible chromosomal mechanism in carcinogenesis. Cancer Research, 43, 3814-3821.

TSUTSUI, T., MAIZUMI, H., and BARRETT, JC. (1984). Colcemid-induced neoplastic transformation and aneuploidy in Syrian hamster embryo cells. Carcinogenesis, 5, no. 1, 89-93.

VED BRAT, S., VERMA, R.S., and DOSIK, H. (1979). Sister chromatid exchange (SCE) induced by caffeine and anthramycin in the euchromatin and heterochromatin of Indian muntjac. Mammalian Chromosomes Newsletter, 20, 1/2, 4-5.

VIAGGI, S., BONATTI, S., and ABBONDANDOLO, A. (1987). New evidence for the presence of chromosomes in micronuclei of human and Chinese hamster cells. Mutagenesis, 2, no. 5, 367-370.

VIG, B.K. (1988). Micronuclei from whole chromosomes. From HSE sponsored workshop on micronuclei: origins, applications and methodologies. 23-25th May. Manchester.

VIG, B.K. (1986). Sequence of centromere separation: Multicentric chromosomes, repetitive DNA and aneuploidy. In, Genetic Toxicology of Environmental Chemicals. Part A: Basic principles and mechanisms of action. 343-350. Ramel, C., Lambert, B. and Magnusson, J. New York. Alan R. Liss, Inc.

WATERS, M.D., STACK, F.H., MAVOURNIN, K.H. and DELLARCO, V.L. (1985). Special committee report, Part II: Quantitative evaluation of chemicals that induce aneuploidy using the Genetic Activity Profile method. In Aneuploidy, Etiology and Mechanisms. 455-490 Dellarco, Voytek and Hollaender. New York and London. Plenum Press.

WILLIAMS, D.A. (1982). Extra-binomial variation in logistic linear models. Applied Statistics, 31 144-148.

WISSINGER, W.L., ESTERVIG, D.N., and WANG, R.J. (1981). A differential staining technique for simultaneous visualization of mitotic spindle and chromosomes in mammalian cells. Stain Technology, 56, no. 4, 221-226.

WOLFE, S.L. (1981). Biology of the cell. 404-410. Second Edition. J.C., Carey. California. Wadsworth Publishing Company.

WURSTER, D.H. and BENIRSCHKE, K. (1970). Indian muntjac, Muntiacus muntjak: A deer with a low diploid chromosome number. Science, 168, 1364-1366.

ZIMMERING, S., MASON, J.M. and OSGOOD, G. (1986). Current status of aneuploidy testing in Drosophila. Mutation Research, 167, no. 1/2, 71-87.