UNIVERSITY OF SOUTHAMPTON

THE GENETIC TOXICOLOGY OF CADMIUM AND CADMIUM/MUTAGEN MIXTURES

Thesis submitted for the degree of Doctor of Philosophy
by Simon David Bouffler

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CONTENTS

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ABSTRACT

FACULTY OF SCIENCE
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Cultured muntjac cells have been used to investigate the genetic toxicology of Cd(II) ions and the influence of the cation on the genotoxicity of five other mutagens (ethyl methanesulfonate, mitomycin C, acridine orange, Hoechst 33258 and nickel (II) ions).

Cd(II) ions could only induce chromosomal aberrations in cells treated for short periods followed by long recovery periods. Sister chromatid exchanges (SCEs) were not induced by the cation, possibly due to the removal of SCE-inducing lesions by excision repair. In treated cells, most intracellular cadmium was located in the cytosol bound to a low molecular weight molecule. This molecule was not metallothionein but might be glutathione.

In general, co-treatment or pretreatment of cells with Cd(II) ions tended to decrease the chromosomal effects of alkylating agents and increase those of other compounds. Experimental evidence suggesting an involvement of the following in these interactions has been produced: (i) inhibition of excision repair by Cd(II) ions leading to increased chromosomal effects of non-covalent DNA-binding agents, (ii) a Cd(II)-inducible repair system reducing the effects of alkylating agents on SCE, (iii) increased cytosolic free Cd(II) ions enhancing chromosomal aberration formation.

The effects of the five mutagens on a cadmium resistant strain of muntjac cells, able to grow in 5μM cadmium acetate, were also investigated. The increased resistance of these cells may involve lipid production and/or increased Cd binding to a cytosolic glutathione-like molecule. Each of the mutagens induced fewer SCEs in resistant cells than in normal cells. However, due to the higher baseline SCE frequency in resistant cells, mutagen treated cells had higher absolute SCE frequencies than similarly treated normal cells. The reduction in sensitivity may involve the conjugation of mutagens with the cytosolic glutathione-like molecule so protecting the nucleus from damage.

These findings imply that exposure to cadmium might alter the susceptibility of humans to chemically induced cancers. Whether susceptibility would be increased or decreased being dependent upon the carcinogen considered and the nature of cadmium exposure.

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Abbreviations

AO	acridine orange
BUdR	5-bromodeoxyuridine
Cd^{2+}	cadmium (II) ions
CHO	Chinese hamster ovary
con A	concanavalin A
CR5	Cd^{2+} resistant variant of muntjac cells
DEAE	diethylamino ethyl
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
EMS	ethyl methanesulfonate
HBSS	Hanks balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
H33258	Hoechst dye 33258
MMC	mitomycin C
Ni^{2+}	nickel (II) ions
PBS	phosphate buffered saline
RNA	ribonucleic acid
SCE	sister chromatid exchange
Tris	tris (hydroxymethyl) aminomethane
UDS	unscheduled DNA synthesis
UV light	ultra violet light

Chapter 1. Introduction

1.1. Overview and aims of this project

The environment contains a multitude of natural and synthetic chemicals, some of which may be carcinogenic to humans. Identification of risk chemicals usually involves short term tests for genotoxicity, that is the ability of a chemical to alter the structure or function of genetic material, and/or long term whole animal carcinogenicity tests. Irrespective of the assay used, in most cases a single compound is tested in isolation, since people come into contact with a whole range of substances, concurrently and sequentially, this is a simplification of environmental exposure. More recently there has been interest in the testing of complex mixtures, for example, food extracts, vehicle exhausts, polluted waters. These studies are more realistic in terms of environmental exposure, but it cannot be ascertained whether the active components are acting independently or interacting to give an overall effect. Interaction between compounds can only be unequivocally determined if defined combinations of chemicals are used. Such systems also allow detailed analysis of the mechanisms of interaction. This approach would be impractical for the study of all possible combinations of genotoxins but, given sufficient knowledge of interactions between model compounds, predictions concerning novel compounds should be possible.

As will become clear later (see Chapter 2), cadmium is a pollutant to which everybody is exposed to a greater or lesser extent. So it is important to know if cadmium can alter the action of other toxic agents. Whether or not cadmium is a human carcinogen is disputed; epidemiological studies have produced positive and negative results as have investigations of chromosomal aberration among environmentally exposed persons. This variability could be due to interactions between cadmium and other genotoxins. The need for further research in this area was realised by a work group of the Workshop/conference on the Role of Metals in Carcinogenesis held in Atlanta, Georgia, 1980:

> "Possible enhancing or inhibitory effects of exposure to various metal compounds in combination with other carcinogens or

cocarcinogenic compounds should be examined in appropriate animal, cellular, 'in vitro' and epidemiological studies. The mechanisms by which metals cause such effects should be elucidated" (Frieberg et al., 1981).

This project sets out to define and examine the mechanisms of cadmium ion-genotoxin interactions at the cellular level. A prerequisite of this aim is to thoroughly investigate the genotoxicity of the cadmium ion itself

1.2 Experimental approach

1.2.1. Assessment of genotoxicity

Two extensively validated assays, sister chromatid exchange (SCE; Latt et al., 1981) and chromosomal aberration (Preston et al., 1981), were employed to assess genotoxicity in this study. Both parameters were analysed in cell cultures of male Indian muntjac cells. This cell type is particularly well suited to cytogenetic work as it has seven easily distinguishable chromosomes (Wurster and Benirschke, 1970). The effect of Cd^{2+} on SCE has not been fully investigated, previous analysis of Cd^{2+} induced chromosomal aberration has yielded variable results (see Section 2.4.3.); so both are worthy of study in themselves. Still less is known of the influence the ion has on the effects of other chemicals in these assays (see Section 2.4.4.).

1.2.2. Treatment protocols

Although any cell culture system is, of necessity, considerably removed from the 'in vivo' situation, some attempts at simulating different types of environmental exposure are possible.

Two treatment protocols were used to parallel the exposure the general population might encounter: (i) concurrent exposure to Cd^{2+} and another compound, (ii) Cd^{2+} followed by a second compound. Most people are constantly in contact with low levels of cadmium and may be exposed to other genotoxins before, with or after cadmium. However, it is unlikely that many cases of 'genotoxin before cadmium' exposure occur as the metal

accumulates in the body from birth.

Some individuals are occupationally exposed to cadmium at high levels. The 'in vitro' counterpart to this situation is the treatment of cadmium resistant cells with the other compounds. The Cd^{2+}-resistant variant of muntjac, produced by long term culture of cells in the presence of the metal, tolerates 5 μM Cd^{2+}. This level of resistance was chosen as, although still high, it is more realistic in terms of the body burdens found in cadmium workers than the 100 uM Cd^{2+} tolerant variants of other cell types which have been described (see Section 2.2).

Relatively low concentrations of cadmium and the other genotoxins have been used throughout this work. These were purposely chosen to try and bring treatments more in line with environmental exposure. Also, it was reasoned that the effect of Cd^{2+} on SCE and chromosomal aberration induced by other compounds would be more readily detected against a relatively low 'other genotoxin only' level of induction.

1.2.3 Studies of the mechanism of Cd^{2+}/genotoxin interactions

As will be seen from reading the next chapter, there is a vast number of ways in which Cd^{2+} might affect the action of other chemicals. Investigation of the uptake and subcellular distribution of Cd^{2+} and, where possible, the other genotoxin has been made in the present work. These experiments should clarify if (i) the extent of chemical uptake, (ii) the extent of nuclear binding, (iii) the extent of organelle and/or cytosol binding, or (iv) the nature and extent of binding to cytosol proteins play any part in the interactions observed. In addition, unscheduled DNA synthesis has been quantified to assess the role of DNA repair.

1.2.4 Choice of other genotoxins

Carcinogens are a heterogeneous group of agents which can be classified into a number of broad categories. The main division is into epigenetic and genotoxic carcinogens. As their name suggests, epigenetic

carcinogens (some steroid hormones for example) are not carcinogenic because of nuclear effects. Such compounds are not considered in this work. Genotoxins can be further classified into physical (e.g. ultra violet light, x-rays), free radical producing (e.g. bleomycin) and DNA binding agents. In the present study the effects of Cd^{2+} on only the last group of compounds are considered.

The DNA binding agents used were as follows:

1. Ethyl methanesulfonate (EMS), a monofunctional alkylating agent.
2. Mitomycin C (MMC), a bifunctional alkylating agent.
3. Acridine orange (AO), an intercalating agent.
4. Hoechst 33258 (H33258) which binds to the grooves of the DNA helix.
5. Nickel (Ni^{2+}), a well documented carcinogenic, genotoxic metal.

These compounds, with the exception of Ni^{2+} do not pose a significant problem as environmental carcinogens. Rather, they should be regarded as model compounds reflecting a range of chemical classes found among environmental carcinogens as a whole. One advantage of using these model compounds is the availability of a great deal of background information as to their effects. A discussion of the mechanisms of action and effects of these chemicals will be found in Section 2.6.

Chapter 2. Literature Review

2.1. Cadmium, environmental occurrence and human exposure

Cadmium is widely distributed throughout the environment, albeit at low levels. Soils and sediments contain less than 1 mg/kg (Peterson and Alloway, 1979), concentrations in unpolluted waters and air are typically less than 1 µg/kg and 2.5 ng/m^3 respectively (Mohlenberg and Jensen, 1980; Cawse, 1977). Cadmium is not found as the native metal and only rarely as a pure mineral, most cadmium is obtained as a by-product of zinc refining. Zinc ores, on average, contain 0.3% cadmium (Fleischer et al., 1974).

Despite the low level natural occurrence of the metal, many industries increase the environmental mobilization of, and environmental contamination by, cadmium. Man's contribution to biologically available cadmium is estimated to be nineteen times that from natural sources (Galloway et al., 1982).

At birth cadmium is essentially absent from the body, the placenta forms an effective barrier to cadmium (Denker, 1975). Throughout life cadmium accumulates in the body up to a total of 15 - 30 mg in the normal adult (Frieberg et al., 1974). For most people, food is the main source of cadmium, 10 - 30 µg/person/day is the average intake for the British population (Ministry of Agriculture, Fisheries and Food, 1973). Given a figure of 8% for the proportion of ingested cadmium absorbed through the gut (Yost, 1984), the average Briton's body burden is increased by 0.8 - 2.5 µg Cd/person/day. Inhalation is not a major route of cadmium uptake for normal individuals, less than 0.1 µg Cd/person/day is absorbed (Yost, 1984). Humans absorb about 30% of inhaled cadmium, so in some industrial situations, where airborne cadmium can be between 5 and 200 ug Cd/m^3 air, 30 - 1200 µg Cd/person/day can be added to the body burdens.

2.2. Cadmium uptake, distribution and metabolism

The fate of cadmium in the body is an extensive subject but will only

be covered briefly here as several reviews are available (Frieberg et al 1974; Bremner, 1979; Bernard and Lauwreys, 1984). Greater attention is paid to intracellular cadmium metabolism, the intracellular distribution of the metal has a major bearing on its toxicity.

A large proportion of ingested cadmium initially associates with the intestinal mucosa. However, little of this is further transported into the body, the bulk is lost through epithelial desquamation. Having crossed the intestinal mucosa or alveolar epithelium, or in experimental situations, been injected, cadmium enters the blood stream for a short period. Binding is to both plasma proteins and cells. With time there is a shift of binding from the former to the latter. Two or three days after a single dose to rodents, the highest proportion of Cd^{2+} is found in the liver. Over a period of months the hepatic cadmium burden decreases while renal concentrations increase. In the mouse, equal renal and hepatic cadmium concentrations are reached in about one and a half months; by four months the kidney contains three times the liver concentration.

In normal humans the kidney contains the greatest percentage of the cadmium body burden (Hallenbeck, 1984). However, those occupationally exposed have a high liver:kidney cadmium ratio, in some cases liver concentrations exceeding those of the kidney (Frieberg et al., 1974). Thus, liver cadmium is taken as a measure of recent exposure.

There are probably three major routes of Cd^{2+} uptake into cells: (i) zinc channels, (ii) calcium channels, (iii) pinocytosis/phagocytosis. Failla and Cousins (1978) found Cd^{2+} to reversibly inhibit Zn^{2+} uptake in the perfused rat liver. Moreover, Cd^{2+} uptake is temperature dependent, requires free sulfhydryl groups and is enhanced by glucocorticoid hormones (Failla et al., 1979), all of which are characteristics of Zn^{2+} uptake. Similarly, Cd^{2+} uptake into isolated rat hepatocytes requires sulfhydryl groups (Gerson and Shaikh, 1984). These pieces of evidence strongly suggest Cd^{2+} enters cells via Zn^{2+} channels. The similarity of ionic charge :ionic radius ratio of Cd^{2+} and Ca^{2+}, 2.06 and 2.02 charges/Å respectively (Jacobson and Turner,

1980), has led several authors to suggest the two ions may interact in living systems. Experimental evidence for the involvement of Ca^{2+} channels in Cd^{2+} uptake is indirect and from diverse sources. Firstly, Cd^{2+} can displace Ca^{2+} in artificial phospholipid monolayers (Suzuki and Matsushita, 1968) and rat brain synaptosome membrane preparations (Kamino et al., 1975). The active transport of Ca^{2+} involves membrane bound ATPases, Cd^{2+} inhibits Ca^{2+}, Mg^{2+} - ATPase (Sugawara and Sugawara, 1975). Direct competition between Ca^{2+} and Cd^{2+} for uptake has been demonstrated in the crab (Carcinus maenas) (Wright, 1977) and laboratory animals (Ando et al., 1977; Chartok et al., 1981; Gruden, 1977; Hamilton, 1978 and Koo et al., 1978). However the interaction is not necessarily at the level of the Ca^{2+} channel (Gruden, 1982). As mentioned above much of the Cd^{2+} in the blood is protein bound, the same is probably true in cell culture medium containing serum. Thus, pinocytosis and phagocytosis of these Cd^{2+} loaded proteins could be a major route of Cd^{2+} entry into cells. Webb (1979a) points out that protein bound cadmium would enter cells as membrane bound vesicles with which lysosomes may fuse. The proteins would be degraded by the lysosomal enzymes and the free Cd^{2+} released would tend to enter the cytosol due to the pH gradient between the lysosome and cytosol. Despite these reasons, lysosomal uptake of Cd^{2+} appears to be a minor component of the total cellular Cd^{2+} uptake (Frazier and Kingsley, 1976; Murakami et al., 1977). So it may be that protein bound Cd^{2+} in blood and culture medium is released close to the surface of cells or directly transfered to cell surface proteins.

Once in the cell, Cd^{2+} can bind to all the major organelles. The extent of binding depends on many factors such as dose, time after dosing, organ or cell type being considered and species. Most studies concern the rodent liver. Yoshikawa and Suzuki (1976) found Cd^{2+} uptake into mouse liver following intraperitoneal injection of 3mg/kg $CdCl_2$ to be essentially complete by four hours, 41 - 43% of the dose was located in the liver. However between four and twenty four hours post-injection the subcellular distribution of the hepatic Cd^{2+} changed dramatically. Initially 28% was located in the nuclear fraction, 11% bound to mitochondria, 16% to microsomes and 46% in the cytosol. By twenty four hours 80% of the liver Cd^{2+} was in the cytosol; nuclear, mitochondrial

and microsomal binding had decreased to 11%, 6% and 3% respectively. Likewise, there is a time dependent increase in cytosolic Cd^{2+} and concomitant decrease in organelle binding between two and six hours in rat liver after intravenous injection of 200ug $CdCl_2$ (Frazier and Puglese, 1978). The latter pattern of Cd^{2+} binding appears stable, at least up to 144 hours after dosing (Stoll et al., 1976). When isolated rat liver cells are treated with 3μM $CdCl_2$ the temporal trend of increasing cytosolic and decreasing organelle bound Cd^{2+} is again observed (Failla et al., 1979). While this general pattern of Cd^{2+} subcellular distribution has been demonstrated in organisms phylogenetically dissimilar to mammals, e.g. Amoeba proteus (Al-Atia, 1980), there are several exceptions. In the rat and fowl testis, nuclear fractions can contain 45-50% of the total testicular Cd^{2+} burden after intravenous injection of $^{109}CdCl_2$, in this case 40-45% of the cadmium was in the cytosol and 2.5% bound to mitochondria and microsomes (Johnson et al., 1972; Chen et al., 1974). The extent of nuclear binding of Cd^{2+} is even more marked in muscle tissue from rat and domestic fowl (Johnson et al., 1972) and in Cd-induced rhabdomyosarcomas (Heath and Webb, 1967). Mitochondria appear to be the main site of Cd^{2+} binding in the adrenal glands (Shanbaky et al., 1978). Some marine organisms such as oysters, mussels and sea lions also show considerable nuclear and mitochondrial binding of Cd^{2+} (Coombs, 1979).

Much attention has focused on the nature of the cytosol binding of Cd^{2+} and the cause of the shift from organelle to cytosol binding. Piscator (1964) observed that large amounts of a specific metalloprotein, now known as metallothionein, were induced in rabbit livers following repeated administration of Cd^{2+}. Metallothionein was originally isolated from the horse kidney cortex by Margoshes and Vallee (1957). It was Kagi and Vallee (1960) who first coined the name metallothionein in a paper which further characterized the protein as a Cd^{2+} and Zn^{2+} binding species with a high (4%) sulphur content due to the presence of cysteine residues. Since these original studies, metallothionein has been characterized by a number of other criteria, see Table 2.1.

Table 2.1 Some properties of metallothioneins*

MOLECULAR WEIGHT:	6500-6900 (thionein 6100) Appears as 10-14,000 on gel filtration columns.
ELEMENTAL COMPOSITION:	16.5% N 10.9% S 6-11% metal
AMINO ACID COMPOSITION:	30-35% cysteine, 1g atom metal per 3cys. No tryptophan, phenylanaline, tyrosine or histidine. No disulphide bridges.
STRUCTURE:	Primary:- single chain Secondary:- tightly folded chain
UV ABSORBANCE:	None at 280 nm Maxima at - 250nm (Cd-thionein) 215nm (Zn-thionein) 270nm (Cu-thionein, Hg-thionein)
ISOFORMS:	Rat liver metallothionein exists as two forms (I α II). Separable by anion exchange chromatography, electrophoresis, isoelectro focusing.

* after Kagi α Nordberg, 1979.

In the following paragraphs I will describe some aspects of metallothionein biochemistry and its biological and toxicological roles. For a more detailed account of these topics, the reader is referred to Webb (1979b), Kagi and Nordberg (1979) and Foulkes (1982).

Since Piscator's (1964) experiments, metallothionein synthesis has been reported to occur in the livers of rodents in response to Cd^{2+} administration by many authors (Shaikh and Lucis, 1970; Nordberg et al., 1971, Webb, 1972, Sugawara, 1977). 'De novo' protein synthesis is involved in the induction as injection of cyloheximide into Cd^{2+} dosed animals blocks the response (Webb, 1972a). Actinomycin D, administered prior to Cd^{2+}, also inhibits metallothionein induction (Squibb and Cousins, 1974). Thus, the induction of metallothionein is associated with increased synthesis of mRNA. In some cell culture systems a correlation between the decrease in nuclear-bound Cd^{2+} and cytosol metallothionein levels has been demonstrated (Bryan and Hidalgo, 1976; Failla et al., 1979), this is suggestive of a direct induction of the metallothionein gene(s) by Cd^{2+}.

Of the Cd^{2+} found in cytosol fractions of livers from Cd^{2+} treated animals and cell cultures, initially there is extensive binding to high molecular weight proteins (see, for example Cempel and Webb, 1976; Failla et al., 1979). Following a lag period of a few hours, thionein (metal-free metallothionein) synthesis is initiated. This newly synthesized thionein provides high affinity Cd^{2+} binding sites so the Cd^{2+} tends to shift from the lower affinity binding sites on high molecular weight proteins to the thionein, forming metallothionein or more specifically, Cd-thionein. The bulk of cytosolic Cd^{2+} is metallothionein-bound in the rat liver by eight hours after intravenous injection of Cd^{2+} (Cempel and Webb, 1976), a similar time course is seen in cultured rat liver cells after the addition of Cd^{2+} (Failla et al., 1979; Gerson α Shaikh, 1982).

It has been suggested that the binding of Cd^{2+} to metallothionein is a detoxification process (Kagi and Vallee, 1960), preventing binding of the cation to more sensitive targets such as enzymes. The work of Nordberg (1971), Nordberg et al. (1971), Squibb and Cousins (1974) and Stonard and Webb (1976) provides support for this hypothesis. Prolonged low level exposure of rats to Cd^{2+} by injection or ingestion allowed accumulation of very high body burdens of the metal without toxic

effects. Had the same amount of Cd^{2+} accumulated following acute exposure, marked toxicity would have been evident. In the former (low level) case, most Cd^{2+} was found in association with metallothionein. Furthermore, pretreatment of animals with a sub toxic thionein-inducing dose of Cd^{2+} protects aginst a subsequent, otherwise toxic, dose of the ion (Nordberg, 1971; Webb, 1972b; Suzuki and Yoshikawa, 1974; Squibb et al., 1976; Lebar and Miya, 1976 and Probst et al., 1977a,b).

Cell lines can be made resistant to the toxic effects of Cd^{2+}. To achieve this, cells are passaged in the presence of sub-toxic concentrations of the cation or challenged with progressively higher, approximately 90% lethal, concentrations of Cd^{2+} and the survivors grown up. Cells obtained by these methods are able to grow in the presence of Cd^{2+} levels toxic to the parental cell line. Cd^{2+} resistant variants of the following cell lines have been obtained: human skin epithelium, NCTC 2545 (Rugstad and Norseth, 1975); mouse L cells, clones 1D and A9 (Rugstad and Norseth, 1978), Chinese hamster ovary cells (Hildebrand et al., 1979; Gick and McCarthy, 1982); mouse friend leukaemia cells (Beach and Palmiter, 1981). In all of these cells the content and induction capacity of metallothionein is increased as compared to the parental cells. These alterations to metallothionein induction can be accounted for by gene amplification and increased transcriptional activity of the metallothionein gene (Beach et al., 1981; Walters et al., 1981). Methylation also appears to be involved in the regulation of the induction response. Treatment of cells with the demethylating agent, 5-azacytidine can increase the frequency of selection of Cd^{2+} resistant CHO cells (Hildebrand et al., 1982) and allow the selection of resistant mouse thymoma W7 cells which are not obtainable by treatment with Cd^{2+} alone (Compere and Palmiter, 1981).

There is some evidence suggesting metallothionein is not only the means of Cd^{2+} detoxification. The resistant mouse thymoma W7 cells mentioned above do not have amplified metallothiionein genes, nor is metallothionein mRNA detected after Cd^{2+} treatment (Beach et al., 1981).

Phenobarbital can reduce the toxicity of several metals, including Cd^{2+} (Yoshikawa and Ohsawa, 1975) yet phenobarbital does not induce metallothionein (Yoshikawa and Suzuki, 1976). The significance of metallothionein in Cd^{2+} detoxification, at least in the neonatal rat, is brought into doubt by the experiments of Klaassen and Wong (1982). These investigators found Cd^{2+} to be more toxic to 4-day old rats than adults despite the younger rats having considerably greater hepatic metallothionein levels. It appears from the available evidence that the synthesis of metallothionein can only be considered an intracellular protective mechanism. Cadmium bound to thionein is more toxic than an equivalent concentration of free Cd^{2+} to both whole animals (Cherian et al., 1976; Webb and Etienne, 1977) and kidney cell cultures (Cherian, 1982). This increased toxicity could be due to Cd-thionein facilitating a greater relative uptake of Cd^{2+}. As previously discussed, this protein bound Cd^{2+} would enter cells by endocytosis, be degraded in lysosomes releasing free Cd^{2+} which could then interfere with sensitive cellular targets (Webb α Etienne, 1977; Webb, 1979c; Cain and Holt, 1983). It has also been argued that Cd-thionein toxicity is caused by a direct effect of the protein moeity on membranes (Cherian et al., 1976; Cherian, 1982).

Cd^{2+} is not the only stimulator of metallothionein production. Zinc, copper, mercury and silver induce thioneins containing the respective metals, while lead, manganese, nickel, chromium and indium increase hepatic Zn-thionein levels in rodents (see review by Webb, 1979d). Miscellaneous organic compounds such as glucocorticoid hormones (Karin et al., 1980; Mayo and Palmiter, 1981), tetrachloromethane (Oh et al., 1978), alkylating agents (Kotsonis and Klaasson, 1979), terpentine (Sobocinski α Canterbury, 1982), bacterial endotoxin (Sabocinski α Canterbury, 1982; Durnham et al., 1984) and α-mercapto-β-(2-furyl)acrilic acid (Giroux and Lachmann, 1984) also appear able to raise hepatic metallothionein content. The induction by the latter compounds may be due to their stressing cells; Oh et al., (1978) found physical stress to be effective at inducing hepatic metallothionein synthesis in the rat.

Despite the large, and still growing, literature concerning metallothionein, surprisingly little is known of its natural function.

Webb (1979e) has argued against metal detoxification as being the major role of the protein. Current consensus favours the theory that involvement in copper and zinc homeostasis, especially in the neonate, is the 'normal' function of metallothionein (see, for example Webb and Cain, 1982).

2.3 Selected aspects of Cd^{2+} toxicity

A number of adverse health effects have been attributed to cadmium exposure including osteomalacia (Itai-Itai disease), renal failure, emphysema, hypertension and cancer (see review by Hallenbeck, 1984). In spite of the vast amount of research, the cellular lesion underlying these effects has yet to be established. The following paragraphs will consider some biochemical and cellular aspects of Cd^{2+} toxicity with particular attention being paid to those cytoplasmic effects that could modulate the toxicity of the metal or other compounds. The carcinogenicity and genetic toxicology of cadmium will be discussed in detail in Section 2.4.

In common with other toxic agents, a knowledge of the chemistry of cadmium enables a more complete appraisal of its toxic action. The salient chemical characteristics of Cd^{2+} and the essential cations Zn^{2+} and Ca^{2+} are seen in Table 2.2. Note that in some respects

Table 2.2 Chemical characteristics of Cd^{2+}, Zn^{2+} and Ca^{2+}*

	Cd^{2+}	Zn^{2+}	Ca^{2+}
PERIODIC GROUP	IIB	IIB	IIA
ATOMIC NUMBER	48	30	20
ATOMIC WEIGHT	112.4	65.37	40.08
IONIC RADIUS (Å)	0.97	0.74	0.99
CHARGE/RADIUS RATIO (Charges/Å)	2.06	2.7	2.02
POLARIZABILITY ($Å^3$)	1.8	0.8	1.1
SOFTNESS PARAMETER ($\bar{\sigma}p$)	0.081	0.115	0.18

* Data from Jacobson & Turner (1980).

Cd^{2+} is similar to both Zn^{2+} and Ca^{2+}. The most important points are: (i) Cd^{2+} and Zn^{2+} are in the same group of the periodic table thus show several other chemical similarities; (ii) the charge/radius ratios of Ca^{2+} and Cd^{2+} are very similar; (iii) softness parameter - this is a measure of the relative affinity of an ion for various ligands. Soft ions ($\sigma p < 0.1$) form their most stable complexes with phosphorus, sulphur and chlorine, hard ions show a preference for nitrogen, oxygen and fluorine ligands. Cd^{2+} is considered to be soft, Zn^{2+} intermediate and Ca^{2+} hard; (iv) polarizability - the higher this value the more covalent in character are its bonds to other atoms.

When considering the binding of Cd^{2+} to amino acids, histidine and cysteine are of particular interest. Of these two molecules, Cd^{2+} has a greater affinity for cysteine, but the affinity of Zn^{2+} for both these ligands is more than that of Cd^{2+} (Sillen and Martell, 1971). The affinity of these two ions for all other amino acids is at least an order of magnitude less than for cysteine and histidine. In all cases the Zn^{2+} complexes are energetically favoured (Sillen and Martell, 1971). When in proteins the metal binding properties of amino acids can be altered. Thus, Cd^{2+} readily displaces Zn^{2+} in the cysteine clusters of metallothionein (Kagi α Vallee, 1960, 1961). Studies of metal binding to bovine serum albumin have shown Cd^{2+} to be bound with a greater affiity than Zn^{2+} (Hashimoto, cited by Jacobson and Turner, 1980). On this protein, Cd^{2+} was found to bind mainly to the thiol groups while Zn^{2+} bound to amino and carboxyl groups (Hashimoto and Watanabe, 1976 cited by Jacobson and Turner, 1980). So given the correct conditions, Cd^{2+} can replace Zn^{2+} in proteins as well as binding to free thiol and other ligands.

As Ca^{2+} and Cd^{2+} are similar in only one property, substitution probably only rarely occurs 'in vivo'. However, displacement of Ca^{2+} by Cd^{2+} can occur in calmodulin 'in vitro'. The Cd^{2+} complex has decreased phosphodiesterase and membrane binding activities (Habermann et al., 1983).

The binding of, and/or substitution by, Cd^{2+} in many enzymes is known to alter their function. Vallee and Ullmer (1972) reviewed the literature and listed some thirty enzymes the activities of which could be increased or decreased by Cd^{2+}. Since then, there have been further reports of Cd^{2+} induced alterations to enzyme function (for example, Rajanna et al., 1983; Tokushige et al., 1984; Sirover and Loeb, 1976a).

Clearly, the enzymes of nucleic acid metabolism are of great importance when considering the genetic effects of a chemical. DNA polymerases are zinc metalloenzymes (Slater et al., 1971; Springgate et al., 1973), if Cd^{2+} replaces Zn^{2+} in the enzyme it is not active (Springgate et al., 1973). It is not then surprising that Cd^{2+} can decrease the rate of DNA synthesis (Friedman+Staub, 1976; Kacew et al., 1976; Stoll et al., 1976; Cihiak and Inoue, 1979; Popenoe and Schmaeler, 1979; Hyde and Poirier, 1981). The fidelity of DNA synthesis is also adversely affected by Cd^{2+} (Sirover α Loeb, 1976; Miyaki et al., 1977). The flow cytometric studies of Costa et al (1982) showed the progression of cells through S-phase to be blocked by Cd^{2+}. Therefore it could be argued that a compound which only damages cells in S-phase would be more toxic in the presence of Cd^{2+}. RNA polymerase, at least from Escherichia coli, is also a zinc enzyme (Scrutton et al., 1971; Chatterji et al., 1984). 'In vitro' RNA synthesis is inhibited by Cd^{2+} (Hoffman and Niyogi, 1977) yet 'in vivo' a biphasic effect is seen (Stoll et al., 1976), low metal concentrations stimulating and higher concentrations inhibiting the process. The reduction in RNA biosynthesis may, in part, account for the depression of protein synthesis by Cd^{2+} 'in vitro' (Stoll et al., 1976; Norton and Kench, 1977) and 'in vivo' (Hidalgo et al., 1976).

If the production or activity of microsomal drug metabolizing enzymes were reduced by Cd^{2+} treatment there may be 'knock-on' effects on the activation/detoxification of xenobiotics. Indeed, there are reports of alterations in mixed function oxidase activity 'in vivo' (Kransy and Holbrook, 1977; Schnell et al., 1979; Murti et al., 1981; Lui et al., 1982; Williams+Menzel, 1982) and 'in vitro' (Colby et al., 1981, 1982,

1984; Fukuhara & Takabatake, 1982) which are irreversible on removal of Cd^{2+} by dialysis (Colby et al., 1982, 1984). The critical effect could be inactivation of the cofactor required for many of the drug metabolizing reactions, cytochrome P_{450} (Colby et al., 1984) rather than direct enzyme interaction.

Unlike compounds which are genotoxic due to the formation of DNA-reactive metabolites which could be affected by the above mechanism, another class of genotoxin promotes the production of superoxide radicals. These highly reactive radicals are able to cause DNA strand breakage. Superoxide radicals are generated by reactions involving the reduction of molecular oxygen (Fridovich, 1975, 1979) and are considered the main cause of oxygen toxicity (McCord et al., 1971). The cells of aerobic organisms are protected from superoxide radicals by superoxide dismutase. This enzyme reduces two superoxide ions in a reaction with two protons to hydrogen peroxide and molecular oxygen. Superoxide dismutase contains a copper (II) ion bound at its active site and a structural zinc ion (Hasson, 1980). Despite Cd^{2+} being able to replace Zn^{2+} in the enzyme 'in vitro' with no loss of activity (Forman and Fridovich, 1973), 'in vivo' Cd^{2+} inhibits superoxide dismutase function (Minami et al., 1982). Thus, the toxicity of superoxide radical producing agents could be potentiated by Cd^{2+}.

Membrane degradation by lipid peroxidation is mediated by free radicals (see, for example, Slater, 1979). Cd^{2+} can cause lipid peroxidation in isolated rat hepatocytes (Stacey et al., 1980) but, in this case, the role of free radicals appears not to have been investigated. Membrane degradation usually leads to leakage of enzymes and solutes from cells, this too occurs after addition of Cd^{2+} to liver cell cultures (Stacey et al., 1980, Santone et al., 1982). Membrane damage appears not to be the major cause of Cd^{2+} cytotoxicity as concomitant treatment of cells with the cation and chelating agents reduces its effect on viability but this does not correlate with a decrease in lipid peroxidation (Stacey et al., 1980).

The studies of Stacey et al. (1980) and Santone et al., (1982)

indicated that metabolic integrity, assessed by lactate:pyruvate ratios, was a more sensitive indicator of Cd^{2+} cytotoxicity than lipid peroxidation. The increased cellular lactate suggests mitochondrial function is impaired by Cd^{2+}. There are many reports of the uncoupling of oxidative phosphorylation by Cd^{2+} in isolated mitochondria (see review by Webb, 1979a). Although not directly linked to genotoxicity, any decrease in energy production would affect many other cellular functions by restriction of energy supply. For example, DNA synthesis and repair require a substantial energy input, therefore, the repair of DNA lesions will be less effective under conditions of restricted energy availability.

One further cellular system worth mentioning here is the cytoskeleton. Not only are cytoskeletal structures involved in the maintenance of cell shape, but also are essential for cell division. Free thiol groups are requied for the 'in vitro' assembly of microtubules from tubulin subunits (Timasheff and Grisham, 1980) and zinc has been implicated as a mediator of tubulin polymerization 'in vivo' (Hesketh, 1982). So, as one might expect, Cd^{2+} inhibits tubulin polymerization 'in vitro' (Wallin et al., 1977) and has a stathmokinetic (metaphase blocking) effect (Levan, 1945).

2.4 The carcinogenicity and genotoxicity of Cadmium

2.4.1 Studies of human populations

In 1965, Potts reported of an unusually high incidence of prostate cancer among workers in a nickel/cadmium battery plant. His recommendation for further studies into the correlation between cadmium exposure and cancer (especially of the prostate) has been followed up by several authors, see Table 2.3.

There are several points to be drawn from this table. Most importantly, several surveys suggest a link between cancer and cadmium exposure, however, the more recent studies do not provide confirmation. In all cases the exposed persons came into contact with chemicals other than cadmium, among these, many such as nickel, arsenic, benzene and cigarette smoke are known carcinogens. In the industrial studies, follow-

Table 2.3 Epidemiological surveys of cancer incidence among humans exposed to cadmium

AUTHOR	TYPE OF EXPOSURE (DURATION)	CANCERS CORRELATING WITH EXPOSURE	COMMENTS
Potts, 1965	Industrial (10yrs+)	Prostate, bronchus, carcinomatosis	Cd/Ni battery plant
Kipling and Waterhouse, 1967	Industrial (1 yr+)	no overall excess, but excess prostate	includes subjects from Potts, 1965
Humperdinck, 1968	Industrial (?)	none	
Holden, 1969	Industrial (?)	prostate, bronchus	
Kolonel, 1976	Industrial (?)	kidney	renal cancers only seen in smokers
Lemen et al., 1976	Industrial (2 yrs+)	all neoplasia, respiratory tract	also exposed to arsenic
McMichael et al., 1976	Industrial (?)	stomach, rectum, prostate, leukaemia, lymphosarcoma	also exposed to organics, e.g. benzene
Kjellstrom et al., 1979	Industrial (?)	none	Cd/Ni battery plant
Holden, 1980	Industrial (1 yr+)	no overall excess, but increased leukaemia	Cd/Cu alloy plant
		respiratory system genito-urinary tract	also exposed to As, Ag, Ni
Armstrong and Kazantzis, 1983	Industrial (11yrs+)	none	mixed exposure
Sorahan and Waterhouse, 1983	Industrial	none	follow up to Kipling & Waterhouse, 1967
Berg and Burbank 1972	Environmental water supply	gastro-intestinal tract, breast, bladder, myeloma lymphoma	correlation between metal levels in drinking water and cancer incidence drawn
Bako et al., 1981	Environmental water supply	prostate	
Inskip et al., 1981	Environmental	none	Shipham population
Lauwerys & DeWalls, 1981	Environmental atmosphere	prostate	survey of cancer incidence in Liege, an area with Cd pollution
Iwao et al., 1983	unknown, if any	none	determined Cd content of liver and kidneys of cancer patients

up periods were short considering the long latent periods needed before the expression of many cancers. Notwithstanding these confounding factors, the IARC (1976) consider cadmium a probable human carcinogen.

2.4.2. Animal Studies

Ryan et al (1982) have reviewed the literature concerning the carcinogenicity of cadmium in experimental animals up to 1980. Studies up to that time demonstrated the following effects of cadmium:

i) Subcutaneous, intramuscular or intratesticular injection of cadmium metal or salts causes injection site tumours.
ii) Subcutaneous injection of high doses of Cd^{2+} can cause testicular tumours distal to the injection site.
iii) Tumours mentioned in i) and ii) are not malignant.
iv) Several long term feeding and inhalation studies could not demonstrate an increase of tumours in rodents or dogs.

More recently two further reports have been published. Poirier et al (1983) confirm findings (i) and (ii), in addition pancreatic island tumors were induced distal to the injection site in rats. Takenaka et al (1983) conducted a long term inhalation study in rats, unlike previous investigations, animals inhaling $CdCl_2$ aerosols had a much higher incidence of lung cancers than controls.

There is no doubt that cadmium and its salts can cause injection site tumors. However, the relevance of such experiments to human exposure is questionable. Many, otherwise non carcinogenic, agents (e.g. inert plastics) can induce implantation/injection site tumors. The recent positive findings of Takenaka et al (1983) are possibly more relevant than previous inhalation studies which have been criticized as being inadequate (IARC, 1976).

2.4.3 The genotoxicity of cadmium

Genotoxicity is defined as the alteration of the structure and/or function of DNA by a chemical or physical agent. Such changes are now

considered essential for cell transformation and carcinogenesis.

Metal-induced alteration to the molecular structure of DNA 'in vitro' has been reviewed extensively by Daune (1974) and Jacobson and Turner (1980). Magnesium, calcium, zinc, copper, iron and manganese ions are found naturally in DNA and are considered necessary for the maintenance of its structure. Mg^{2+} and Ca^{2+} coordinate with phosphate groups stabilizing the double helical structure; Zn^{2+}, Ca^{2+}, Fe^{2+} and Mn^{2+}, being softer in character, are able to complex with both phosphate groups and bases. Cd^{2+} is softer than the latter group of ions so preferentially binds to bases. Cd^{2+} binding to DNA can take the form of internal chelates, inter- and intra-strand crosslinks, and base-phosphate crosslinks. The effect of these complexes is to destabilize the double helix and so decrease the thermal stability of DNA. Detailed analysis of the Cd-base complexes has been made by X-ray diffraction studies of Cd-cytidine monophosphate and Cd-inosine monophosphate crystals. The structure of the Cd-cytidine monophosphate crystal is such that, if similar complexes formed in DNA, normal hydrogen bonding between complementary bases would not occur. As a consequence, base mispairing could occur 'in vivo'.

Such metal DNA interactions are one of the possible causes of Cd^{2+} induced infidelity of DNA synthesis (Zakour et al., 1981). Work from the laboratory of Sirover and Loeb (reviewed by Zakour et al., 1981) has demonstrated the ability of several metal ions, including Cd^{2+}, to increase the frequency of errors (incorporation of non complementary nucleotides) during the replication of artificial DNA templates 'in vitro'. Similar perturbations in the synthesis of natural DNA templates are induced by manganese cobalt and chromium (Cr^{3+} and Cr^{6+}) ions. Metal binding to nucleotide precursors or DNA polymerase (see Section 2.3) could also account for this infidelity. Whatever the mechanism, the fact that Cd^{2+} can cause errors in DNA replication may underlie some of the other genotoxic phenomena described below.

The techniques of alkaline sucrose gradient centrifugation and alkaline elution have been used to detect structural alterations to DNA in

bacteria and cell cultures. Both methods determine the extent of DNA strand breakage, the former by the buoyant density of DNA molecules and the latter by the retention characteristics of DNA on polycarbonate filters. The experiments of Zasukhina et al (1977) demonstrated the capacity of Cd^{2+} to degrade the DNA of human cells. Mitra and Bernstein (1978) used alkaline sucrose gradient centrifugation to show DNA single strand breakage occurred in E. coli treated with Cd^{2+}. These two early reports have been extended recently by Robison et al (1984). Cultures of Chinese hamster ovary cells were exposed to particulate (crystalline CdS, 10μg/ml, 24 hours) or soluble ($CdCl_2$, 1-100μM, 3-4 hours) cadmium compounds. Both these treatments led to decreases in the average molecular weight of nuclear DNA as determined on alkaline sucrose gradients. A more detailed analysis of the nature of these DNA strand breaks utilized the technique of alkaline elution. The DNA from lysates of V79 cells treated with 20-100μM Cd^{2+} for two hours showed a decreased retention time only after proteinase K digestion (Ochi and Ohsawa, 1983). This is indicative of DNA-protein cross-links being present which mask the strand breaks until removed by the enzyme treatment. The strand breaks appeared to be entirely single stranded in nature (Ochi et al., 1983b). Strand breakage has also been observed in isolated rat hepatocytes after exposure to 0.03 - 0.3mM $CdSO_4$ for three hours (Sina et al., 1983). It has been suggested that these single strand breaks are not a direct effect of the Cd^{2+} but due to oxygen free radicals (Ochi et al., 1983a). No strand breakage was seen in anaerobically grown cells. The implication of this work is that Cd^{2+} causes or calalyses the production of oxygen free radicals, or prevents their inactivation by superoxide dismutase (see Section 2.3).

The mechanism involved in the repair of these cadmium induced lesions is a subject of contention; furthermore, whether or not the damage is repaired at all is disputed. Repair deficient strains of Bacillus subtilis and E. coli are more prone to killing by Cd^{2+} (Nishioka, 1975; Kanematsu et al., 1980; De Flora et al., 1984). The recovery of viability of E. coli after Cd^{2+} exposure correlates with a return to the normal molecular weight of the bacterial DNA (Mitra and Bernstein, 1978). So, bacteria do seem able to repair Cd^{2+}-induced DNA damage; on the other

hand, the situation in eukaryotes is less clear. DNA damage to human cells is apparently not repaired despite repair systems for gamma radiation damage being intact (Zasukhina et al., 1977). Similarly, unscheduled DNA synthesis is not observed in Cd^{2+} treated human fibroblasts (Hollstein and McCann, 1979). However, unscheduled DNA synthesis does occur in Cd^{2+} treated Petunia pollen (Jackson and Linskins, 1982) and Robison et al (1984) found the cation to be a very effective inducer of excision repair in Syrian hamster embryo cells. Ochi et al (1983b) have also produced evidence that Cd^{2+} induced strand breakage is repaired by an excision repair pathway. Yet, in another paper, Ochi and co-workers (1983a) argue that excision repair is not involved as the damage is oxygen free radical induced so should be repaired by an X-ray repair-like system. In support of the latter hypothesis are the experiments of Mitra and Bernstein (1977). Repair enzyme-deficient mutants of E. coli were employed to investigate the mechanism of accomodation to growth in Cd^{2+} containing media. Most wild type treated cells died and the viability of the surviving fraction was much reduced; with time, viability increased and division recommenced. If bacteria lacking functional DNA polymerase I were treated, the accommodation process occurred as in wild type cells. DNA ligase deficient mutants, however, could not accommodate to growth in Cd^{2+}. Excision repair is thought to require both polymerase and ligase enzymes while X-ray-like repair needs only DNA ligase.

Studies of the effects of Cd^{2+} on chromosome structure have produced mixed results. Experiments with several plant species (thoroughly reviewed by Degraeve, 1981) demonstrated Cd^{2+} to disrupt the mitotic spindle and cause chromosome breakage. The concentrations of Cd^{2+} used in these studies, where stated, tend to be high and are probably acutely toxic. These positive findings in plants have not always been borne out in mammalian cell studies (see Table 2.4). As in cancer epidemiology studies (Section 2.4.1), the effect of Cd^{2+} exposure on aberration induction in those who have contacted the ion in their environment is obscured by concurrent exposure to other chemicals. Further doubt is cast by the 'in vivo' assays, these are on the whole negative. 'In vitro' studies also have generally yielded negative data,

Table 2.4 Chromosomal aberration induction in mammalian cells by cadmium

AUTHOR	CELL TYPE	EXPOSURE PROTOCOL	EFFECT
(i) 'in vitro'			
Shirashi et al., 1972	human lymphocytes	0.3 μM $CdSO_4$	+ numerous types
Patton and Allison, 1972	human fibroblasts MRC5 and W138, human lymphocytes	2 unspecified concentrations, 24, 48 or 72 hours, several weeks	-
Rohr and Bauchinger, 1976	Chinese hamster 'Hy' cells	0.01 - 500 μM $CdSO_4$, 1 hour + various recovery periods	+ mainly chromatid type
Zasukhina et al., 1977	primary rat embryo cells	3.5 - 25 μM $CdCl_2$	-
	as above, virus infected	3.5 - 25 μM $CdCl_2$	+
Deknudt and Deminatti, 1978	human lymphocytes	5 - 250 μM; Cd^{2+}, 48, 72 hours	-
Umeda and Nishimura, 1979	mouse carcinoma, Fm3A	10 - 50 μM, Cd^{2+} 24, 48 hours	-
Deavan and Campbell, 1980	Chinese hamster ovary	1 μM $CdCl_2$ 48 hours	+ chromosome shattering by 48 hrs.
		0.1 μM $CdCl_2$ 12 weeks followed by 2 uM $CdCl_2$ 48 hours	-
ii) 'in vivo'			
Doyle et al., 1974	sheep lympho cytes	diet containing 60 μg Cd/g, 191 days	± numerical aberrations only
Gillavod and Leonard, 1975	mouse spermatocytes	0.5 - 3 mg $CdCl_2$/kg, subcutaneous	- no increase in translocations
Shimida et al., 1976	mouse oocytes	2 - 6 mg $CdCl_2$/kg, subcutaneous	± numerical aberrations at 12 hrs
Bruce and Heddle, 1979	mouse bone marrow	1-6 mg/kg subcutaneous	- no increase in micronuclei
Deknudt and Gerber, 1979	mouse bone marrow	0.06% Cd^{2+} in diet, 1 month	-
Watanabe et al., 1979	golden hamster oocytes	1 - 4mg/kg subcutaneous	± minimal aberrations only
iii) environmental exposure			
Shiraishi and Yosida, 1972 Shiraishi, 1975	human lymphocytes	12 Itai-Itai disease patients	+ significant increases in chromosome breaks and exchanges
Deknudt et al., 1973	human lymphocytes	14 zinc industry workers with signs of lead poisoning	+ probably due to lead
Leonard et al., 1974	cattle lymphocytes	cattle fed hay grown close to a metal refinery	-
Bui et al., 1975	human lymphocytes	4 Itai-Itai disease patients and 5 industrially exposed workers	-
Deknudt and Leonard, 1976	human lymphocytes	35 workers exposed to Cd, Pb & Zn	+ high frequency of gross chromosomal aberrations
Bauchinger et al., 1976	human lymphocytes	24 zinc workers with elevated blood Cd + Pb	+ mainly chromatid type
O'Riordan et al., 1978	human lymphocytes	Cd pigment workers	-

but there are some positive findings. Some of the variability in 'in vitro' experiments may be due to the amount and type of serum used in the culture medium as suggested by Deavan and Campbell (1980). Differences in exposure protocol may also play a role as might species and cell type differences. Factors such as metallothionein induction in different species and cell types have not been correlated with chromosomal aberration induction. There is only one mention in the literature of the effect of Cd^{2+} on sister chromatid exchange. Deavan and Campbell (1980) did not detect any increases in SCE frequency in CHO cells treated with 0.4 μM $CdCl_2$.

The classic test for mutagenesis, the Ames test, is not good at detecting mutagenic metals (Sirover, 1981). With the range of Salmonella strains available, this test has consistently given negative results for Cd^{2+} (Heddle and Bruce, 1977; Kalinina and Polukhina, 1977; Milvy and Kay, 1978; Bruce and Heddle, 1979; DeFlora et al., 1984; Sokolowska and Jongen, 1984). A host mediated Salmonella mutagenicity assay in mice has given a positive response (Kalinina et al., 1977 cited by Degraeve, 1981). Reversion to tryptophan prototrophy is not induced by Cd^{2+} in E. coli (Vennit and Levy, 1974). Eger and Doko (1980) found Cd^{2+} to cause gene conversion in yeast, however other investigators have failed to repeat this observation or show the metal to induce mutation in yeast (Putrament et al., 1977; Singh, 1983). An early report suggested Cd^{2+} was not mutagenic to mammalian cells (Nishimura and Umeda, 1978). Subsequent studies have shown many metals, including Cd^{2+} to be mutagenic at the HGPRT locus, located on the X chromosome (Hsie et al., 1979; Oberly et al., 1982, Ochi and Ohsawa, 1983) and the TK locus (Amacher and Paillet, 1980) of mammalian cells. 'In vivo' recessive lethal mutations are not seen in Cd^{2+} exposed rodents (reviewed by Degraeve, 1981) nor are morphologically abnormal sperm (Heddle and Bruce, 1977; Bruce and Heddle, 1979). One sperm abnormality assay cited by Wyrobeck et al (1983), however, does show a positive effect of Cd^{2+}.

The most consistent 'in vitro' assays for metal carcinogens are the cell transformation systems (Costa, 1980). Cadmium salts can enhance viral transformation of Syrian hamster embryo cells (Casto et al., 1976,

1979) and the spontaneous transformation of these cells (DiPaolo and Casto, 1979). Although in some ways similar to the process of carcinogenesis, 'in vitro' cell transformation is considered: "a highly artefactual system designed to detect alterations in cell growth parameters which may or may not be related to carcinogenesis 'in vivo'". (Heck and Costa, 1982). None the less, there is a good correlation between 'in vitro' transformation and whole animal carcinogenesis for metal compounds (Heck and Costa, 1982).

The investigations cited above demonstrate that Cd^{2+} shows genotoxic activity in many systems such as DNA binding and strand breakage, reduced fidelity of DNA synthesis, mammalian cell mutation and 'in vitro' transformation. The more established assays, for example, mutation in Salmonella and chromosomal aberration induction have tended to yield negative or inconclusive results. It must be borne in mind that most 'in vitro' testing has involved large organic molecules, thus the assays have probably been selected for their ability to detect the effects of these compounds rather than those of small metal ions.

2.5 Can Cd^{2+} alter the effect of other genotoxins?

The discussion in section 2.3 pointed to many effects of Cd^{2+} which could affect the action of other chemicals, especially genotoxins. There is some evidence, as yet rather fragmented, suggesting Cd^{2+} does indeed alter the effects of other genotoxins.

Recently, much interest has focused on the toxicity of cancer chemotherapeutic drugs to cells with increased metallothionein levels. As metallothionein is rich in nucleophilic thiol clusters and many chemotherapeutic drugs are active electrophiles, the protein could react with, and trap, the drugs in the cytoplasm. The nucleus is the target for anticancer drugs so the above mechanism should reduce their toxicity. This idea is basically an extension of that put forward by Connors (1968) who argued that thiols are able to protect cells from alkylation damage by thiol-alkylator binding. Thus, the alkylatiang agent would be deactivated prior to causing DNA damage. The anti-cancer drugs used in these

experiments have all been alkylating agents. Cis-dichlorodiammine-platinum (Bakka et al., 1981), iodoacetate (Tobey et al., 1982b), chlorambucil (Endresen et al., 1983), melphalan (Tobey et al., 1982a, 1983) and prednimustine (Endresen, 1984) are all less toxic to uninduced or zinc-induced Cd^{2+} resistant cells. In the case of cis-dichlorodiammine-platmium, about 70% of the drug which entered the cells was bound to metallothionein (Bakka et al., 1981). Thus, there appears to be a role for metallothionein in the reduction of toxicity. 'In vitro' binding of alkylating agents to metallothionein has also been demonstrated (Cagen and Klaassen, 1980) as has induction of the protein in rat liver (Kotsonis and Klaassen, 1979) and normal Hepa 1A mouse hepatoma cells in culture (Durnham and Palmiter, 1984) by alkylating agents. However, Tobey et al (1982a) were unable to directly correlate cytosol metallothionein content with reduced iodoacetate toxicity in variant CHO cells with a range of metallothionein induction capacities. A better correlation has been found between melphalan induced cell killing and cellular glutathione levels (Seagrave et al., 1983).

In contrast to the above, studies quoted by Degraeve (1981) showed plants simulataneously treated with Cd^{2+} and ethyl methanesulfonate or gamma radiation had synergistic yields of chromosome aberrations and chlorophyll mutations respectively. In contrast Cd^{2+} antagonized the clastogenic action of ethylenimine in plants and had no effect on the incidence of dominant lethal mutation in mice caused by gamma rays. In relation to the radiation effects, Kominami et al (1976) found Cd^{2+} to reduce the formation of radicals and strand breaks in gamma irradiated DNA. This finding is hard to reconcile with the observed increases in chlorophyll mutations in irradiated Cd^{2+} treated plants. Clearly, the interactions of Cd^{2+} with radiation and alkylating agents are not simple.

More recent work has demonstrated that cadmium acetate can synergistically enhance the transformation of Syrian hamster embryo cells by benzo(a)pyrene, N hydroxy-2-acetylaminofluorine and 4-nitroquinoline-1-oxide (Rivedal and Sanner, 1981). These authors also showed that treatment of cells with benezo(a)pyrene followed by Cd^{2+} had a greater

transforming activity than the reciprocal treatment, so implying that Cd^{2+} is a more effective promoter, rather than initiator, of transformation. It would appear that mutation and cell transformation are independent, treatment of V79 cells with Cd^{2+} after benzo(a)pyrene only has an additive effect on HGPRT mutation (Ochi and Osawa, 1983), no interaction was observed between benzo(a)pyrene and Cd^{2+} in the Ames test (Sokolowska and Jongen, 1984). However, chromate mutagenicity is decreased by concurrent exposure of Salmonella tester strains to cadmium salts (Sokolowska and Jongen, 1984). Cd^{2+} can also protect against aflatoxin (a potent hepatocarcinogen) induced liver damage (Cain and Griffith, 1980). Treatment of mice with combinations of Cd^{2+} and dimethylnitrosamine, on the other hand, has a synergistic effect on the formation of micronuclei in bone marrow cells (Watanabe et al., 1982).

The work discussed in the preceeding paragraphs has shown that answer to the question posed in the title of this section to be yes. However, the nature of the interactions seems to vary with the chemical considered and the assay used. Clearly, there is a need for a systematic survey of cadmium/genotoxin interactions.

2.6 DNA binding and effects of the genotoxins other than cadmium used in this study

Having discussed the relevant aspects of cadmium toxicology, consideration will now be given to the other compounds used in the present work. These compounds may be broadly categorized as those that form covalent bonds to nucleic acids (i.e. the alkylating agents, ethyl methanesulfonate, mitomycin C), organic molecules which bind non covalently (acridine orange, Hoechst 33258) and the metal ion Ni^{2+}.

2.6.1 Alkylating agents

Alkylating agents are defined as chemicals which can form covalent bonds to DNA, attaching a methyl or higher alkyl group. The term is sometimes used to cover compounds which link more complex aliphatic, heterocyclic and aromatic groups to DNA. The mechanism and effects of DNA

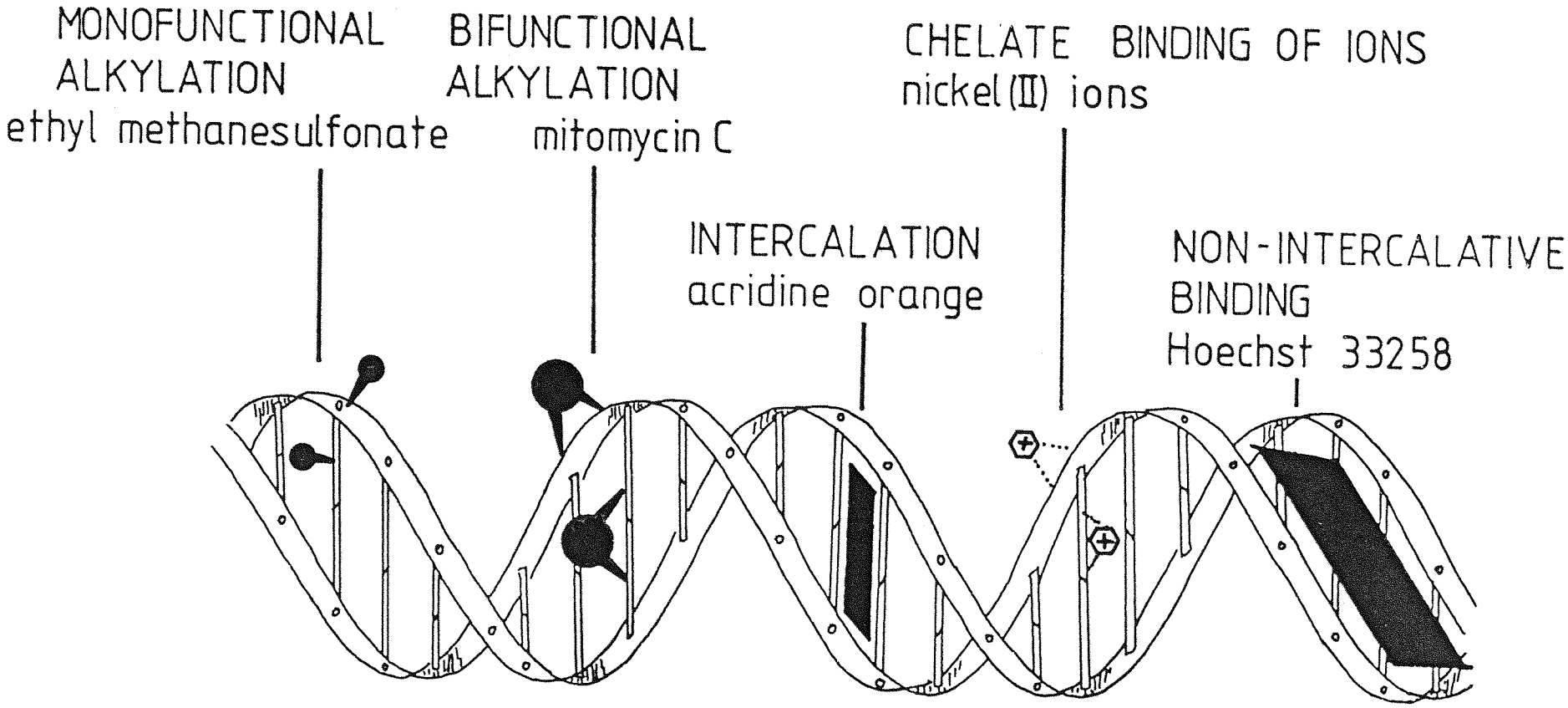
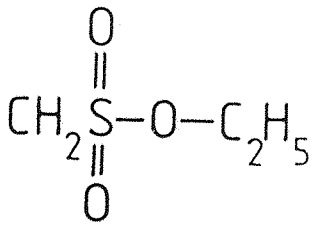
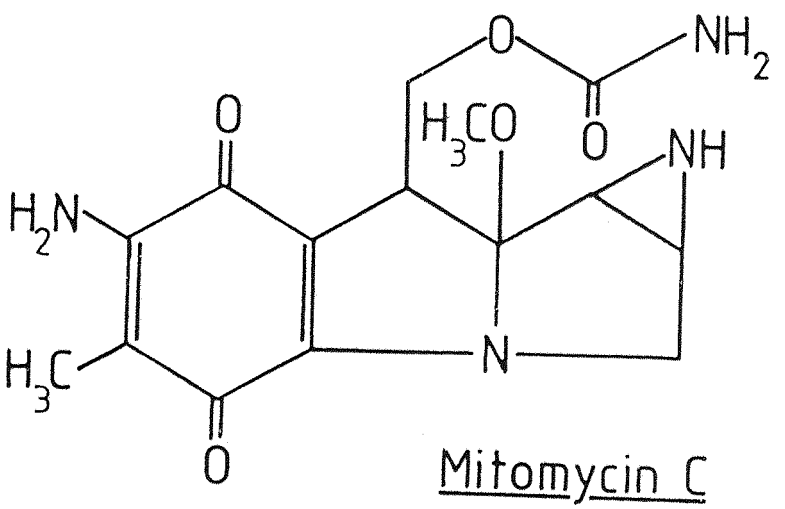


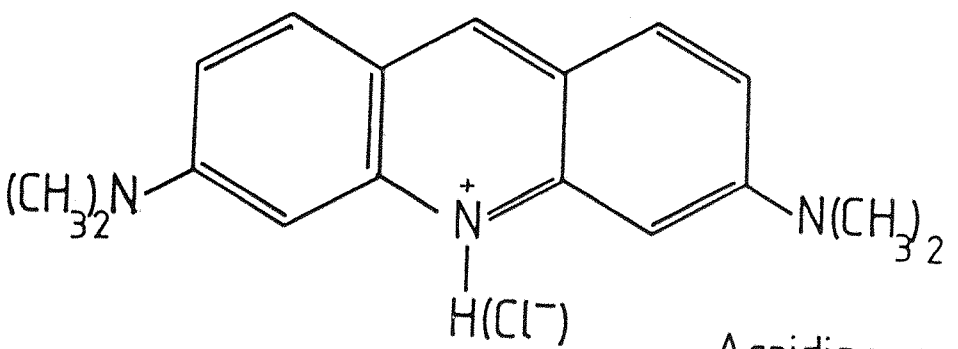
Figure 2.1. Diagramatic representation of the binding of EMS, MMC, AO, H33258 and Ni^{2+} to DNA.



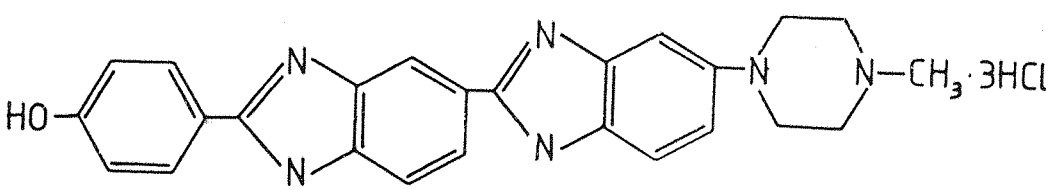
Ethyl methanesulfonate



Mitomycin C



Acridine orange



Hoechst 33258

Figure 2.2. Structural formulae of ethyl methanesulfonate, mitomycin C, acridine orange and Hoechst 33258.

alkylation have been covered in some detail by Lawley (1980) and Hemminki (1983).

By nature, alkylating agents are strong electrophiles so tend to react with nucleophilic sites in nucleic acids. The N7 position of guanine is the most nucleophilic centre in DNA; other ring nitrogens, hydroxyl groups and amino groups in all the bases of DNA are also nucleophilic in character. As well as the nucleophilicity and steric availability of a nucleophilic centre, the rate of its alkylation can depend on the electrophile alone (SN1 mechanism) or both electrophile and nucleophile (SN2 mechanism). Compounds which act by an SN2 type reaction preferentially react with centres of high nucleophilic strength such as N7 guanine. On the other hand, agents which react by SN1 kinetics are less selective for nucleophilic strength so can alkylate exocyclic O and N groups.

In aqueous solution EMS dissociates into an ethyl ion ($C_2H_5^+$) and a negatively charged methyl sulfonate ion ($CH_3SO_3^-$). The ethyl ion is the active alkylating species. Alkyl sulfonates react by the SN2 mechanism but, with increasing alkyl chain length, the reactions gain more SN1 character. Thus, EMS produces a wide range of alkylation products: 81% of the alkylations are to N7 guanine, 2.2% to N3 adenine, 0.7% to N7 adenine, 0.8% to N3 guanine, 0.3% to O^6 guanine, and 0.1% to both N1 adenine and N3 cytosine.

MMC is an antibiotic produced by Streptomyces Caespilosus and S. verticillatus. In itself, MMC is not an alkylating agent but requires metabolism, as shown in figure 2.3, to the active form. These reactions apparently are not restricted to certain cell types but are common to prokaryotic and eukaryotic cells (Szybalski and Iyer, 1964). Metabolized MMC has two nucleic acid - reactive sites, thus is able to form DNA-DNA, DNA-protein and protein-protein crosslinks. Less is known of the chemistry of alkylation by MMC, O^6 guanine adducts are thought to be formed but N7 guanine is probably not a major product.

Table 2.5 summarizes the results of several studies of the

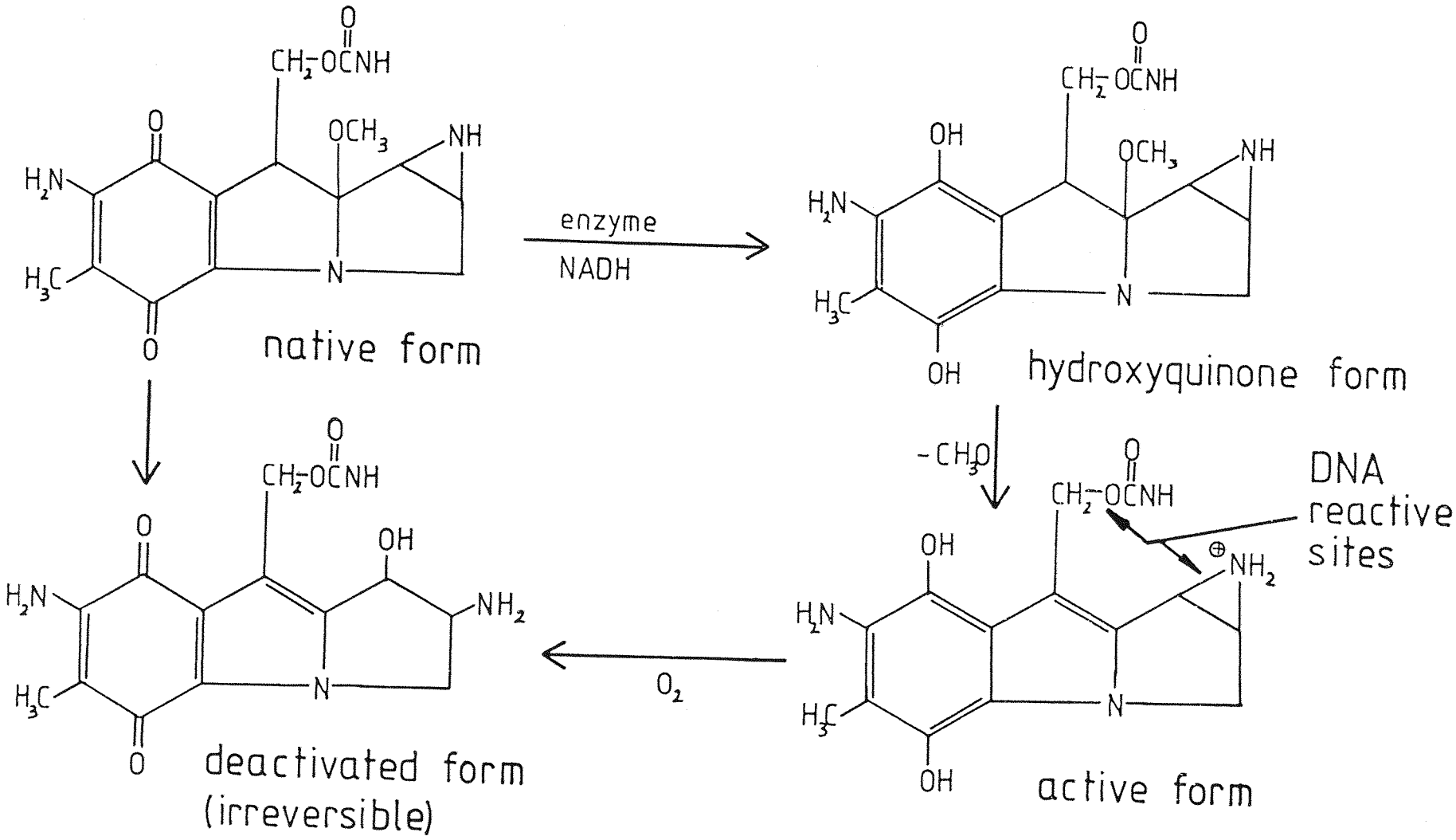


Figure 2.3. Metabolic activation and deactivation of mitomycin C.

genotoxicity of EMS and MMC. Clearly, both are mutagenic and able to induce chromosomal aberration and SCE.

2.6.2 Acridine orange and Hoechst 33258

Acridine orange and Hoechst 33258 represent two classes of non-covalent DNA binding agents. AO is an example of an intercalating agent while H33258 binds to DNA in a non-intercalative fashion.

Physical chemical studies of the AO-DNA interaction (reviewed by Nasim and Brychcy, 1979) have demonstrated two forms of DNA binding. The first mode of binding is relatively strong and involves one AO molecule per 4-5 DNA base pairs. This interaction leads to increased rigidity, viscosity and radius of gyration of DNA - all indicative of DNA intercalation. For every AO molecule intercalated the DNA chain increases by 3-4 Å in length, an equivalent extension would occur with the addition of another base pair. Thus, the 'reading frame' of DNA is altered during replication and transcription. The second form of AO-DNA binding is weaker and only occurs at high AO/DNA ratios, so is probably not important 'in vivo'. It involves interaction with two non complementary bases on the same DNA strand.

Reference to table 2.5 shows that AO has been tested in several systems and has many genotoxic effects. On the other hand, H33258 has been less well studied but is known to cause SCE's and chromosome despiralization. H332558 has been used more as a probe of DNA and chromosome structure (Latt, 1978).

The binding of H33258 and several other non-intercalative DNA binding agents has been reviewed by Krey (1980). The H33258 molecule apparently binds along the grooves of the DNA helix and shows some preference for A:T rich sequences.

2.6.3 Nickel ions

Although in the class of non covalent DNA binding agents, nickel (II)

ions warrant separate consideration. The DNA binding and effects of metal ions are different from organic compounds in many respects.

In Section 2.3 the concept of hard and soft ions and its relevance to the interaction of metal ions with biological systems was discussed. Ni^{2+} is considered intermediate ($\overline{\sigma p}$ = 0.126) on the hard/soft scale, so tends to bind to DNA phosphate groups. As well as causing DNA strand breakage (table 2.5), nickel compounds can form DNA-protein crosslinks (Ciccarelli et al, 1981).

Ni^{2+} has been found to be mutagenic to mammalian cells (Table 2.5) but, in common with other metal ions, variable results have been obtained in a number of other genotoxicity assays. Nevertheless, some nickel compounds are carcinogenic to rodents and respiratory tract cancers are unusually common among nickel refinery workers (see review by Leonard et al., 1981). The carcinogenicity of nickel compounds inversely correlates to their solubility in aqueous solutions (Leonard et al., 1981; Kasprzak et al., 1983). The chemical and physical form of nickel also affects its 'in vitro' cell transformation activity (see review by Heck and Costa, 1982). Thus, crystaline NiS, αNi_3S_2 and Ni_3Se_3 are more potent transforming agents than amorphous NiS, Ni metal, Ni_2O_3 and NiO. The crystalline compounds have been shown to be more readily phagocytosed by cells in culture so it has been suggested that the transforming activity of the crystalline compounds is greater due to their entering cells more readily. A model has been proposed (Evans et al., 1982) that once in the cell, the nickel particles slowly dissolve releasing Ni^{2+} which is the active species.

Table 2.5 Activity of test compounds in some genotoxicity assays

ASSAY \ CHEMICAL	EMS	MMC	AO	H33258	Ni^{2+}
Bacterial Mutation	+ (1)	±(1,2)	+ (1)		± (3,4)
Yeast Mutation	+ (5)	+ (5)			
Yeast Gene Conversion	+ (6)	+ (6)	+ (6)		
Mammalian Cell Mutation	+ (7)	+ (7)			+ (3)
Sperm Morphology	+ (8)	+ (8)			
Cell Transformation	+ (9)		+ (9)		+ (9)
In vivo Micronucleus formation	+ (10)	+ (10)			
In vivo Chromosome aberration	+ (11)	+ (11)			- (12,13)
In vitro Chromosome Aberration	+ (11)	+ (11)	+ (14)	± (15)	± (16,17)
In vitro Sister Chromatid Exchange	+ (18)	+ (18)	+ (18)	+ (18)	+ (19,20)
DNA Strand Breakage in Mammalian Cells	+ (22)	+ (22)			+ (22)
DNA repair synthesis	± (23)	± (23)			± (3,24,25)
Fidelity of DNA synthesis					+ (26)
DNA damage in repair defficient bacteria	+ (21)	+ (21)	+ (21)		- (21,4)

KEY

+ positive response
± marginal response or variable data
- negative response

NUMBERS IN BRACKETS REFER TO THE FOLLOWING REFERENCES:

1. McCann et al (1975)
2. Purchase et al (1978)
3. Hollstein and McCann (1979)
4. DeFlora et al (1984)
5. Brockman et al (1984)
6. Zimmerman et al (1984)
7. Clive et al (1983)
8. Wyrobek et al (1983)
9. Heidelberger et al (1983)
10. Heddle et al (1983)
11. Preston et al (1981)
12. Mathur et al (1978)
13. Waksvik and Boysen (1983)
14. Nasim and Brychcy (1979)
15. Rocchi et al (1976)
16. Nishimura and Umeda (1979)
17. Christie and Costa (1983)
18. Latt et al (1981)
19. Wulf (1980)
20. Newman et al (1982)
21. Leifer et al (1981)
22. Sina et al (1983)
23. Mitchell et al (1983)
24. Jackson and Linskins (1982)
25. Robison et al (1984)
26. Zakour et al (1981)

Chapter 3. Materials and Methods

3.1 Treatment Chemicals

3.1.1 Sources and preparation of solutions

Cadmium acetate (Analar grade; B.D.H., Poole) was prepared as a 0.1 M solution in 0.1 M HCl, further dilutions to 10^{-3}, 10^{-4} and 10^{-5} M were made in distilled water. All cadmium solutions were stored in polyethylene bottles at 4°C to prevent absorbtive loss of Cd^{2+} to the vessel surfaces. Fresh solutions were prepared every three weeks. Over this time the concentration of Cd^{2+} remained constant as judged by atomic absorption spectrophotometry (assays courtesy Dr. H.T. Delves, Department of Chemical Pathology, Southampton General Hospital).

Ethyl methanesulfonate (Koch-Light Laboratories, Colnbrook) was made up as 1M and 0.1M solutions in dimethyl sulfoxide and stored at 4°C. Solutions of not more than one month old were used.

Working solutions of 1.8×10^{-5} M and 1.8×10^{-6} M mitomycin C (Sigma, Poole) were made up in distilled water from a concentrated aqueous stock of 1.8×10^{-3} M (0.61 mg/ml). The working solutions were prepared within one hour of use.

Aqueous stock solutions of 10^{-2} M acridine orange (Sigma) were stored at 4°C in the dark for up to three months. Dilution to 10^{-3} and 10^{-4} M was made within one hour of use.

Hoechst dye 33258 (Calbiochem, La Jolla, USA) stocks of 10^{-3} M in distilled water were kept at 4°C in the dark for not more than two months. Dilution in distilled water to 10^{-5} M was made within an hour of use.

Nickel sulfate ($NiSO_4 . 7H_2O$, Analar grade, BDH) solutions were prepared within one hour of use. Aqueous working solutions of 10^{-3} and 10^{-4} M were made from an initial solution of 0.1M in 0.1M HCl.

All chemicals were sterilized by filtration through 0.22μm pore size Millipore filters prior to addition to cell cultures. In all cases treatments were added in a volume of 100μl or less per 5ml culture medium.

3.1.2 Handling precautions

All the above chemicals are known or suspected carcinogens, thus, care must be exercised in their handling and disposal. The guidelines of Ehrenberg and Wachtmeister (1977) were followed.

3.2 Cells and Cell Culture

A permanent cell line of dermal fibroblasts derived from a male Indian muntjac (Muntiacus muntjak; American Type Culture Collection number CCL 157) was obtained from Flow Laboratories (Irvine, Scotland).

3.2.1 Routine Maintenance

In order to maintain sterile culture conditions, all handling of tissue cultures was carried out in a Holten-Lamin Air HLB 2448 tissue culture cabinet.

Cell monolayers were grown in 25cm^2 area plastic tissue culture flasks (Sterilin, Teddington) containing 5 ml HEPES buffered Eagles Minimal Essential Medium with Earles Salts (Flow) supplemented with 10% foetal calf serum (Sera Labs, Crawley Down or Imperial Labs, Salisbury) and 1% antibiotic-antimycotic solution (Gibco, Paisley, Scotland; final concentrations: 100iu/ml penicillin, 100μg/ml streptomycin, 2.5μg/ml Fungizone). The cultures were kept in a 37^{o}C incubator. Culture medium was changed every 3 - 4 days. At weekly intervals the cells were subcultured by the following method:

1. Remove old medium from flask.
2. Wash cells twice with Ca^{2+}, Mg^{2+} - free Hanks balanced salt solution (HBSS, Flow).

3. Add 0.5 ml trypsin-EDTA solution (Gibco, 0.05% 1:250 trypsin + 0.02% EDTA) incubate at 37^{o} for about ten minutes.
4. When all cells have detached from their substratum (this may be checked under an inverted phase contrast microscope) add 24.5ml complete culture medium and disperse cells by gentle pipetting.
5. Place 5ml aliquots of cell suspension into each of four new flasks. Weekly subculture at a 1:5 split ratio gave an initial cell density of 1.5-2 x 10^{4} cells per flask.

3.2.2 Frozen stocks

A stock of cells was kept in a liquid nitrogen freezer. The methods described below are modified from Paul (1975).

To freeze cells down, log-phase cultures were trypsinized as in Section 3.2.1, HBSS was used to suspend the cells instead of culture medium. The suspension was transferred to a sterile centrifuge tube and the cells pelleted at 1200 rpm for 5 minutes in a bench centrifuge. After removal of the supernatant, cells were suspended in culture medium containing 10% (v/v) sterile dimethyl sulfoxide. 1 ml aliquots of this suspension were sealed in sterile plastic freezer vials (Flow). Cell viability is highly dependant on the rate of freezing so this step was carefully controlled. The samples were placed in a thick walled expanded polystyrene box either in a -80^{o}C freezer for two hours or kept at 4^{o}C for one hour then -20^{o}C for two hours. Following initial freezing, the samples were removed from the polystyrene box and stored in the vapour phase of liquid nitrogen.

To recover cells from the frozen state, vials were rapidly thawed at 37^{o}C, the cell suspension pipetted into a sterile centrifuge tube and pelleted as before. Cells were resuspended in culture medium and plated out.

3.3 Cell Growth and Viability Studies

To determine suitable concentrations of Cd^{2+} to use in subsequent

experiments, long and short term cell growth and viability studies were carried out.

2.5 mls of 4 x 10^3 cells/ml cell suspension in complete medium were plated in 3.5cm diameter plastic tissue culture petri dishes (Sterilin). After 24 hours incubation to allow cells to settle and spread, cadmium acetate was added to give final concentrations of 0 - 10^{-4} M. Following varying culture times, cells from replicate dishes were trypsinized and suspended in a known volume of HBSS. A small volume (1 drop or less per ml) of trypan blue (Flow) was added to the cell suspension. The total number of cells and those stained blue (dead) were determined in a Neubauer counting chamber under a phase contrast microscope. Cells were counted within ten minutes of placing in trypan blue as the stain can penetrate viable cells after prolonged incubation.

For short term experiments, samples were taken daily for seven days; long term assays extended over twenty eight days and sampling was less frequent.

3.4 Production and Characterization of Cadmium Resistant Cells

3.4.1 Production

A cadmium resistant variant of muntjac cells was produced by long term culture of cells in low Cd^{2+} concentrations followed by selection in higher concentrations of the ion.

For four weeks cells were grown in 10^{-7} M Cd^{2+} followed by four weeks in 5 x 10^{-7} Cd^{2+} then a further four weeks in 10^{-6} M Cd^{2+}. When the metal concentration was raised to 2 x 10^{-6} M, considerable cell death was seen but a few cells continued to proliferate. Once the cells were growing well in 2 x 10^{-6} M Cd^{2+} the concentration was raised to 3 x 10^{-6} M Cd^{2+}. The metal concentration in the culture medium was increased in a similar fashion until the cells were adapted to 5 x 10^{-6} M Cd^{2+}. This cadmium resistant variant of muntjac has been denoted CR5.

3.4.2 Characterization

As well as being used in the experiments described in the following sections, the CR5 cells were characterized by a number of other criteria.

3.4.2.1 Anchorage independent growth

The technique of Barrett (1978) was used to compare the growth of normal and CR5 cells in semi-solid medium. Briefly, the method involves the following steps:

1. Prepare 1.2% agar (Oxoid) + 0.4% bactopeptone (Oxoid) in distilled water, autoclave, cool and equilibrate to 45^{o}C in a water bath.
2. Prepare 2x concentrated culture medium, equilibrate to 45^{o}C.
3. Mix the agar and 2x medium 1:1, pour 4 ml into 5cm diameter petri dishes and allow to solidify at room temperature for 15 minutes. This forms the base layer of 0.6% agar.
4. To the mixture prepared in step 3 add an equal volume of cell suspension at 5 x 10^{4} cells/ml in 1x medium.
5. Immediately pipette 4ml of this suspension onto the base layers. Allow to firm at room temperature for 1 hour then incubate at 37^{o}C. Each dish will contain 10^{5} cells in 0.3% agar.
6. After desired culture period (2-4 weeks), stain plates with 0.01% crystal violet in 10% formaldehyde. Examine macroscopically and microscopically.

Note: if dishes are cultured beyond 2 weeks, 1 ml medium should be added per dish each week to prevent drying.

3.4.2.2 Lectin agglutinability

The ability of the lectin, concanavalin A (con A) to agglutinate normal and CR5 cells was assayed by the method of Borland and Hard (1974):

1. Prepare a suspension of cells at 4 x 10^{6} cells/ml.

2. Make up solutions of 10 and 100µg/ml con A (Sigma) in PBS. Solutions must be fresh.
3. Mix 0.1 ml cell suspension with 0.1 ml con A solution or PBS (control).
4. Incubate at room temperature for 5 minutes.
5. Observe under phase contrast. Determine agglutination on following criteria: less than 4 cells/clump, negative; 4-40 cells/clump positive; 40+ cells/clump, strong positive.

3.4.2.3 Cytoskeletal development

The settling behaviour and development of the cytoskeleton of freshly trypsinized cells was followed in coverslip cultures. Cells were seeded onto sterile, grease-free coverslips in 35cm diameter petri dishes and incubated for 5 minutes to 48 hours. Triton-extracted cytoskeleton preparations were made by the method of Pena (1980):

1. Wash coverslips in HBSS.
2. Treat for 5 minutes with 4M glycerol (BDH), 0.5% v/v Triton X-100 (BDH) and 0.25% w/v glutaraldehyde (EMscope, Ashford) in HBSS.
3. Fix in 3% w/v glutaraldehyde in HBSS for 15 minutes.
4. Stain for 15 minutes in 0.2% coomassie blue (R250) in methanol/glacial acetic acid/water, 46.5:7:46.5.
5. Rinse coverslips several times in distilled water, air dry, mount in Permount and observe under a microscope.

3.4.2.4 Time lapse video analysis

Differences in the behaviour and division of normal and CR5 cells were examined by time lapse video analysis.

Cells growing in 5cm diameter tissue culture dishes were placed on the stage of an Olympus inverted phase contrast microscope. The microscope was within a polythene 'tent' containing a small fan heater which maintained the stage at a temperature of 37 ± 1 ^{o}C. A Hitachi

Denshi HV175K video camera was linked to the microscope via its phototube. The video signal was passed through a ForA VTG 33Y video timer to a National Panasonic time lapse video recorder (Model NV 8030). The image being recorded was viewed on a Sanyo VM4219 monitor. Recordings were made at the 80 hour setting of the recorder on Sony high density video tapes, and usually were of 24 hours duration.

For analysis tapes were played back at normal speed and stopped at intervals to facilitate the tracing of cell outlines on acetate sheets.

3.4.2.5. Electron microscopy

The ultrastructure of CR5 and normal muntjac cells was examined by electron microscopy. I am indebted to Dr. M.J. Ord for performing these studies. The methods used for fixation, sectioning and staining the material are detailed in Collins et al (1981).

3.5 Cytogenetic Studies

3.5.1 Sister chromatid exchange

To allow the visualisation of SCEs, cells must have passed through two S-phases in the presence of 5-bromodeoxyuridine (BUdR). Due to the semi-conservative nature of DNA replication, cells which have completed two successive rounds of replication in BUdR will have chromosomes in which one chromatid is substituted with the base analogue in both the strands of its DNA duplex, while the other chromatid will have BUdR in only one of its DNA strands. This difference can be detected in metaphase preparations following appropriate staining.

3.5.1.1 Cell culture

Approximately 2×10^4 cells were seeded into tissue culture flasks and incubated for twenty four hours. BUdR (Sigma), in aqueous solution was then added to a final concentration of 3µg/ml. Following incubation for 46 hours, colchicine (BDH), 5µg/ml final concentration was added and the cells cultured for a further two hours. Colchicine treatment

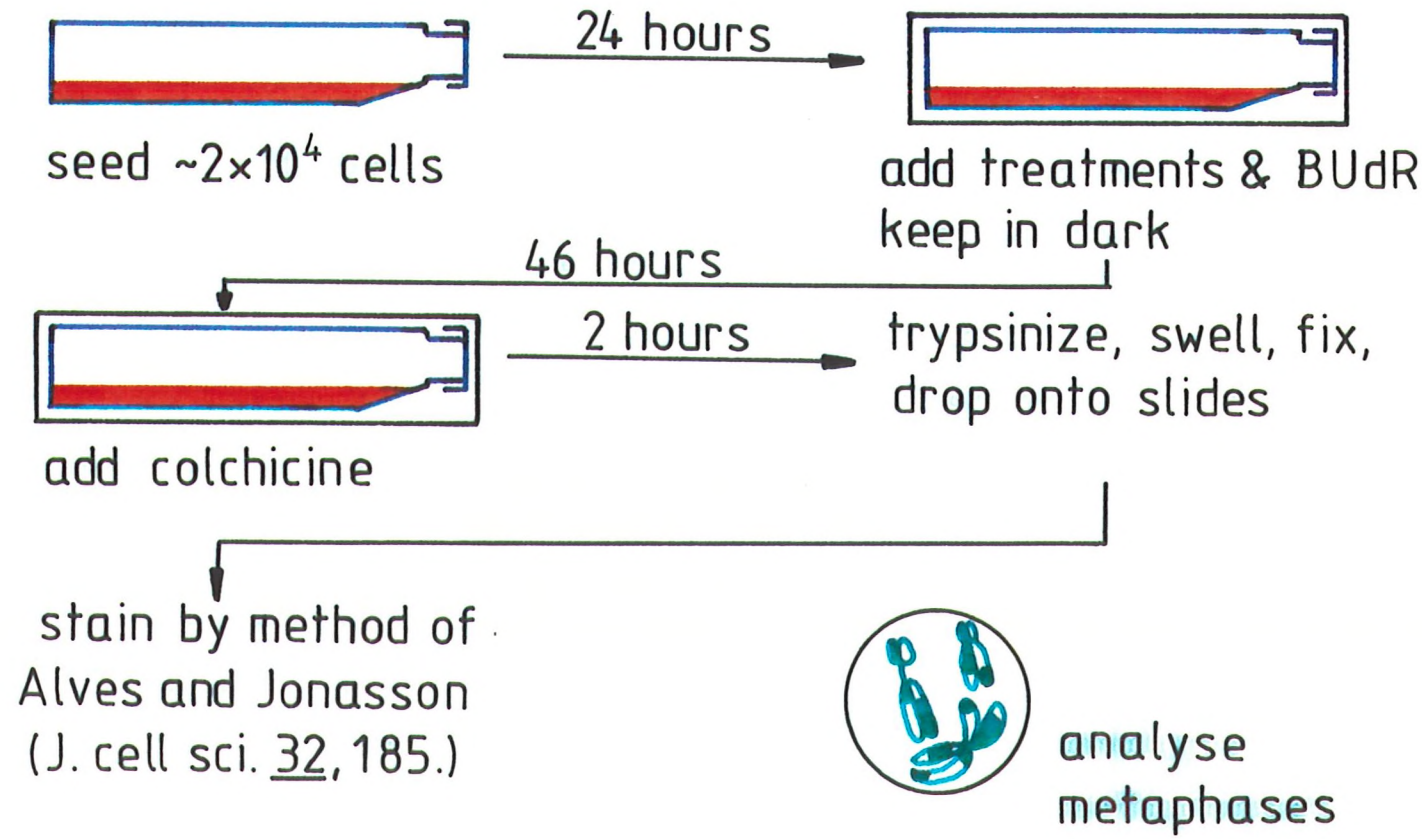


Figure 3.1. Method for producing cells stained for sister chromatid exchange.

increases the yield of metaphase cells by preventing mitotic spindle formation, thus, cells cannot proceed beyond metaphase.

Treatment chemicals could be added at any time during, or prior to, the culturing in BUdR; in general, treatments were for the entire 48 hour culture period.

When BUdR was present in cultures, care was taken to minimize their exposure to light. This was achieved by wrapping the flasks in aluminium foil and handling only under a red safe light. Such precautions are necessary as it has been shown that visible light can act in synergy with BUdR and some other chemicals in the formation of SCEs (Ikushima and Wolff, 1974; Schvartzman et al., 1979).

3.5.1.2 Harvesting procedure

The following method was used to produce metaphase spreads:

1. Remove medium and wash monolayer twice with HBSS.
2. Trypsinize cells as in Section 3.2.1, when cells are detached, suspend them in 2ml HBSS and transfer to a centrifuge tube.
3. Pellet cells at 1200 rpm for 5 minutes in a bench centrifuge. Discard supernatant.
4. Suspend cells in 2 mls prewarmed (37^{o}C) 0.075M KCl, incubate at 37^{o}C for 10 minutes.
5. Following centrifugation and removal of the supernatant as before, add 2 mls cold, freshly prepared 3:1 methanol/glacial acetic acid (fixative) dropwise while the centrifuge tube is being agitated on a whirlimix.
6. After two further centrifugations, fresh fixative being added after removal of the supernatants, suspend cells in approximately 0.5ml fixative.
7. Take a slide from an alcohol jar, wipe dry and breathe on it to provide a misted surface (this aids spreading of cells across the slide). Pipette a drop of cell suspension onto the slide from a height of 30 cm or more.

8. As the drop ceases to spread and interference colours appear, add two or three drops more fixative.
9. Allow each slide to dry slowly on a hot plate at minimal heat.

3.5.1.3 Staining method

The method of Alves and Jonasson (1978) was used to stain for SCEs. This has the advantage over more widely used techniques such as fluorescence-plus-Giemasa (Perry and Wolff, 1974) in that it is quicker and simpler.

Slides were stained with freshly prepared 2% Giemsa (improved R66, Gurr) in 0.3M disodium hydrogen orthophosphate (Na_2HPO_4), pH 10.3 - 10.6. The optimum pH for staining was determined empirically for each batch of slides. After staining for ten minutes, slides were rinsed by dipping into distilled water three times, blotted with filter paper and dried on a hot plate. The preparations were cleared in xylene and mounted in DePex, Canada balsam or Permount.

3.5.1.4 Scoring of SCEs

Under a Wild microscope slides were scanned at X 150 magnification, well stained and spread metaphases were examined at X 1000 or X 1500 oil immersion. The following information was recorded for each metaphase cell:

1. Position as defined by vernier scale readings.
2. The number of cell cycles it had completed in the presence of BUdR. This is determined by the staining pattern (see figure 3.2).
3. The number of SCEs and their location on each chromosome (well stained and spread second round metaphases only).
4. The presence of any gross chromosomal aberrations and the type of aberration.

For each culture, a minimum of 25 metaphases were scored for SCE.

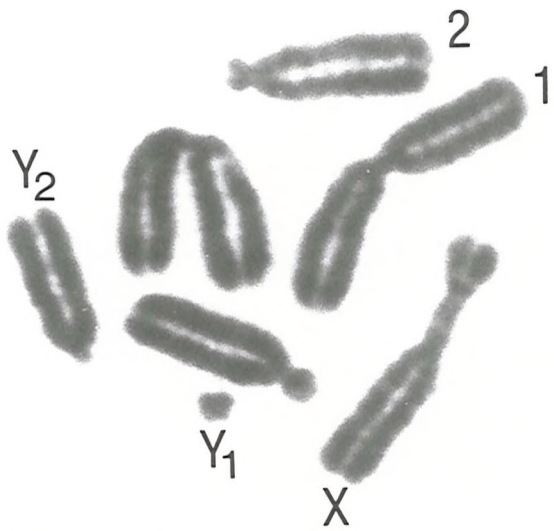
Figure 3.2

Staining patterns in metaphases of cells which have completed one two or three S phases in the presence of BUdR.

1. First cell cycle metaphase. Standard nomenclature of chromosomes also shown. Bar = 10μm.

2. Second cell cycle metaphase, note differential staining of chromosomes. Arrows point to examples of sister chromatid exchanges. Bar = 10μm.

3. Third cell cycle metaphase. Bar = 10μm.



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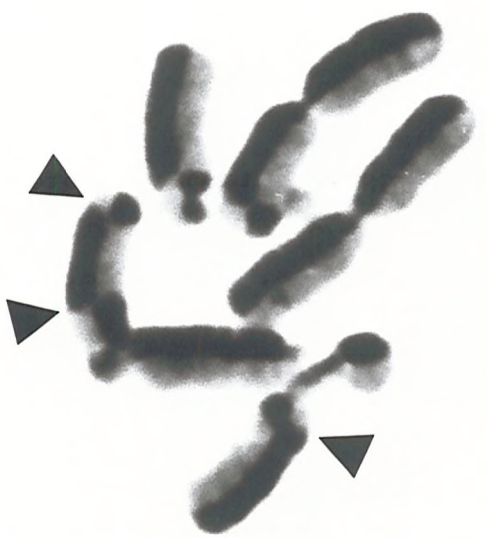

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Data were analysed by students 't' tests.

3.5.2 Chromosomal aberration

As well as being scored in SCE cultures, chromosomal aberration was sometimes investigated in cells prepared specifically for the purpose.

The culture method used was as for SCE analysis (Section 3.5.1.1) except BUdR was omitted and cells could be harvested at any time after treatment. Harvesting was as in section 3.5.1.2.

Slides were stained for 5 minutes in 5% Giemsa in phosphate buffer, pH 6.5 (BDH), briefly rinsed in buffer, cleared and mounted as before.

Aberrations were classified according to Savage (1979) after examination of metaphases under X 1500 oil immersion. Statistical comparison of treatments was made by means of chi-squared tests.

3.6 Assessment of DNA Repair

DNA repair was quantified by autoradiographic determination of unscheduled DNA synthesis. This assay measures the extent of non-S phase incorporation of nucleotides into nuclei.

Cells were seeded onto sterile, grease-free coverslips in 3.5cm diameter bacterial grade petri dishes (Sterilin) and grown to confluence. Treatment chemicals were added, without changing the culture medium, for two hours prior to addition of radiolabelled thymidine. A final concentration of 10 µg Ci/ml (methyl)-^{3}H thymidine (28 µCi/m mole; Amersham International) was used. Incorporation of radioactive thymidine was allowed to continue for 2 hours at 37^{o}C.

Note that fresh medium was not added prior to chemical treatment or addition of radiolabel. Thus, there was no stimulation of cells into S-phase by the presence of fresh serum. This technique obviates the need to treat cells with metabolic inhibitors to reduce replicative incorporation

of thymidine.

Coverslips were prepared for autoradiography while still in their dishes by the following method:

1. Remove medium, wash twice with HBSS.
2. Treat with 1% sodium citrate for 10 minutes at room temperature. This swells nuclei so aiding grain counting later.
3. Fix for five minutes in 3:1 ethanol/glacial acetic acid. Rinse with distilled water.
4. Extract three times (30 minutes each) with ice cold 5% trichloroacetic acid. Follow each extraction with a rinse in distilled water.
5. After extraction wash coverslips three times in distilled water, dry on hotplate.
6. Mount coverslips, cells uppermost, on glass slides.

When the mountant had set, the slides were coated with a 1:1 aqueous dilution of Ilford L-4 nuclear research emulsion. To do so, slides were dipped into the diluted emulsion (maintained at 45^{o}C), removed and allowed to dry vertically. These operations were carried out in a dark room. The dry slides were transferred to a slide box containing desiccant, the box was then sealed inside two black plastic bags. Autoradiographs were exposed for one week at 4^{o}C.

Slides were developed in 50% D-19 (Ilford) for 3.5 minutes and then fixed for 4 minutes in 20% sodium thiosulfate. When washed and dried, the autoradiographs were stained in Giemsa or crystal violet and mounted in DePex.

For each culture, the grains over 100 non-S phase nuclei were counted. The background activity was determined by grain counting in non-nuclear areas using a squared graticule. After measurement and calculation of average nuclear area, the background could be expressed as mean grains per nucleus. This figure was subtracted from each experimental nuclear count. Students 't' tests were used for statistical analysis of the data.

3.7 Uptake and Subcellular Distribution of Cd^{2+} and Ni^{2+}

Metal uptake and subcellular distribution was determined by use of $^{109}CdCl_2$ (194 μCi/ml, 0.192 μg Cd/ml, Amersham International) and $^{63}NiCl_2$ (1.38 mCi/ml, 86 μg Ni/ml, Amersham International).

Approximately 2.5×10^5 cells were plated into 5 cm diameter tissue culture dishes (Sterilin). Following incubation for 24 hours the medium was replaced with fresh medium containing 1 or 0.5 μCi/ml $^{109}CdCl_2$ or 1μCi/ml $^{63}NiCl_2$, the molar concentration of metal required was made up with unlabelled cadmium acetate or nickel sulfate. Other treatment chemicals required were also added at this time.

The cells were harvested and fractionated according to the proceedure detailed below (Section 3.7.1) after incubation for 1 - 48 hours. The number of cells obtained from each culture was determined by counting in a Neubauer counting chamber. The activity in samples of homogenate and each fraction were counted on a Beckman LS 7500 scintillation counter in Aquasol-2 liquid scintillation fluid (New England Nuclear, Southampton).

^{109}Cd was counted between channel limits 0 and 550 and ^{63}Ni between channel limits 317 and 600. As can be seen from these counting windows, no double-labelling experiments were possible. Appropriate quench curves were constructed for each isotope using the scintillation counter's data reduction facility. Thus, results were printed out in disintegrations per minute and so could be calculated back to μg metal/ number of cells.

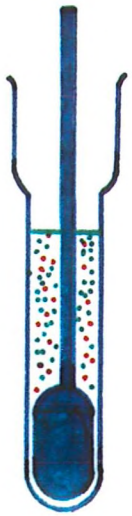
Some of the cytosol fractions produced from these experiments were saved for use in the column chromatography work (Section 3.8). These samples were stored at -80°C after lyophilizing in an Edwards EF03 freeze dryer.

3.7.1 Cell fractionation procedure

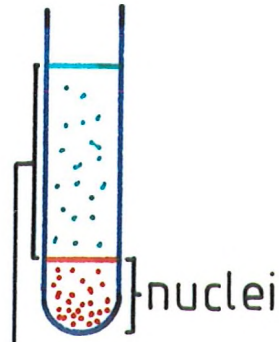
As there is no published method for the subcellular fractionation of muntjac cells, the following procedure was developed. The composition and purity of each of the fractions generated was determined by light microscopy and marker enzyme analysis (see Section 3.7.2).

1. Remove cells from dishes by trypsinization (Section 3.2.1) suspend in HBSS, pellet in a bench centrifuge.
2. Resuspend cells in 2mls ice cold homogenisation buffer (0.25M sucrose in 5mM tris-HCl buffer pH 7.3 plus 0.5mM phenyl methyl sulfonyl fluoride, a protease inhibitor).
   From this point all manipulations must be carried out on ice to prevent autodigestion of cellular components.
3. Homogenise cell suspension with 5 strokes of a Potter-Elvehjem homogeniser driven at approximately 2500 rpm. If necessary, the degree of cell disruption can be determined by examination of an aliquot of homogenate under a phase contrast microscope.
4. Transfer homogenate, diluted to 4 - 5 mls, to 6.5 ml polycarbonate centrifuge tube (MSE, Crawley). Underlay with 0.5 ml 2.3M sucrose in 5mM tris-HCl, pH 7.3.
5. Place loaded tubes in the buckets of a MSE 3 x 6.5 ml titanium swing out rotor. Balance tubes. Spin for one hour at 60,000 x $g_{av.}$ (27,500 rpm) in a MSE Superspeed 75 Centrifuge.
6. Transfer material at and above the interface of the two sucrose solutions to another centrifuge tube. Retain sucrose pad, this is the nuclear fraction (note: 1:4 dilution with water is necessary to make this strength sucrose solution miscible with scintillation fluid).
7. Spin supernatant at 110,000 x $g_{av.}$ (37,000 rpm) for one hour in the same centrifuge and rotor described in step (5).
8. The supernatant of this centrifugation represents the cytosol fraction, and the pellet organelles and membranes.

1. Seed cells, grow 48 hours.
2. Add ^{109}Cd or ^{63}Ni.
3. Trypsinize, wash.



4. Homogenize in 0·25 M sucrose / 5mM tris-HCl pH 7·3



5. Overlay on 2·3M sucrose pad, spin at 60000 × g 1 hour.

6. Spin 110000 × g 1 hour.

Figure 3.3. Subcellular fractionation proceedure.

3.7.2 Determination of composition and purity of fractions

The methods below were used to analyse each of the fractions produced by the above fractionation procedure.

Protein was assayed using the Sigma protein determination kit

The distribution of nuclei was determined by counting the nuclei in an aliquot of each fraction by means of a Neubauer counting chamber. This also allowed assessment of the integrity of the nuclei and the presence of any cytoplasmic 'tags'.

Plasma membrane was followed with the enzyme 5'nucleotidase, the method of Michell and Hawthorne (1965) being used.

The mitochondrial distribution in the fractions was evaluated by succinate dehydrogenase activity, Green and Narahara's (1980) method was employed.

Lysosomal distribution was assayed by acid phosphatase activity, the kit from Sigma being used.

The microsomal and cytosol components in each fraction were determined by the methods of Chambers and Rickwood (1978); Glucose-6-phosphatase and pyrophosphatase were the respective marker enzymes.

All spectrophotometric determinations were carried out on a Pye Unicam SP6-550 uv/vis spectrophotometer.

Many of the above assays depend on the determination of inorganic phosphate released from substrates by the various enzymes; therefore, to avoid contamination from extraneous phosphate ions, well rinsed, acid washed glassware was used throughout.

These assays were used on three separate runs of the subcellular fractionation procedure. Averaged results are shown in figure 3.4. It

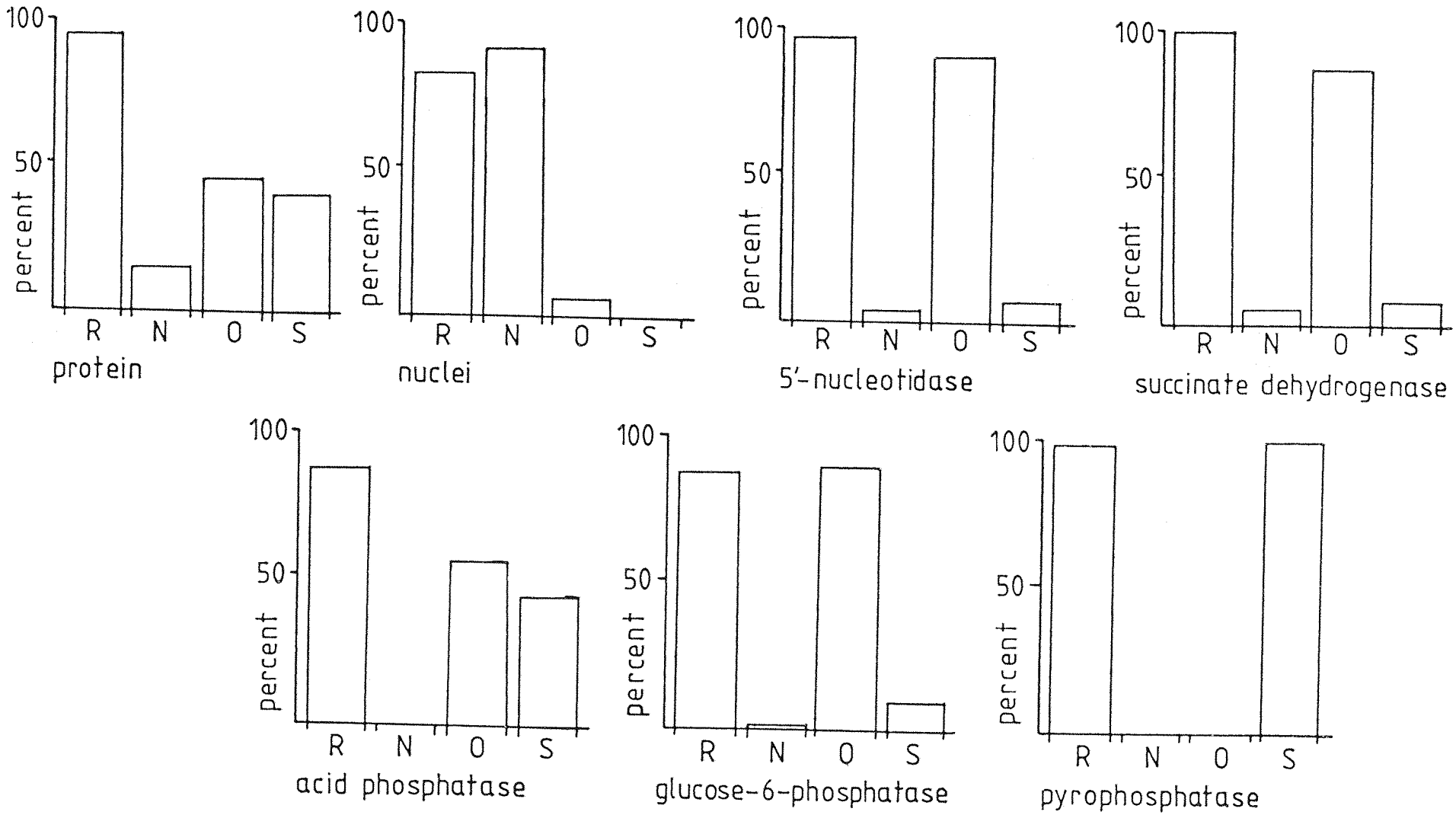


Figure 3.4. Analysis of subcellular fractions. R- percent recovery; N, O, S - percent of recovered activity in nuclear, organelle/membrane and cytosol fractions respectivly.

can be seen that the nuclear fraction contains only low levels of contamination; small percentages of membrane, mitochondria and microsomes appeared to be present. The organelle/membrane pellet contains the bulk of the plasma membranes, mitochondria and microsomes. Acid phosphatase activity is nearly equally distributed in the cytosol and organelle/membrane fractions. This activity in the cytosol fraction is likely to be due to release of the enzyme from lysosomes by their rupturing during homogenisation or separation. So the cytosol fraction contains some lysosomal contents and small numbers of the other organelles (except nuclei).

3.8 Cadmium Binding to Cytosol Components

This work was carried out at the MRC Toxicology Unit, Carshalton with the help of Dr. K. Cain.

3.8.1 Gel filtration chromatography

Gel filtration columns are packed with porous beads through which a buffer solution is pumped. There are two fluid phases in the column: the bulk flow phase around the beads and the stationary phase within the pores of the beads. Large molecules which cannot enter the beads will move through the column fastest (i.e. entirely in the bulk flow phase). Smaller molecules will spend a portion of their time in the stationary phase, where they are subject only to Brownian motion, so will move more slowly. The smaller the molecule, the longer it will stay in the stationary phase. Consequently, a hetrogenous mixture of molecules can be separated on the basis of size. Molecules are eluted in order of decreasing size.

Gel filtration media are available with several different pore sizes, each is suited to separation of molecules in a specified molecular weight range (molecular weight is proportional to molecular size in most cases). As the original aim of these experiments was to investigate the binding of Cd^{2+} to metallothionein, Sephadex G-75 columns were used (see, for example, Cain and Holt, 1979).

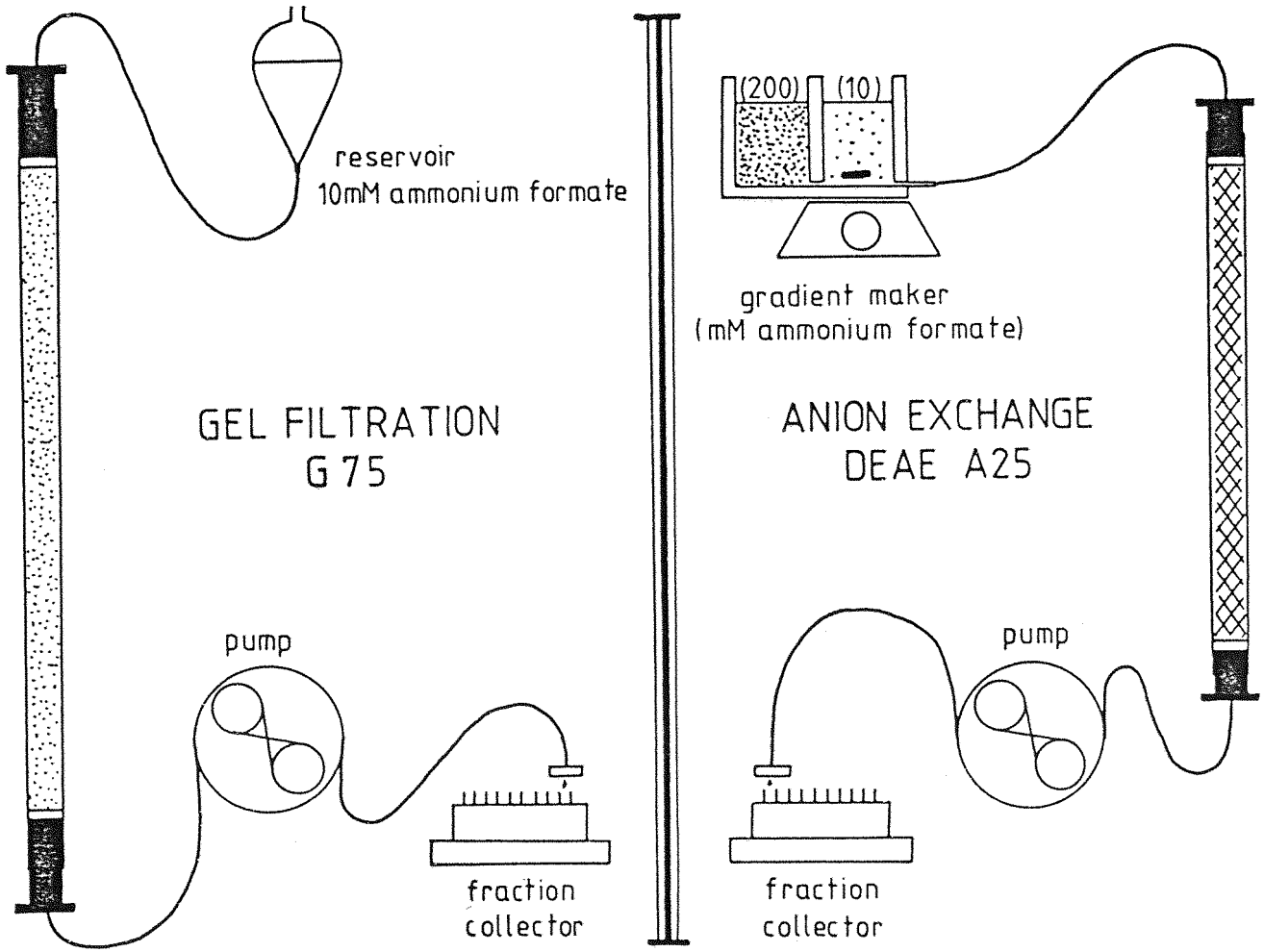


Figure 3.5. Schematic representation of column chromatography apparatus.

Glass columns (90 x 1.5cm) were packed with swollen, degassed Sephadex G-75 (Pharmacia GB Ltd., Hounslow) and equilibrated with 10 mM ammonium formate buffer, pH8. This buffer was pumped through the columns from a reservoir above by a peristaltic pump, flow rate approximately 25 ml/hour. During the course of each run, one hundred fractions of 3 ml were collected from the base of the columns in an LKB 2112 Readirak fraction collector. All columns were run at room temperature.

The void volume of each column was determined by eluting 2 ml samples of blue dextran. The void volume is the volume of buffer required to elute a molecule unable to enter the pores of the resin and is characteristic for each column. Unknown samples run down different columns can be compared on the basis of the ratio of elution volume (Ve) of their component molecules to the void volume (Vo).

One column was calibrated with the molecular weight standards ribonuclease A (m.w. 13,700), chymotrypsinogen A (m.w. 25,000) ovalbumin (m.w. 43,000) and bovine serum albumin (m.w. 67,000). The standards were from a Pharmacia low molecular weight gel filtration calibration kit. 20 mg ribonuclease A, 6mg chymotrypsinogen A, 14 mg ovalbumin and 14 mg albumin were dissolved in 2 ml 10 mM ammonium formate buffer and applied to the column which was run overnight. The protein content of each fraction was determined by its absorbance at 280nm on a Pye-Unicam spectrophotometer. The elution pattern of these proteins is shown in figure 3.6A. A linear calibration curve can be derived from this data by plotting log molecular weight against K_{av}.

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$

where: Ve = elution volume of the protein.
Vo = void volume (elution volume of blue dextran)
Vt = total bed volume

The calibration curve can be seen in figure 3.6B.

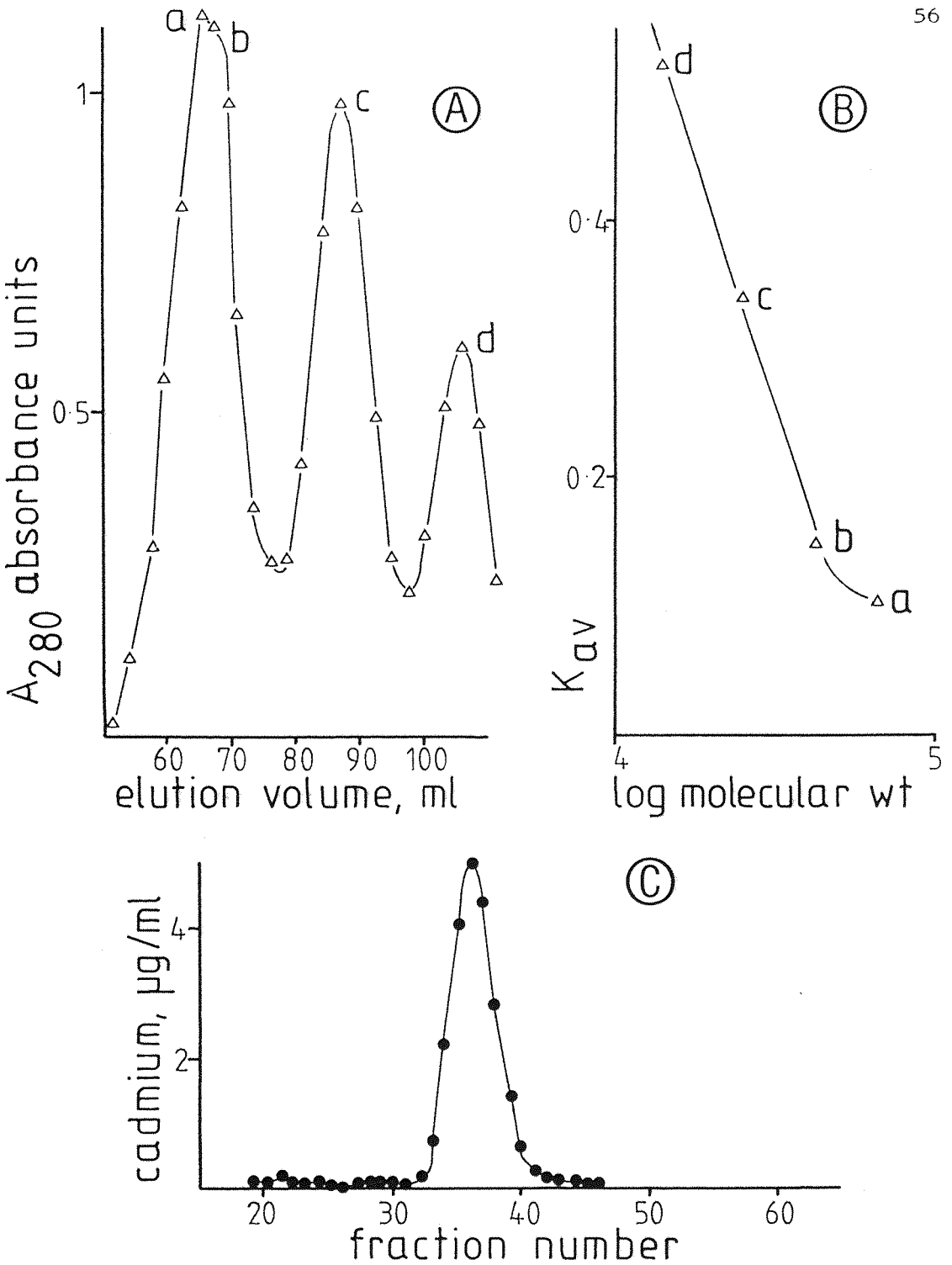


Figure 3.6. Calibration of G75 columns. Ⓐ elution of molecular weight markers, a – albumin, b – ovalbumin, c – chymotrypsinogen, d – ribonuclease A. Ⓑ calibration curve derived from Ⓐ. © rat liver metallothionein run through another G75 column. Data for © courtesy Dr. K. Cain, MRC, Carshalton.

Another column was calibrated with rat liver metallothionein. This experiment was performed by Dr. K. Cain. A sample of cytosol prepared from a cadmium dosed rat liver was run and the metal content of the fractions analysed by atomic absorbtion spectrophotometry. This data is plotted in figure 3.6C. The Ve/Vo of the cadmium binding peak (i.e. metallothionein) is 2.14.

For experimental runs, freeze dried cytosol fractions were reconstituted in 10 mM ammonium formate buffer, applied to columns and run overnight as described above. When each run was completed, the ^{109}Cd activity in each fraction was determined on a Packard 5330 AutoGamma spectormeter (settings: window 2, lower limit 0.5, energy 0.25 MeV).

3.8.2 Ion exchange chromatography

Ion exchange resins consist of beads similar to those used in gel filtration but, in this case, they have charged groups covalently linked to them. Anion exchange resins have negatively charged functional groups while cation exchange resins have positively charged functional groups. At the start of an ion exchange experiment, the resin is equilibrated with a low ionic strength buffer. Thus, all the charged sites are occupied by a 'counter ion'. When the sample to be separated is applied to the resin, some of the counter ions are displaced by the sample molecules of the same charge as the counter ions, so initially all the sample molecules are bound to the resin. The affinity of the bound molecules for the ion exchanger can be controlled by varying conditions such as pH or ionic strength. So, as a gradient of increasing ionic strength is passed through the resin, bound molecules are released in order of increasing charge.

In the present experiments 30 x 1.5 cm columns were packed with DEAE-cellulose D-52 resin. DEAE, diethylaminoethyl, is a positively charged group, hence these are anion exchange columns. Initially the columns were equilibrated with 10 mM ammonium formate, pH 8. Elution was with a linear gradient of 10 - 200 mM ammonium formate. Samples of interest, i.e. peaks obtained from the gel filtration columns were loaded onto the columns.

The top of the columns was then linked to a gradient maker containing 10 mM ammonium formate in the first chamber and 200 mM ammonium formate in the second chamber. The gradient maker was switched on and the gradient pumped through at approximately 25 ml/hour with a peristaltic pump. 130 3 ml fractions were collected from the base of each column per run. When the gradient had completely run through, the fractions were analysed for ^{109}Cd on the gamma counter as before (Section 3.8.1).

Chapter 4 The effects of cadmium ions on the chromosomes of muntjac cells

In Section 2.4.3 published work concerning the effects of Cd^{2+} on chromosome structure was discussed. Studies with plants have, on the whole, shown Cd^{2+} to be clastogenic, in contrast, whole mammal and mammalian cell experiments have mixed results. About half the mammalian studies are positive and half are negative; species, cell type, treatment protocol and culture conditon differences have been suggested to account for this variability. No studies have been made of the chromosomal effects of Cd^{2+} in Muntiatus muntjac. It was for this reason, and because of the need for 'Cd^{2+} alone' data with which to compare combined Cd^{2+}/mutagen treatments, that the experiments described below were performed.

4.1 Selection of Cd^{2+} treatment doses

To enable a suitable choice of Cd^{2+} concentrations to use in the chromosome assays, cell growth and viability tests with the metal ion were carried out. The methods used for these experiments are described in Section 3.3.

Figures 4.1A and B show the short term growth and viability of cells grown continuously in cadmium acetate. Cells rapidly died when grown in 50 or 100μM Cd^{2+}. 10μM Cd^{2+} is on the margin of acute toxicity, observation of living cells indicated that many cells died but a few remained dividing. At 1 and 5μM Cd^{2+} cells continued to proliferate, but at reduced rates and viabilities. 0.5μM Cd^{2+} had little effect on either parameter over seven days.

Figures 4.1C and D show the results of similar growth and viability experiments of longer duration. Cells were able to survive 0.1μM Cd^{2+} but their growth rate was reduced after 12 days of culture. Likewise at 0.5 and 1μM Cd^{2+} growth rate was reduced within two weeks but some cells survived and continued to proliferate for at least 28 days. At cadmium ion concentrations exceeding 1μM, cells died within two weeks.

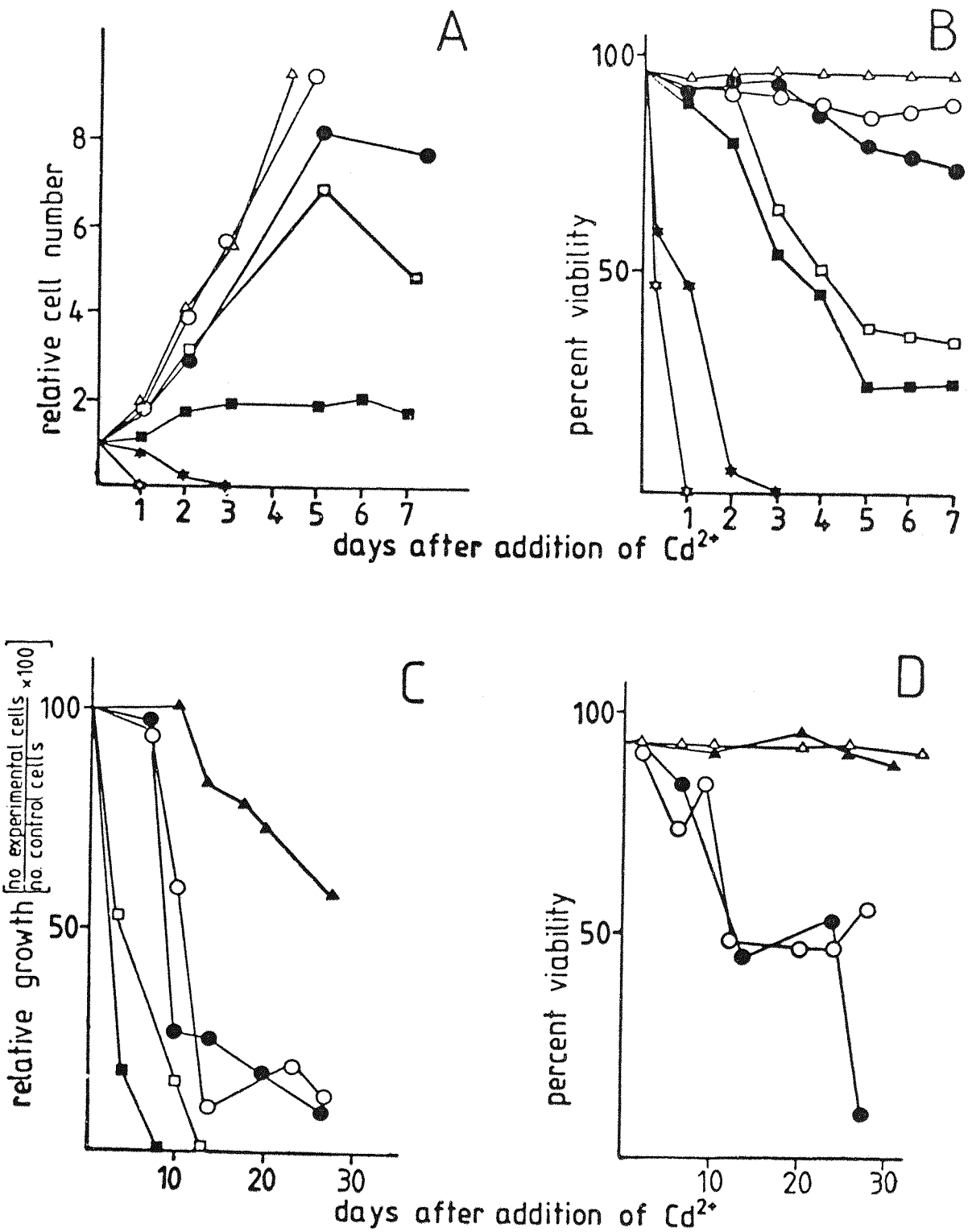


Figure 4.1. Growth and viability of muntjac cells cultured in the presence of cadmium acetate. △ control, ▲ 0.1μM, ○ 0.5μM, ● 1μM, □ 5μM, ■ 10μM, ✱ 50μM, ✲ 100μM. A and B: short term, C and D: long term.

4.2 Sister chromatid exchange induction by Cd^{2+}

On the basis of the evidence above it seemed likely that SCE induction by Cd^{2+} should be able to be assayed up to a concentration of 10uM in experiments of 48 hours duration. This indeed was found to be the case, attempts to produce metaphase spreads after treatment of cells for 48 hours with 20μM Cd^{2+} were unsuccessful.

Table 4.1 shows the results of SCE analysis in cultures treated with 0.1 - 10μM Cd^{2+}. None of these treatments were found to be statistically significantly different from controls on the basis of 't' tests.

Note that the SCE frequencies are expressed as SCEs per chromosome. This figure is the quotient of the number of SCEs and the number of chromosomes, excluding the small Y_1 chromosomes, in a given cell. This measure of SCE frequency was chosen in preference to others such as SCE/cell and SCE/picogram DNA for the following reasons:

1. It allows inclusion of data from cells with more or less chromosomes than in the normal karyotype without too great a distortion of the results. Loss of one chromosome from a muntjac cell represents a proportionately greater loss than the loss of one chromosome from a human metaphase spread.
2. It is easy to calculate, unlike SCE/picogram DNA.
3. For all treatments, the mean SCE/chromosome multiplied by 6 was not statistically significantly different from mean SCE/cell. Thus, cells with abnormal karyotypes did not predominate in the samples.

To extend the concentration range tested, another treatment schedule was used. This involved the exposure of cells to Cd^{2+} for two hours then culturing for 48 hours in BUdR only (recovery period). Harvesting and staining was as in the standard procedure (Section 3.5.1). Again no statistically significant elevation in SCE frequency was found, see

Table 4.1 Sister chromatid exchange induction by Cd^{2+}, 48 hour treatment

Concentration Cd^{2+} (μM)	No. Replicate cultures	No. metaphases scored	Mean SCE/ chromosome	95% confidence limits
0	3	323	1.05	± 0.05
0.1	2	96	1.03	± 0.06
0.5	2	119	0.99	± 0.07
1.0	2	121	1.01	± 0.01
5.0	2	101	1.15	± 0.11
10.0	2	50*	1.11	± 0.12

* Marked reduction in yield of metaphase cells.

Table 4.2 SCE induction by Cd^{2+}, 2 hour exposure + 48 hour recovery

Concentration Cd^{2+} (μM)	No. Replicate cultures	No. metaphases scored	Mean SCE/ chromosome	95% confidence limits
0	3	323	1.05	± 0.05
1	2	78	1.11	± 0.09
10	2	90	1.21	± 0.09
100	2	67	1.15	± 0.11

table 4.2.

4.3 Chromosomal aberration induction by Cd^{2+}

Examples of the types of chromosomal aberration scored are shown in figure 4.2. The classification is based on that of Savage (1979). Metaphases with ten or more aberrations were classed as severely damaged.

The induction of chromosomal aberrations by Cd^{2+} was investigated over the same concentration ranges as for SCE, that is 0 - 10μM Cd^{2+} for 48 hours and 1 - 100μM Cd^{2+} 2 hours plus 48 hours recovery. In addition the effect of a 100μM Cd^{2+} plus 17 hours recovery treatment was also analysed. The results of these experiments can be seen in figure 4.3, the raw data is shown in table 4.3.

As was found with SCE, continuous treatment of cells with Cd^{2+} did not lead to an increased incidence of chromosomal aberration. However, a short period of treatment followed by a 48 hour recovery period gave rise to increases in the yields of several types of aberration. Chi-squared tests showed the increases in chromosome type and chromatid type aberrations to be significantly different ($p < 0.01$ and $p < 0.001$ respectively) from controls. Chromosome breaks, chromatid breaks and dicentric chromosomes are especially prevalent. Moreover, after two hours in 100 μM Cd^{2+} and 48 hours recovery, cells with drastically altered chromosomes were seen (figure 4.4). Several of these severely damaged cell appeared to be polyploid and none had completed more than one cell cycle in BUdR.

The expression of severe damage appears time dependent. After a 17 hour recovery period no severely damaged cells were observed. But the yield of chromosome type aberrations decreases between 17 to 48 hours recovery. Also the frequency of chromosome type aberrations after 48 hours recovery is less at 100μM Cd^{2+} than 10μM Cd^{2+}. These pieces of evidence suggest that a cellular process, possibly a DNA repair system, may lead to the formation of severely damaged cells. As the recovery period continues the process gives rise to chromosome type aberrations

Figure 4.2.

Examples of chromosomal aberrations. Scale bars represent 10μm.

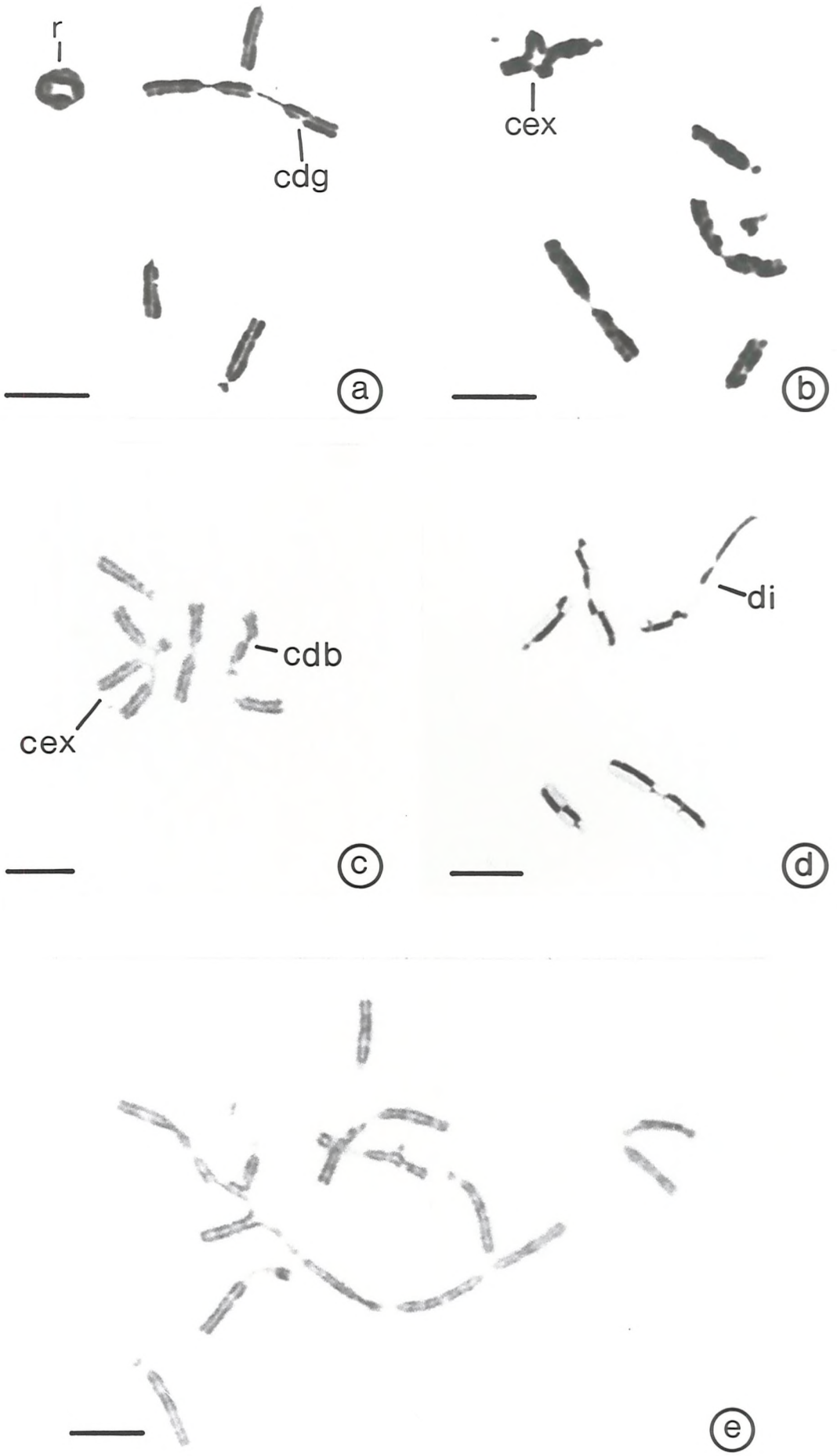
a) r - ring chromosome,
cdg - chromatid gap.

b) cex - asymetrical chromatid type exchange.

c) cex - asymetrical chromatid type exchange,
cdb - chromatid break.

d) di - centric chromosome.

e) Highly damaged cell, note multi - centric chromosomes, exchange figures, gaps and breaks.


r
cdg
a
cex
b
cdb
cex
c
di
d
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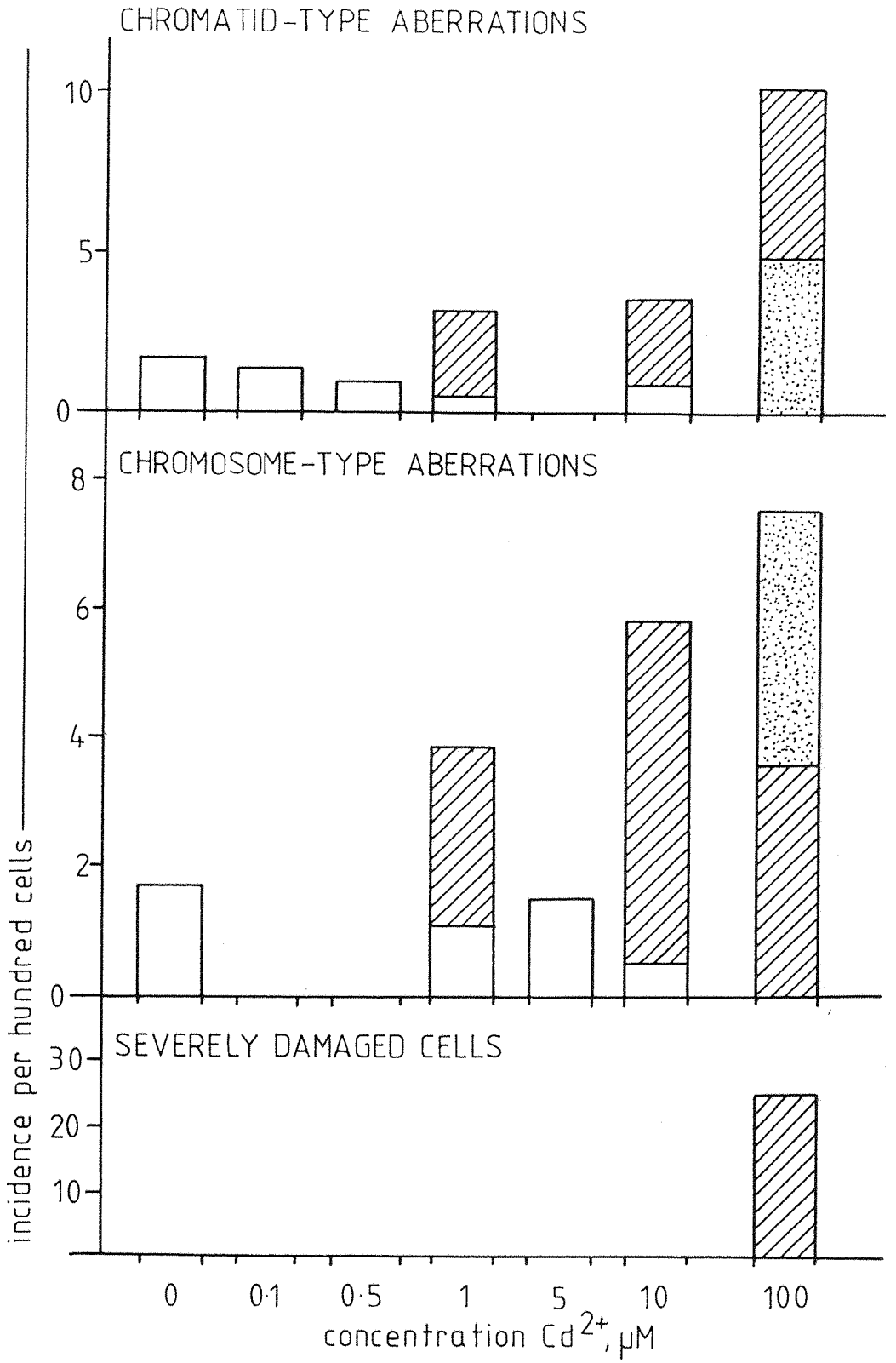


Figure 4.3. Induction of chromosomal aberrations by cadmium acetate. Unshaded bars - continuous treatment, stippled bars - two hour treatment plus seventeen hour recovery, hatched bars - two hour treatment plus forty eight hour recovery.

		continuous exposure						2 + 48			2+17
	$[Cd^{2+}]$ µM	0	0·1	0·5	1	5	10	1	10	100	100
chromosome	gaps										
	breaks	3			2	2	1	4	3	3	10
	dicentrics							2	4	2	5
	rings								1		8
	TOTAL	3	0	0	2	2	1	6	8	5	23
chromatid	gaps				1		2	1		3	13
	breaks	2	3	2				4	5	7	2
	exchanges									4	
	TOTAL	2	3	2	1	0	2	5	5	14	15
severe damage										35	
TOTAL		5	3	2	3	2	3	11	13	54	38
no. cells scored		175	213	209	190	158	211	156	139	139	308

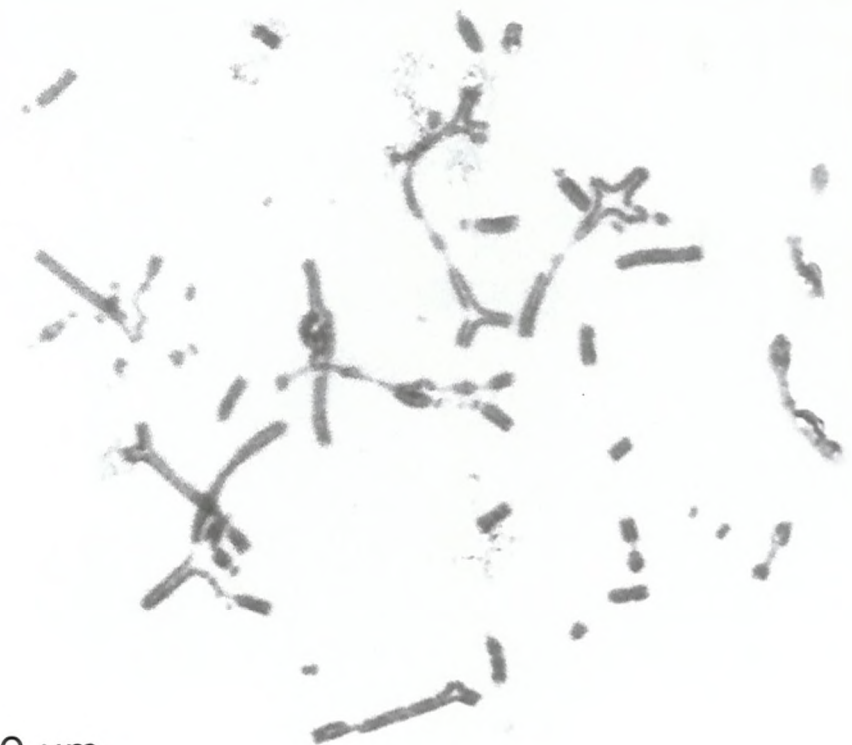
Table 4.3. Cadmium acteate induced chromosomal aberration: raw data. 2+48: two hour treatment plus forty eight hour recovery, 2+17: two hour treatment plus seventeen hour recovery.

Figure 4.4

Comparison of a normal muntjac metaphase (a) from a control culture and a severely damaged polyploid metaphase (b) from a culture treated with 100μM cadmium acetate for two hours followed by a forty eight hour recovery period.

10 µm

a



20 µm

b

initially, then severely damaged cells. Alternatively, the different recovery periods may simply be 'windows' at which time cells with a given degree of chromosomal damage enter division and so can be visualized in metaphase preparations. Whatever the events involved in aberration formation they are either inhibited by the continued presence of Cd^{2+} or damaged cells cannot divide while Cd^{2+} remains in the culture medium.

4.4 Discussion

Deavan and Campbell (1980) found that 0.4 μM $CdCl_2$ did not induce SCE in Chinese hamster ovary cells. This is the only report of the effect of Cd^{2+} on SCE in any system. Experiments described in this chapter confirm and extend this finding. Over a wide concentration range, up to the toxic limit, Cd^{2+} did not elevate baseline SCE levels in muntjac cells cultures continuously exposed to the ion for 48 hours. Similarly a two hour Cd^{2+} treatment, even at 100μM, followed by a 48 hour recovery period did not induce SCEs.

There is a much larger literature concerning the induction of chromosomal aberrations by Cd^{2+} (see Section 2.4.3). Studies most similar to those conducted here, i.e. those involving mammalian cell lines continuously exposed to the metal ion, on the whole agree with the data obtained from the present experiments (Patton and Allison, 1972; Rohr and Bauchinger, 1976; Umeda and Nishimura, 1979). The main exception being the finding of Deavan and Campbell (1980) that 1μM $CdCl_2$ caused chromosome shattering in CHO cells after 48 hours exposure. Chinese hamster Hy cells are similar to muntjac cells in that chromosomal aberrations can only be detected following a 16 hour or longer recovery after Cd^{2+} treatment (Rohr and Bauchinger, 1976).

The following points summarize the experimental findings of this chapter:

1. Cadmium acetate is highly toxic to muntjac cell cultures.

2. Cadmium acetate does not elevate the frequency of SCE after continuous exposure or short exposure followed by a recovery period.

3. Continuous treatment of muntjac cells with cadmium acetate does not increase the frequency of chromosomal aberrations.

4. A two hour exposure of cells to cadmium acetate followed by recovery periods of 17 or 48 hours causes chromosomal aberration. 48 hours after a two hour treatment with 100μM Cd^{2+}, a large proportion (25%) of cells have severely damaged chromosomes.

The data obtained from these experiments provides the 'cadmium alone' controls required for comparison with the 'cadmium plus another mutagen' results presented in the next chapter.

Figure 4.5

Sister chromatid exchange in muntjac cells. Scale bars represent 10μm.

a) Metaphase with five SCEs.

b) Metaphase with nine SCEs. Note presence of sub-sister chromatid exchange involving less than a whole chromatid width (arrow).

c) Metaphase with sixteen SCEs.

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Chapter 5. The influence of Cd^{2+} on the genotoxicity of other mutagens

5.1 Experimental Design

The experimental work described in this chapter attempts to provide evidence to answer the question: can Cd^{2+} alter the genotoxicity of other compounds? This simple question begs several others such as: does Cd^{2+} increase or decrease genotoxicity? To what extent? Are all compounds affected in the same way? What is the mechanism of the Cd^{2+}-induced alterations? The last question is addressed in later parts of this thesis (Chapters 6 and 8), the others are dealt with in the following sections.

Interaction between Cd^{2+} and the other compounds was investigated by analysis of SCE and chromosomal aberration induction by the compounds in the presence and absence of Cd^{2+}. In the preceeding chapter it became clear that exposure of cells to Cd^{2+} for 48 hours did not induce SCE or chromosomal aberration. This makes the interpretation of combined Cd^{2+}/other mutagen results simple. If the presence of Cd^{2+} enhances the levels of SCE or chromosomal aberration induced by a given mutagen the interaction is synergistic; conversely, if the effects are reduced in magnitude the interaction is antagonistic. Treatment of cells with Cd^{2+} prior to the other mutagen may not lead to changes of the same type or extent as concurrent treatment. Thus, experiments where cells were pretreated with Cd^{2+} for varying lengths of time before exposure to the other mutagen (no Cd^{2+} added) were performed. This treatment protocol can be seen as the 'in vitro' counterpart of an individual who is exposed to the metal before coming into contact with the other compound.

5.2 Cd^{2+} and Alkylating Agents

Despite a number of differences in their binding to DNA (see Section 2.6) the alkylating agents EMS and MMC cause a remarkably similar spectrum of cellular effects (Table 2.5). Both are effective inducers of SCE and

chromosomal aberration. The available literature concerning Cd^{2+}/ alkylating agent interactions, reviewed in Section 2.5, is sparce and contradictory. It is hoped that the results obtained in this study will help clarify the situation.

5.2.1 Ethyl methanesulfonate

In figure 5.1A the results of SCE analysis in cultures treated with EMS or EMS + Cd^{2+} for 48 hours can be seen. 0.1mM EMS alone more than doubled the control frequency of SCE, a difference which is statistically significant ($p < 0.001$). Raff and Sugahara (1980) also found EMS to be an SCE inducer in muntjac cells. Unfortunately their use of a different treatment regimen and a virus transformed cell line do not allow direct comparison of results.

When 1μM Cd^{2+} was present with EMS, the increase in SCE frequency above baseline was reduced to approximately 50% of that caused by EMS alone. EMS-induced increases in SCE frequency were also reduced by pretreatment of cells with 0.1μM Cd^{2+}, again a reduction of about 50% was seen after a 48 hour pretreatment (see figure 5.2A).

As Cd^{2+} tended to reduce the effect of EMS on SCE, it is somewhat surprising to find that it potentiated the chromosomal aberration-inducing capacity of the alkylating agent (figure 5.3, table 5.1). However, statistical analysis shows that not all of these increases are significant. The Cd^{2+}/0.1mM EMS treatments are significantly different from the 0.1mM EMS alone treatment ($p < 0.001$ for chromosome type and chromatid type aberrations). The 0.1mM EMS alone treatment is not significantly different from controls. On the other hand, the 1mM EMS induces both aberration types ($p < 0.001$) but the combined Cd^{2+}/1mM EMS treatment is not significantly different from the 1mM EMS alone treatment.

The Cd^{2+} pretreatment experiments also indicated a synergy between the metal and the alkylating agent in the formation of chromosomal aberrations. The pre-treatment lead to significant increases ($p < 0.001$)

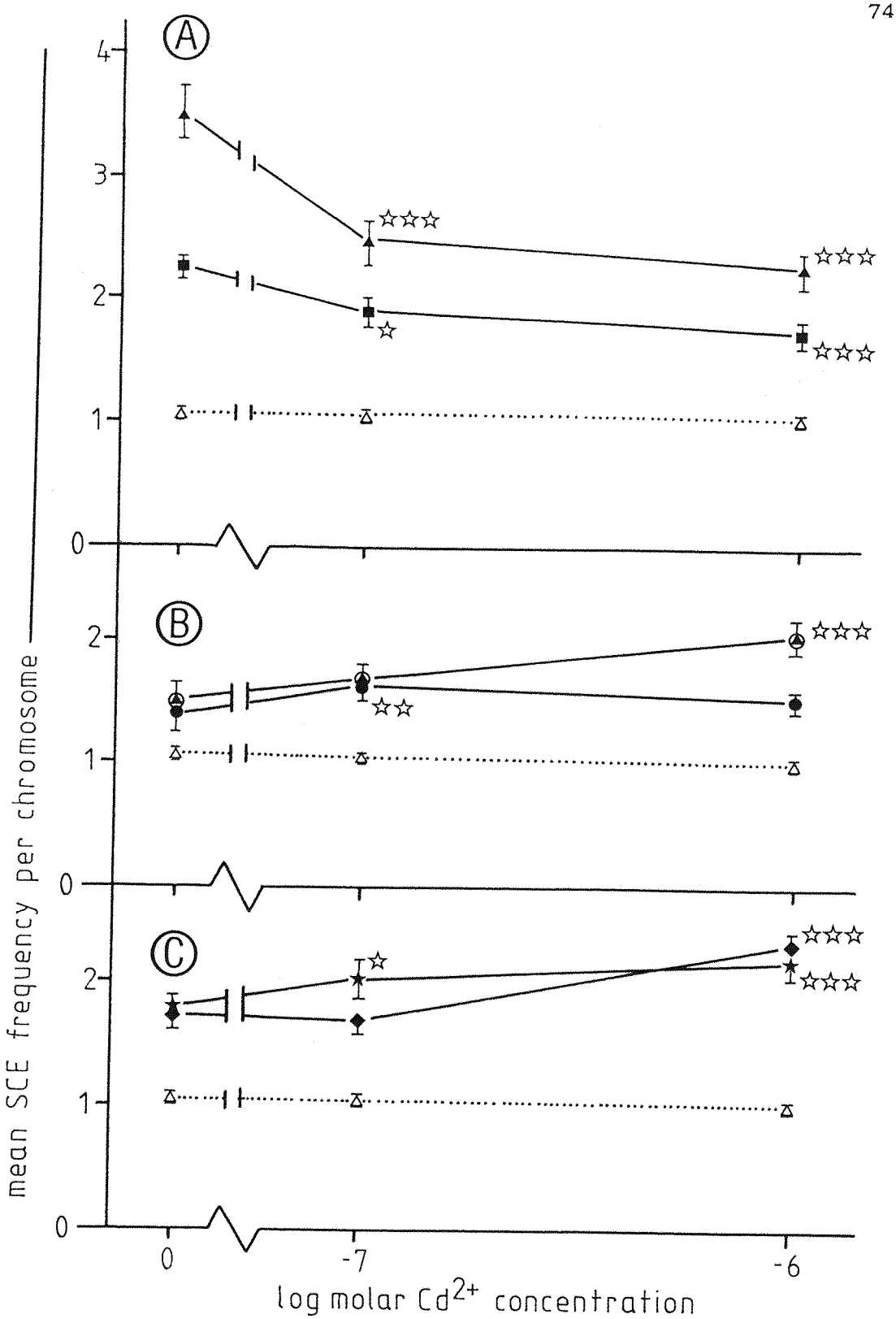


Figure 5.1. Influence of co-treatment with Cd^{2+} on SCE induction by: Ⓐ 0.018μM MMC (▲), 100μM EMS (■); Ⓑ 0.5μM AO (●), 5μM H33258 (◮); Ⓒ 1μM Nickel sulphate (★), 10 μM Nickel sulphate (◆). Controls (Δ). Each point represents data from 3 replicate cultures. Bars: 95% confidence limits. White stars: significant differences from mutagen alone treatments in 't' tests - ☆ $p<0.05$, ☆☆ $p<0.01$, ☆☆☆ $p<0.001$.

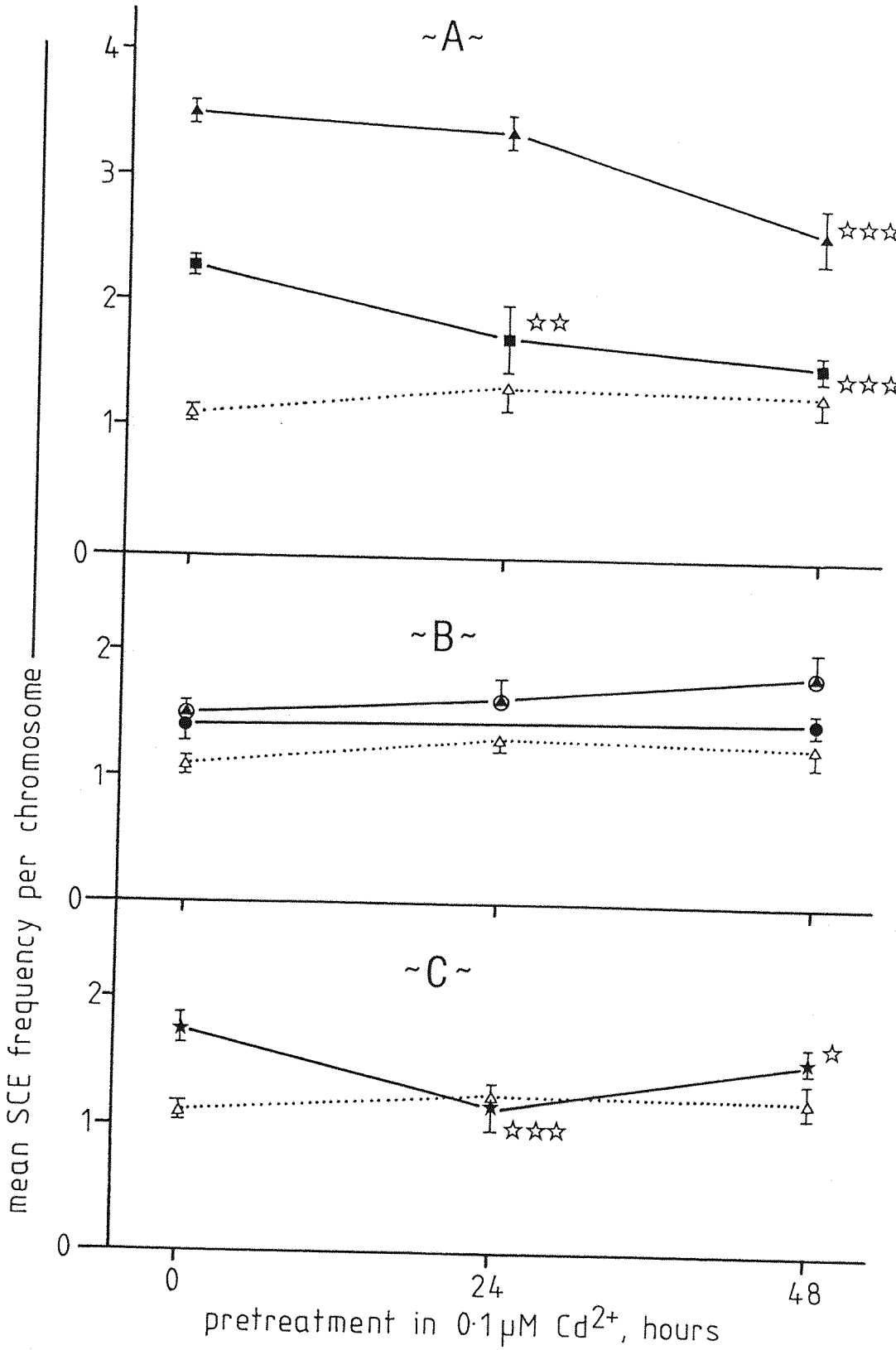


Figure 5.2. Effect of pretreatment with 0.1μM Cd^{2+} on SCE induction by:- A: 0.018μM MMC (▲), 100μM EMS (■); B: 0.5μM AO (●), 5μM H33258 (◬); C: 1μM Ni^{2+} (★). Controls (△). Each point represents data from 3 replicates. White stars: significant differences from mutagen only data, star code as in Figure 5.1.

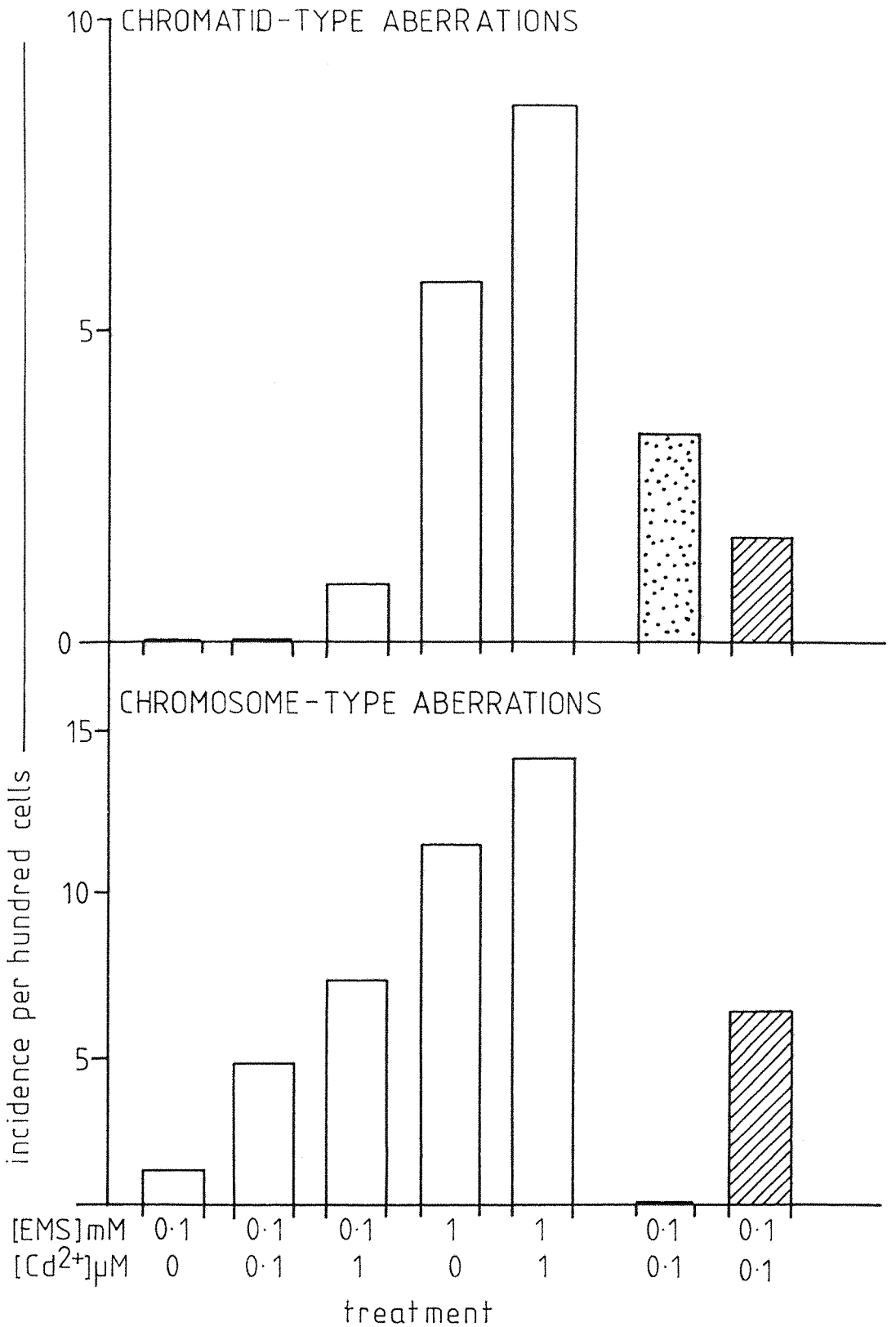


Figure 5.3. Induction of chromosomal aberrations by EMS and Cd^{2+}. Clear bars: co-treatment; stippled bars: 24 hour Cd^{2+} pretreatment; hatched bars: 48 hour Cd^{2+} pretreatment.

		continuous exposure						Cd pre	
[EMS] µM		100			1000			100	
[Cd^{2+}] µM		0	0·1	1·0	0	1·0		0·1, 24	0·1, 48
chromosome	gaps			1		2			1
	breaks	1	4	1	19	2			4
	dicentrics		2	4	5	1			3
	rings			2					
	TOTAL	1	6	8	24	5		0	8
chromatid	gaps				2	2		1	1
	breaks			1	8	1			1
	exchanges				2				
	TOTAL	0	0	1	12	3		1	2
severe damage									
TOTAL		1	6	9	36	8		1	10
no. cells scored		94	132	112	209	35		30	121

Table 5.1. Chromosomal aberration in EMS/Cd^{2+} treated cultures. Cd pre: pretreatment with Cd^{2+}, figures in [Cd^{2+}] rows refer to concentration and duration of pretreatment.

in both aberration types over the EMS treatment without any pretreatment. There was no clear time dependence shown. Some caution should be exercised in interpreting this data as two of the results are from samples of small size.

5.2.2 Mitomycin C

Mitomycin C is a more complex alkylating agent than EMS, being bifunctional it can form crosslinks as well as monofunctional adducts.

Data from concurrent MMC/Cd^{2+} treatment SCE experiments is shown in figure 5.1A. MMC alone increased baseline SCE frequency about 3.5-fold (significant at $p < 0.001$). The work of Carrano and Johnson (1977) is in agreement with this result in that MMC induces SCE in muntjac; but the two sets of data are not directly comparable due to differences in exposure protocol. As was found with EMS, the addition of Cd^{2+} and MMC to cells induced about 50% fewer SCEs than MMC alone. Cd^{2+} pretreatment also tended to reduce the number of SCEs induced by MMC (figure 5.2A), however, the reduction was of a smaller magnitude than seen in concurrent exposure experiments.

Turning attention now to the chromosomal aberration data (figure 5.4, table 5.2), some similarities with the SCE data can be seen. There is a tendency for pretreatment and combined treatment with Cd^{2+} to lessen the effect of MMC. However the reductions in chromosome type and chromatid type aberrations caused by combined treatment with Cd^{2+} and MMC fail to reach significance in chi-squared tests. MMC itself induced a significant increase in only chromosome type aberrations ($p < 0.001$). Following Cd^{2+} pretreatment, the frequency of chromosome type aberrations was significantly lower than in cells treated with MMC alone ($p < 0.001$).

5.3 Cd^{2+} and Non-covalent DNA binding agents

The binding of acridine orange and Hoechst 33258 to DNA is quite different to that of alkylating agents. The linkage between the compounds and DNA is not covalent, weaker forces such as hydrogen bonds and dipole-

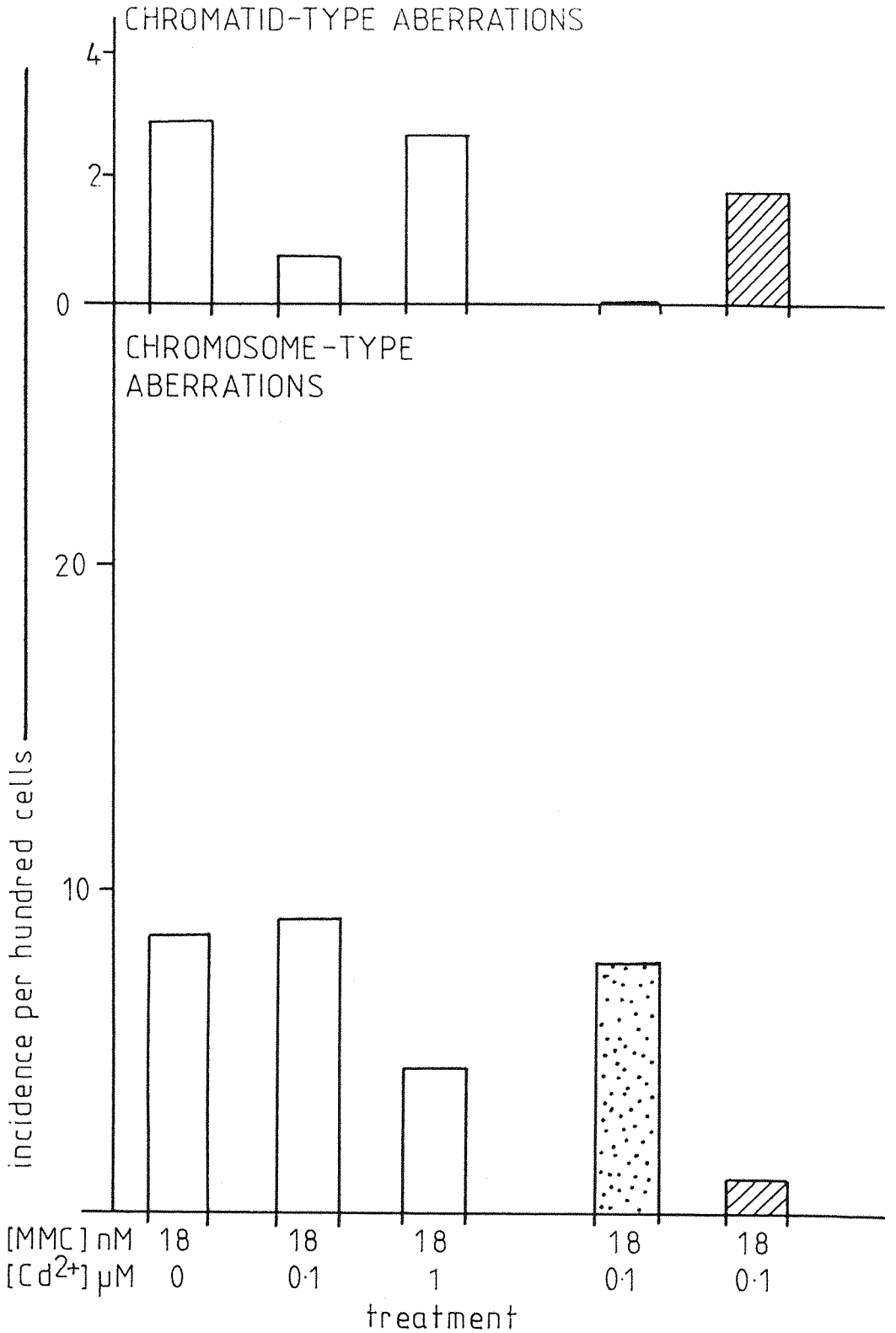


Figure 5.4. Induction of chromosomal aberrations by MMC and Cd^{2+}. Clear bars: co-treatment; stippled bars: 24 hour Cd^{2+} pretreatment; hatched bars: 48 hour Cd^{2+} pretreatment.

		continuous exposure				Cd pre	
[MMC] μM		0·018				0·018	
[Cd^{2+}] μM		0	0·1	1·0		0·1,24	0·1,48
chromosome	gaps						
	breaks	10	15	8		3	3
	dicentrics	6	17	2		7	7
	rings	4	2	1		1	1
	TOTAL	20	34	11		11	11
chromatid	gaps	2	1	2			1
	breaks	5	2	5			1
	exchanges						
	TOTAL	7	3	7		0	2
severe damage						1	
TOTAL		27	37	18		12	13
no. cells scored		242	399	261		142	116

Table 5.2. Chromosomal aberration in MMC/Cd^{2+} treated cultures. Cd pre: pretreatment with Cd^{2+}, figures in [Cd^{2+}] rows refer to concentration and duration of pretreatment.

dipole attractions are important. Chapter 2 deals with the mode of DNA binding and effects of these two compounds.

5.3.1 Acridine orange

Acridine orange, the DNA intercalating agent, caused a small but statistically significant ($p < 0.001$) increase in SCE, see figure 5.1B. Treatment with 0.1μM cadmium acetate and 0.5μM AO significantly increased ($p < 0.01$) the 'AO-alone' level of SCE. When the Cd^{2+} concentration was raised to 1μM the increase over AO-alone treatment was not so great and fails to reach significance in 't' tests. Cd^{2+} pretreatment had no significant effect on AO-induced SCE (figure 5.2B).

The small increase in chromosomal aberration induced by AO alone (see figure 5.5 and table 5.3) is not statistically significant. Concurrent treatment of cells with Cd^{2+} and AO led to an overall increase over AO-alone treatment in the number of aberrations (table 5.3). However, the formation of the two types of aberration was affected differently by the presence of Cd^{2+} (figure 5.5). Despite their absence from combined treatment cultures, the frequency of chromatid type aberrations was not significantly different from that in AO-alone treated cells. Chromosome type aberrations were considerably more common in AO/Cd^{2+} treated cultures than in AO only cultures, these increases are statistically significant ($p < 0.001$). A similar pattern was seen in Cd^{2+} pretreated cells. Chromatid type aberrations were not present but there was a significant ($p < 0.001$) increase in the incidence of chromosome type aberrations due to cadmium pretreatment.

5.3.2 Hoechst 33258

A small but significant ($p < 0.001$) elevation in SCE frequency was induced by Hoechst 33258 (figure 5.1B). If the cell treatment medium contained Cd^{2+} and H33258, the induction of SCE was greater than with H33258 alone (fig. 5.1B). Results from Cd^{2+} pretreatment experiments showed a similar trend of increased SCE with increased pretreatment time (figure 5.2B), however none of the increases were statistically

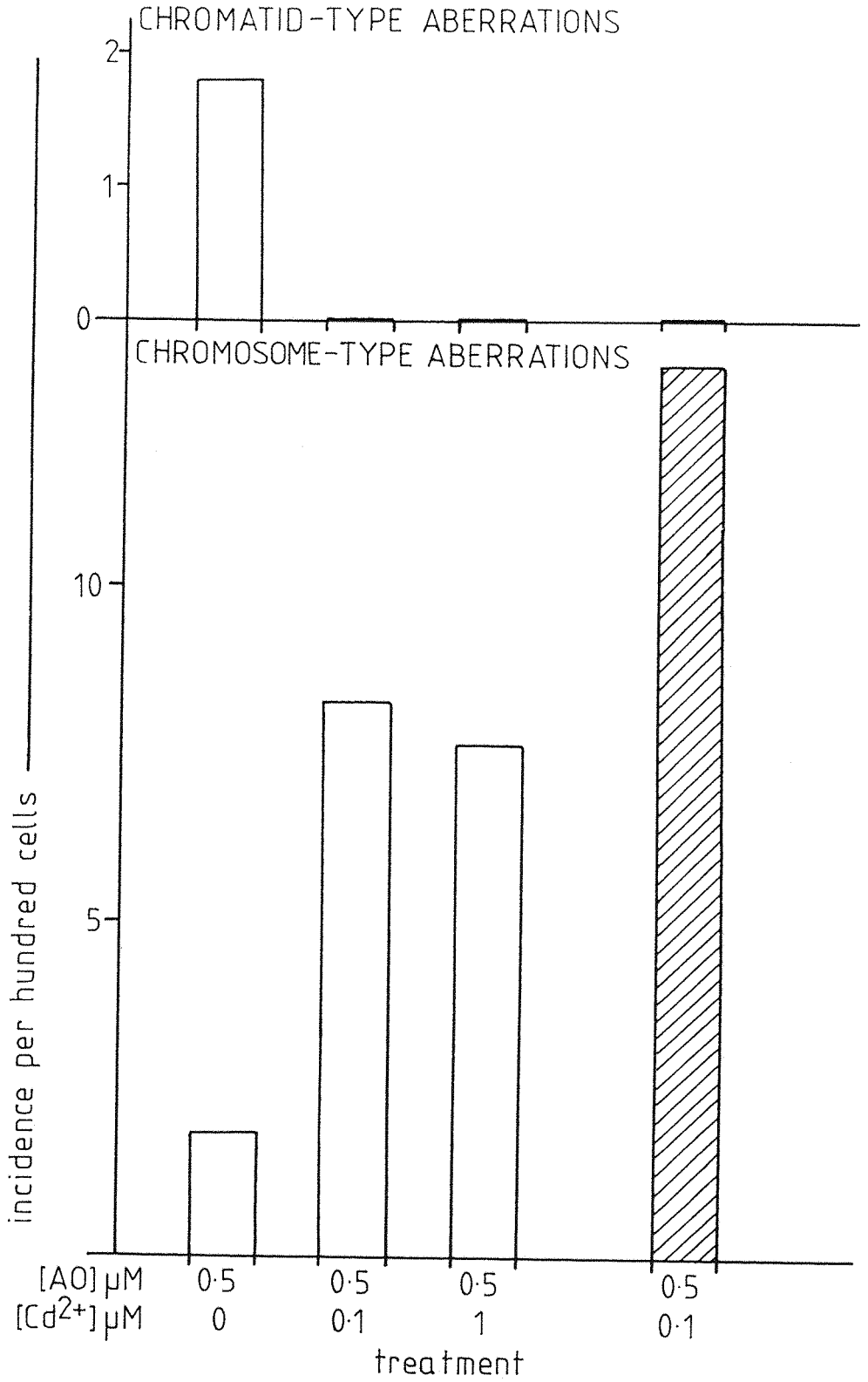


Figure 5.5. Induction of chromosomal aberrations by AO and Cd^{2+}. Clear bars: co-treatment; stippled bars: 24 hour Cd^{2+} pretreatment; hatched bars: 48 hour Cd^{2+} pretreatment.

		continuous exposure					Cd pre
	[AO] μM	0·5			1		0·5
	[Cd^{2+}] μM	0	0·1	1·0	0		0·1, 48
chromosome	gaps			1	1		
	breaks	2	2	3	5		4
	dicentrics	1	1	2			10
	rings		7	4			
	TOTAL	3	10	10	6		14
chromatid	gaps				1		
	breaks	3			1		
	exchanges						
	TOTAL	3	0	0	2		0
severe damage							
TOTAL		6	10	10	8		14
no. cells scored		167	121	131	97		105

Table 5.3. Chromosomal aberration in AO/Cd^{2+} treated cultures. Cd pre: pretreatment with Cd^{2+}, figures in [Cd^{2+}] rows refer to concentration and duration of pretreatment.

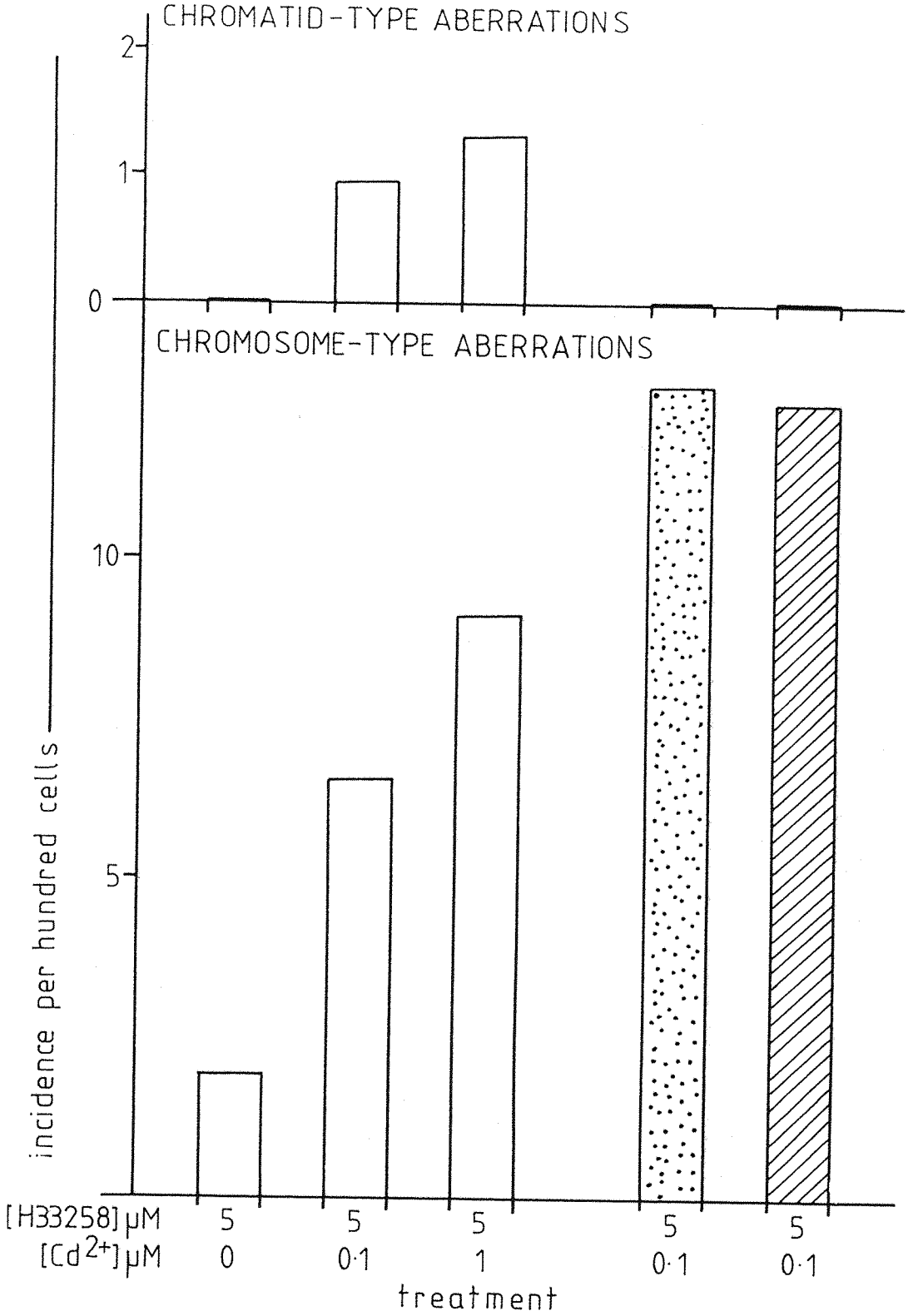


Figure 5.6. Induction of chromosomal aberrations by H33258 and Cd^{2+}. Clear bars: co-treatment; stippled bars: 24 hour Cd^{2+} pretreatment; hatched bars: 48 hour Cd^{2+} pretreatment.

		continuous exposure				Cd pre	
[H 33258]μM		5				5	
[Cd^{2+}]μM		0	0·1	1		0·1, 24	0·1, 48
chromosome	gaps					1	
	breaks	1	7	5		1	4
	dicentrics	1	5	1		6	10
	rings	1	2	1		1	
	TOTAL	3	14	7		9	14
chromatid	gaps		1	1			
	breaks		1				
	exchanges						
	TOTAL	0	2	1		0	0
severe damage							
TOTAL		3	16	8		9	14
no. cells scored		157	214	77		71	113

Table 5.4. Chromosomal aberration in H33258/Cd^{2+} treated cultures. Cd pre: pretreatment with Cd^{2+}, figures in [Cd^{2+}] rows refer to concentration and duration of pretreatment.

significant.

Data from chromosomal aberration experiments is seen in figure 5.6 and table 5.4. H33258 alone did not significantly alter control levels of chromosomal aberration. Concurrent cadmium treatment caused significant elevation of chromosome type aberrations ($p < 0.001$ for combined treatment and pretreatment).

5.4 Cd^{2+} and another metal ion

Many individuals are exposed to a combination of metals, thus it is important to determine the effects of mixtures of metals in genotoxicity assays. Many epidemiological studies of metal-induced cancer and studies of chromosomal aberration in environmentaly exposed individuals have been unable to determine which of a number of metals caused the effects observed, or if it was the combination of metals which was important. Nickel was chosen as the other metal for investigation in this study. Nickel is a known human carcinogen and workers in a number of industries are exposed to cadmium and nickel together.

5.4.1 Nickel ions

Previous reports have shown Ni^{2+} to induce small increases in SCE frequency in human lymphocytes (Wulf, 1980; Newman et al, 1982). This was also found to be true in muntjac cells (figure 5.1C). Cells treated with both 1 and 10μM Ni^{2+} had a significantly higher SCE frequency than control cells ($p < 0.001$ for both concentrations). Co-treatment of cells with both metal ions led to synergistic yields of SCEs (figure 5.1C). When Ni^{2+} exposure followed Cd^{2+} treatment SCE frequencies were less than after Ni^{2+} alone.

Changes in SCE were closely paralleled by changes in chromosomal aberration. Unlike the results obtained with the other chemicals, Ni^{2+} caused more pronounced changes in chromosomal aberrations than SCE (figures 5.7C, table 5.5). Ni^{2+} itself significantly increased the frequency of chromosome type aberrations ($p < 0.001$). On combined

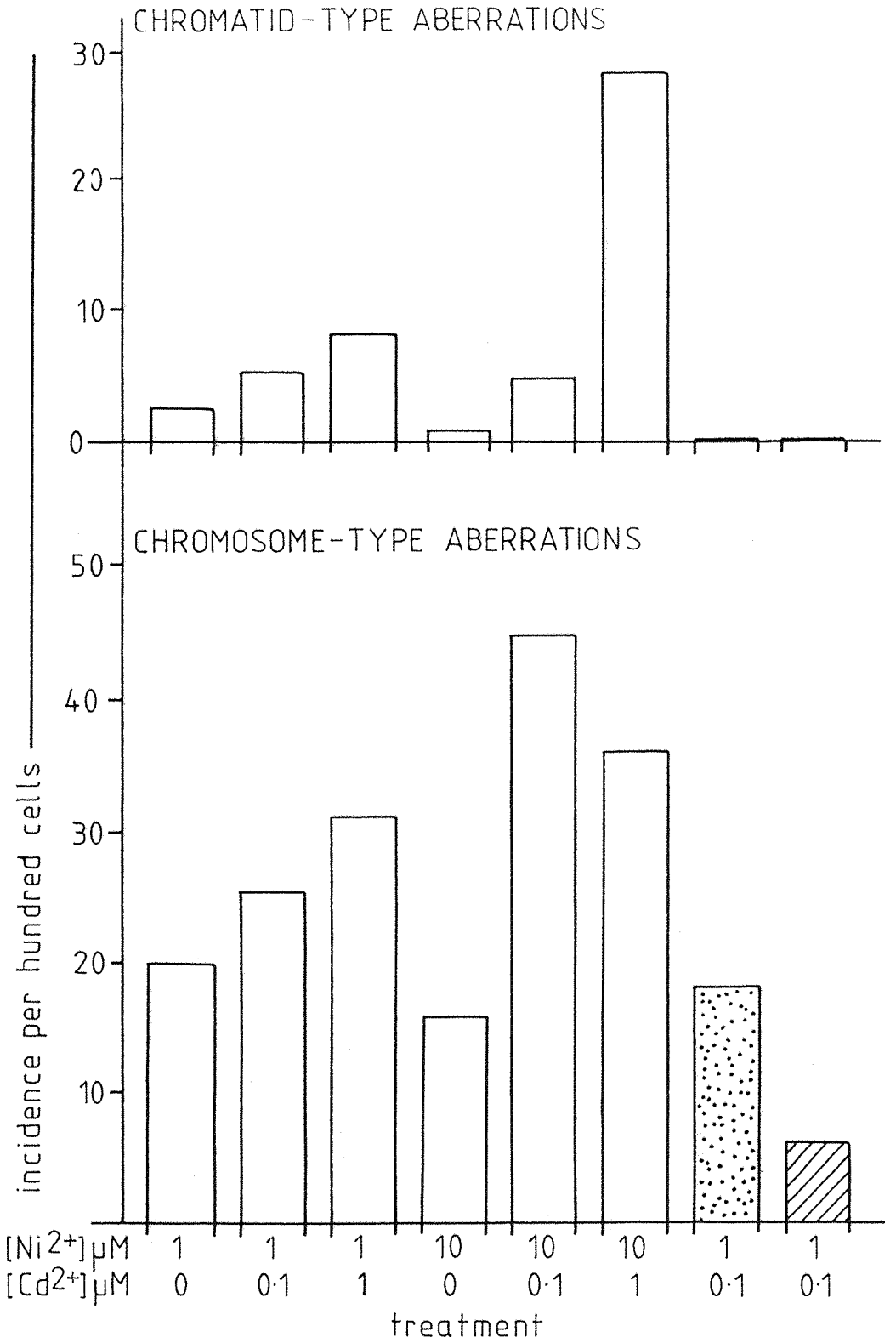


Figure 5.7. Induction of chromosomal aberrations by Ni^{2+} and Cd^{2+}. Clear bars: co-treatment; stippled bars: 24 hour Cd^{2+} pretreatment; hatched bars: 48 hour Cd^{2+} pretreatment.

		continuous exposure							Cd pre	
	$[Ni^{2+}]\mu M$	1·0			10·0				1·0	
	$[Cd^{2+}]\mu M$	0	0·1	1·0	0	0·1	1·0		0·1, 24	0·1, 48
chromosome	gaps			1		3	5		4	
	breaks	7	5	20	14	26	25		7	5
	dicentrics	29	23	32	24	43	27		5	6
	rings	5	2	9	1	3	6		1	
	TOTAL	41	30	62	39	75	64		17	11
chromatid	gaps	2	2	7	1	5	17			
	breaks	2	4	9	1	3	29			
	exchanges	1					4			
	TOTAL	5	6	16	2	8	50		0	0
severe damage				3			6			
TOTAL		45	36	81	41	83	120		17	11
no. cells scored		208	118	199	248	167	177		92	128

Table 5.5. Chromosomal aberration in Ni^{2+}/Cd^{2+} treated cultures. Cd pre: pretreatment with Cd^{2+}, figures in $[Cd^{2+}]$ rows refer to concentration and duration of pretreatment.

treatment there was a significant ($p < 0.001$) synergy between the two ions in the formation of chromosome type and chromatid type aberrations. The occurance of Ni^{2+}-induced chromatid type aberrations was unaffected by Cd^{2+} pretreatment but the formation of chromosome type aberrations was significantly ($p < 0.01$) antagonized.

5.5 Discussion

At the beginning of this chapter a number of questions were posed, the most important being: can Cd^{2+} alter the genotoxicity of other compounds. The data presented in the preceeding sections clearly indicates that the answer is yes. Whether the genotoxic effect of a compound is increased or decreased by Cd^{2+} depends on the nature of the compound. In some cases, the treatment protocol and assay considered had some bearing on the nature of the interaction.

Of the compounds investigated in the present study, only EMS has been considered by other researchers in relation to the effects of Cd^{2+} on its genetic toxicology (see Section 2.5). This work, mainly from Degraeve's laboratory, showed Cd^{2+} to synergistically enhance EMS-induced chromosome breakage in several plant species. The results presented above extend this finding to include muntjac cells. However, Cd^{2+} had the opposite effect on EMS-induced SCE - a clear indication that the two endpoints of genotoxicity arise through distinct mechanisms. Studies employing totally different experimental approaches have reached the same conclusion (reviewed by Kato, 1977). Which then, of the two indicators of genotoxicity is the most reliable? Both have been extensively validated and are reliable predictors of chemical carcinogens (Latt et al., 1981; Preston et al., 1981). However, it has been argued that SCE-induction is the more sensitive assay (Latt et al., 1981). As relatively low concentrations of treatment chemicals were used in the present experiments, one would expect the more sensitive assay to be more reliable. Taking into account (i) the relativly small sample sizes considered in some of the chromosomal aberration experiments and (ii) the greater consistency of the SCE data it appears that the SCE assay is the more reliable indicator of genotoxicity for the present purpose.

In the absence of any proven mechanism of SCE or chromosomal aberration formation suggestions as to the possible causes of the difference in effect of Cd^{2+} on these two phenomena would be entirely speculative.

The Cd^{2+}/MMC results are more consistent than the Cd^{2+}/EMS data. Cd^{2+} pretreatment or combined Cd^{2+}/MMC treatment reduces the effect of MMC on both SCE and chromosomal aberration. In the remaining Cd^{2+}/other mutagen combined treatment experiments, the presence of Cd^{2+} tended to increase the 'other mutagen alone' levels of SCE and chromosomal aberration. In the case of AO and H33258 the effects were less pronounced than found with Cd^{2+}/alkylating agent combinations. The Cd^{2+} induced alterations to Ni^{2+} genotoxicity were most evident in chromosomal aberration experiments.

Of the two types of chromosomal aberration, the induction of chromosome type aberrations by the other mutagens appeared to be affected to a greater extent by Cd^{2+}. The explanation of this observation may lie in some cell cycle specificity in the formation of the two types of aberration. It has been shown that Cd^{2+} causes a slowing in the progression of cells through S phase (Costa et al., 1982). Thus, any S phase dependent event would have a greater probability of occuring when cells are treated with Cd^{2+}.

In the case of Ni^{2+} the effect of Cd^{2+} co-treatment and pretreatment was different (Section 5.4.1). Co-treatment enhanced SCE and chromosomal aberration induction while pretreatment antagonized the formation of the two parameters. Among several possible explanations of this finding are:

1. An increased cellular uptake of one or both metals when they are present in culture medium together.
2. Cd^{2+} pretreatment prevents Ni^{2+} uptake.
3. Differences in the intracellular distribution of the metals - e.g. a decreased level of nuclear binding of Ni^{2+} following Cd^{2+} pretreatment.

Similar explanations could be put forward for any of the Cd^{2+}/other mutagen interactions. In the next chapter, cellular uptake and subcellular distribution are investigated as a possible basis for the interactions. There are, of course numerous other mechanisms which may be involved, many of which were discussed in Chapter 2. In the present study interaction at one other level has been considered, that of DNA repair (Chapter 8). The efficiency and rate of repair of DNA lesions has a major bearing on the genotoxicity of a compound. Thus, DNA repair systems are quite possibly involved in the interactions documented in this chapter.

5.6 Summary

1. The induction of SCE by EMS and MMC is reduced by cotreatment or pretreatment with Cd^{2+}.
2. Cd^{2+} treatment reduces the level of chromosomal aberration induced by MMC but increases that induced by EMS.
3. The induction of SCE and chromosomal aberration by AO and H33258 tends to be increased by Cd^{2+} treatment.
4. SCE and chromosomal aberration induction by Ni^{2+} is potentiated when cells are treated with Cd^{2+} concurrently.
5. Pretreatment of cells with Cd^{2+} reduces the level of Ni^{2+} induced SCE and chromosomal aberration.

Chapter 6 Mechanisms of Interaction I: Uptake and Subcellular Distribution Studies

6.1 Rationale and experimental design

When considering the toxic action of a compound, it is obvious that if the compound in question does not reach its cellular target it is not going to affect the cell. Similarly, the magnitude of the effect will depend upon the amount of the compound reaching its cellular target. The nucleus is the target organelle for the genotoxic effects of Cd^{2+} and DNA-binding agents.

In Chapter 4 it was shown that Cd^{2+} does not induce SCE in muntjac cells, also, the metal only increases the frequency of chromosomal aberrations after a short treatment followed by a recovery period. So, does Cd^{2+} reach its target (the nucleus) immediately or is the recovery period required before nuclear binding will occur? Results detailed in the preceeding chapter demonstrate the ability of Cd^{2+} to alter the chromosomal effects of other compounds. Does the same amount of Cd^{2+} enter cells and their nuclei when treated alone and in combination with another mutagen? Could this be important in the interactions? Likewise, is the cellular and nuclear uptake of the 'other mutagens' the same in the presence and absence of Cd^{2+}? These questions can be answered by means of uptake and subcellular distribution studies.

The use of radioisotopes makes cellular uptake experiments simple. Cells are grown in the presence of the radiolabelled compound, harvested and the activity in the cells determined using a suitable counter. An analysis of subcellular distribution may be made by homogenizing the cells after harvesting and subjecting the homogenate to a centrifugal fractionation proceedure. The activity in each of the fractions can then be determined. Results from such experiments are presented in this chapter. Cells were grown for varying periods in the presence of radiolabelled compounds, subjected to the subcellular fractionation proceedure described in Section 3.7.1. The activity in each of the fractions was determined by scintillation counting (Section 3.7). The

uptake and subcellular distribution of Cd^{2+} was followed using $^{109}CdCl_2$. The effects of EMS and Ni^{2+} on the cellular uptake and partitioning of Cd^{2+} were examined. As previously shown, the presence of Cd^{2+} alters the genotoxicity of these two compounds. Ideally the uptake and subcellular distribution of both EMS and Ni^{2+} should have been investigated. However only radiolabelled Ni^{2+} (as $^{63}NiCl_2$) was readily available, thus experiments were limited to this one other compound.

In Chapter 2 the ability of Cd^{2+} to induce the synthesis of the metal binding protein metallothionein was described. The ways in which metallothionein may modulate the toxicity of Cd^{2+} and other compounds were also considered. So it is of interest to investigate the role of metallothionein in the interactions previously described. Cytosol fractions from the subcellular distribution work were further analysed by gel filtration chromatography. Metallothionein has a characteristic elution profile on G-75 filtration columns so can be tentatively identified. Binding to other cytosolic molecules can also be followed in gel filtration experiments.

6.2 The uptake and subcellular distribution of Cd^{2+}

The results of a time course experiment following the uptake of Cd^{2+} into muntjac cells can be seen in figure 6.1. In this experiment cells were incubated in 0.1μM Cd^{2+}; nine tenths of this concentration was added as $^{109}CdCl_2$ (giving a radioactive concentration of 1μCi/ml) the remainder was unlabelled cadmium acetate.

When cells were treated with 0.1μM Cd^{2+}, uptake into whole cells (i.e. homogenate) was rapid and approximately linear over the first twenty four hours. During the subsequent 24 hours Cd^{2+} uptake was considerably less rapid. The maximum amount of Cd^{2+} to enter the cells was approximately $0.5\mu g/10^9$ cells. The pattern of uptake was somewhat different when cells were treated with 1μM Cd^{2+} (figure 6.2, dotted line). Over the entire 48 hour time course Cd^{2+} uptake was rapid, no

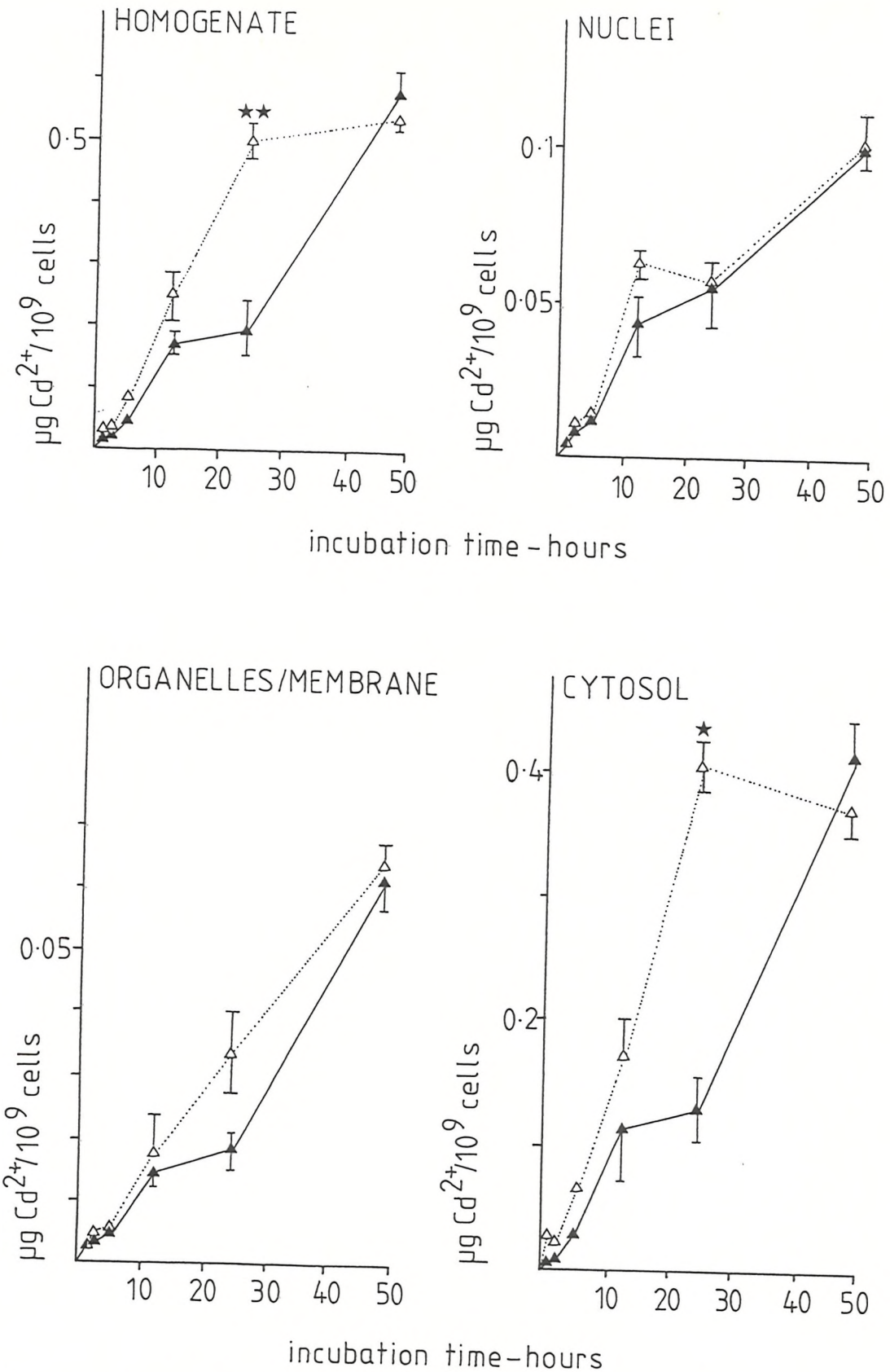


Figure 6.1. Uptake and subcellular distribution of Cd^{2+} in the presence and absence of EMS. △ 0.1μM cadmium acetate only, ▲ 0.1μM cadmium acetate + 100μM EMS. Points are means of three replicates, bars represent ± standard errors. Significant differences between treatments are denoted by stars, star code as in figure 5.1.

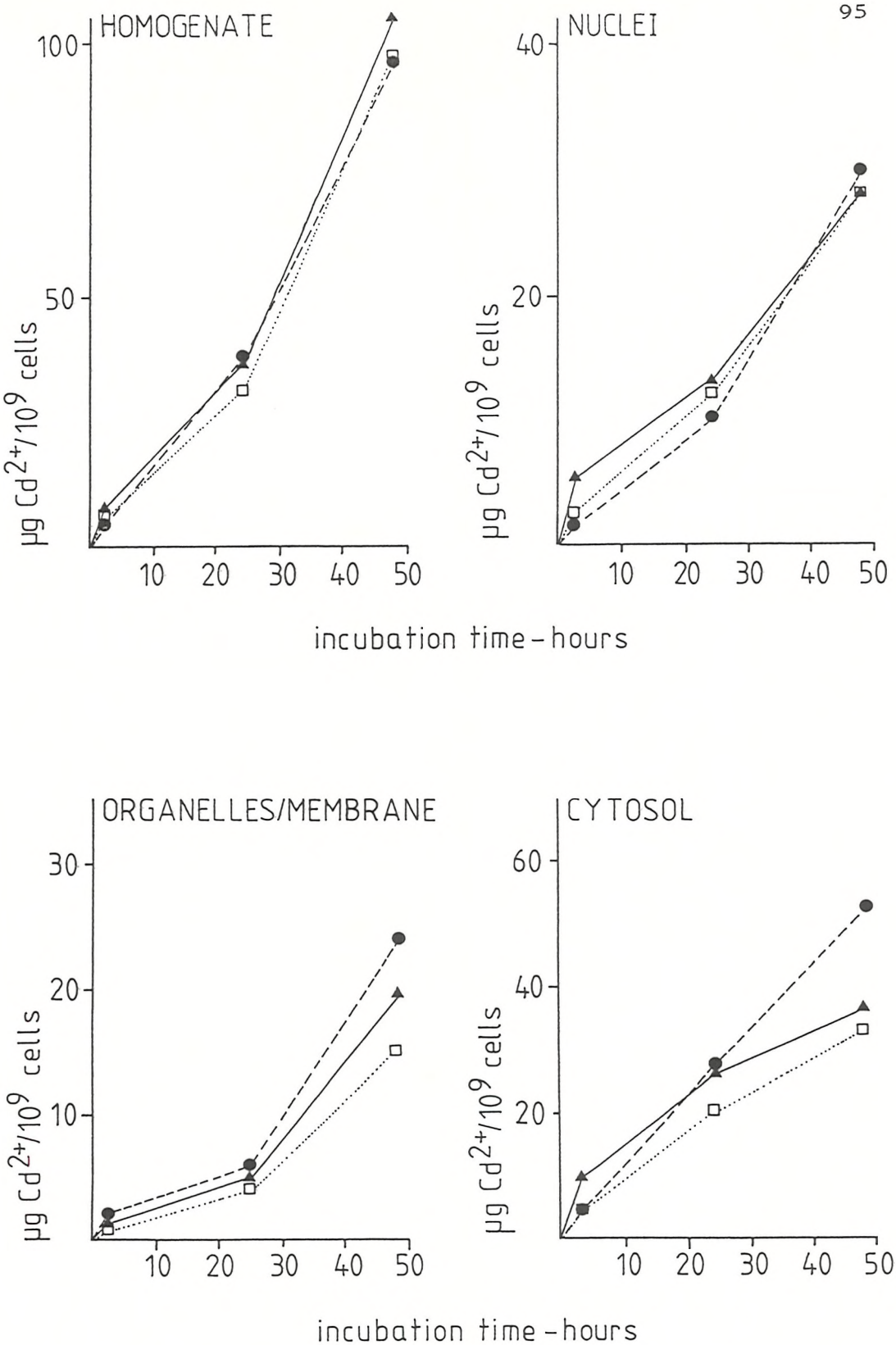


Figure 6.2. Uptake and subcellular distribution of Cd^{2+} in the presence and absence of Ni^{2+} or EMS. □ 1μM Cd^{2+} only, ▲ 1μM Cd^{2+} + 1μM Ni^{2+}, ● 1μM Cd^{2+} + 100μM EMS. Two replicates per point.

reduction in rate was seen. The initial rate of uptake, i.e. over the first 24 hours, was approximately 1.25μgCd/10^9 cells/hour compared with 0.02μgCd/10^9 cells/hour when cells were treated with 0.1μM Cd^{2+}. Thus, a ten fold increase in Cd^{2+} concentration in the treatment medium led to a 60 fold increase in the rate of uptake. From 24 to 48 hours of incubation the rate of Cd^{2+} uptake into cells treated with 1μM Cd^{2+} was still higher (2.7μg Cd/10^9 cells/hour). It would appear then that the processes governing Cd^{2+} uptake are different depending upon the concentration of Cd^{2+} to which cells are exposed. This difference in the pattern of cellular uptake may be due to:

1. Membrane damage occuring at the higher concentration allowing a more rapid uptake of Cd^{2+}.
2. The presence of two transport systems, a more rapid system functioning only at higher concentrations of the metal ion.
3. The uptake of the metal depending on the number of available intracellular binding sites. 1μM Cd^{2+} may induce the synthesis of a Cd-binding molecule (possibly metallothionein) while 0.1μM Cd^{2+} is below the threshold for initiating the synthesis of this molecule. Thus, the number of Cd^{2+}-binding sites would increase allowing a greater and more rapid accumulation of the metal in cells treated with the higher Cd^{2+} concentration.
4. Cadmium/zinc competition. As discussed in Chapter 2, Cd^{2+} can displace Zn^{2+} from proteins and other molecules. Thus, intracellular zinc homeostasis may be disturbed by cadmium. In an attempt to restore Zn^{2+} balance, the cells may 'open' zinc channels. As it seems likely that the uptake of Cd^{2+} into cells is by means of Zn^{2+} channels (Failla et al., 1979; Garson and Shaikh, 1984), if the extracellular Cd^{2+} concentration is high, cells will accumulate Cd^{2+} instead of Zn^{2+}. If extracellular Cd^{2+}/Zn^{2+} ratios were low more Zn^{2+} would be accumulated.

The first possible explanation can be excluded as data presented in Chapter 4 showed that little membrane damage occured in cells exposed to

either 0.1 or 1μM Cd^{2+} for 48 hours. At present it is not possible to determine which of the latter three explanations is correct.

Figures 6.1 and 6.2 (dotted lines) also show data concerning the subcellular distribution of Cd^{2+} in cells. It can be seen that at all times the highest proportion of the total cellular Cd^{2+} was found in the cytosol fractions; this was particularly marked in cells treated with 0.1μM Cd^{2+}. The pattern of Cd^{2+} accumulation in the cytosol was similar to that in whole cells. Little Cd^{2+} was found bound to the organelle/membrane pellets. In cells exposed to 1μM Cd^{2+} the pattern of binding to all three fractions fairly closely resembled the time course of cellular uptake. However, the situation is different in cells treated with 0.1μM Cd^{2+}. The organelle/membrane and nuclear binding of the metal continued to increase between 24 and 48 hours of exposure while whole cell uptake tended to level off. Organelle/membrane binding increased linearly throughout the 48 hours. In nuclei, a slight drop in the amount of Cd^{2+} bound was observed between 12 and 24 hours of incubation; however, this was only temporary as the nuclear content of the metal increased over the final 24 hours of the experiment.

Note that in cells treated with 0.1μMCd^{2+} the absolute amount of Cd^{2+} binding to organelle/membrane and nuclear fractions continued to increase over the 48 hours despite a tendency for the percentage of total cellular Cd^{2+} in these fractions to decrease. This effect is not seen at the higher concentration of the cation.

6.3 The effects of Ni^{2+} and EMS on Cd^{2+} uptake and subcellular distribution

Figure 6.1 compares the uptake and subcellular distribution of Cd^{2+} in cells treated with 0.1μM Cd^{2+} in the presence and absence of 100μM EMS. Uptake of the metal into whole cells was similar at all time points except 24 hours when more was found in cells treated with Cd^{2+} alone. This difference was reflected in the cytosolic accumulation of the metal

ion. Cd^{2+} binding to organelle/membrane and nuclear fractions was not significantly altered by the presence of EMS. The alkylating agent had no effect on Cd^{2+} accumulation in cells treated with 1μM Cd^{2+} either (figure 6.2). Nuclear accumulation of the metal was also similar in the presence and absence of EMS. Binding to organelle/membrane and cytosol fractions was slightly increased when cells were treated with the alkylating agent and cadmium.

Only slight alterations in Cd^{2+} uptake were caused by adding nickel with cadmium to the treatment medium. Figure 6.3 shows the uptake and subcellular binding of Cd^{2+} in cells treated with 0.1μM Cd^{2+} and 1μM Ni^{2+}. Whole cell uptake was unaffected by the presence of nickel ions. Cadmium binding to nuclei was less in cells treated with both metals for 48 hours than cells exposed to Cd^{2+} alone for the same period. This difference, however, is not statistically significant in a 't' test. cytosol accumulation was also the same irrespective of treatment. It was only the organelle/membrane fractions of cells treated for 48 hours that showed any significant differences between the treatments. The organelle-membrane fractions of cells treated with Cd^{2+} alone accumulated a significantly greater amount of the metal than cells treated with the two ions concurrently. However, in absolute terms the difference is small, approximately 0.035ug $Cd^{2+}/10^9$ cells.

The presence of 1μM Ni^{2+} had little effect on the uptake and subcellular binding of 1μM Cd^{2+}, see figure 6.2.

6.4 Uptake and subcellular distribution of Ni^{2+}

Figure 6.4 (dotted line) shows the time course of Ni^{2+} incorporation into whole cells incubated in 1μM Ni^{2+}. Nickel ions appear not to be as readily taken up by muntjac cells as cadmium ions. The maximal cellular Ni^{2+} concentration reached was approximately 0.9μg $Ni^{2+}/10^9$ cells. In contrast, cells exposed to the same concentration of Cd^{2+} incorporated 95μg/$Cd^{2+}/10^9$ cells by 48 hours of incubation. By converting these figures into the number of ions per cell, the bias due

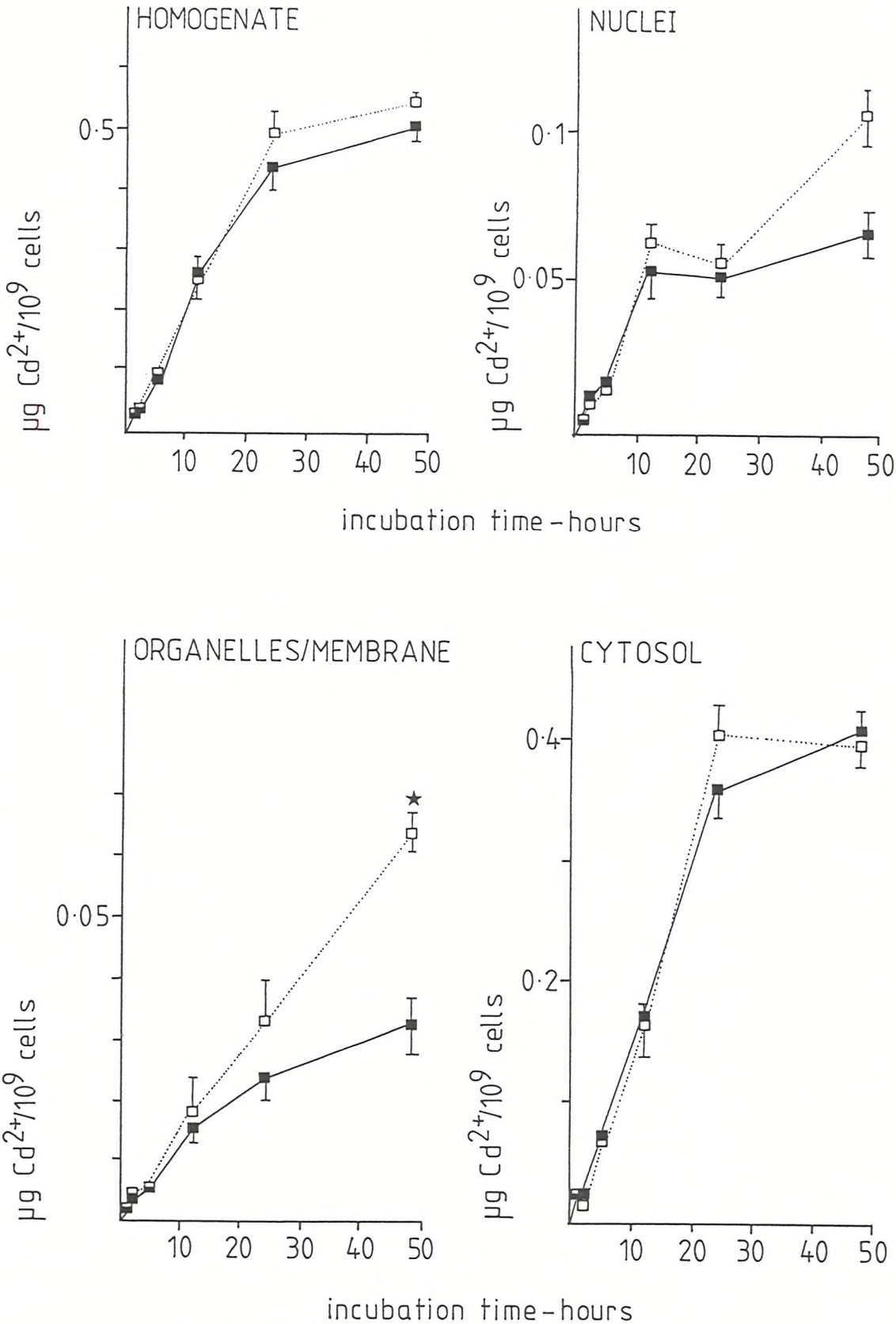


Figure 6.3. Uptake and subcellular distribution of Cd^{2+} in the presence and absence of nickel ions. □ 0.1μM cadmium acetate only, ■ 0.1μM cadmium acetate + 1μM nickel sulphate. Points are means of three replicates, bars represent ± standard errors. Significant differences between treatments are denoted by stars, star code as in figure 5.1.

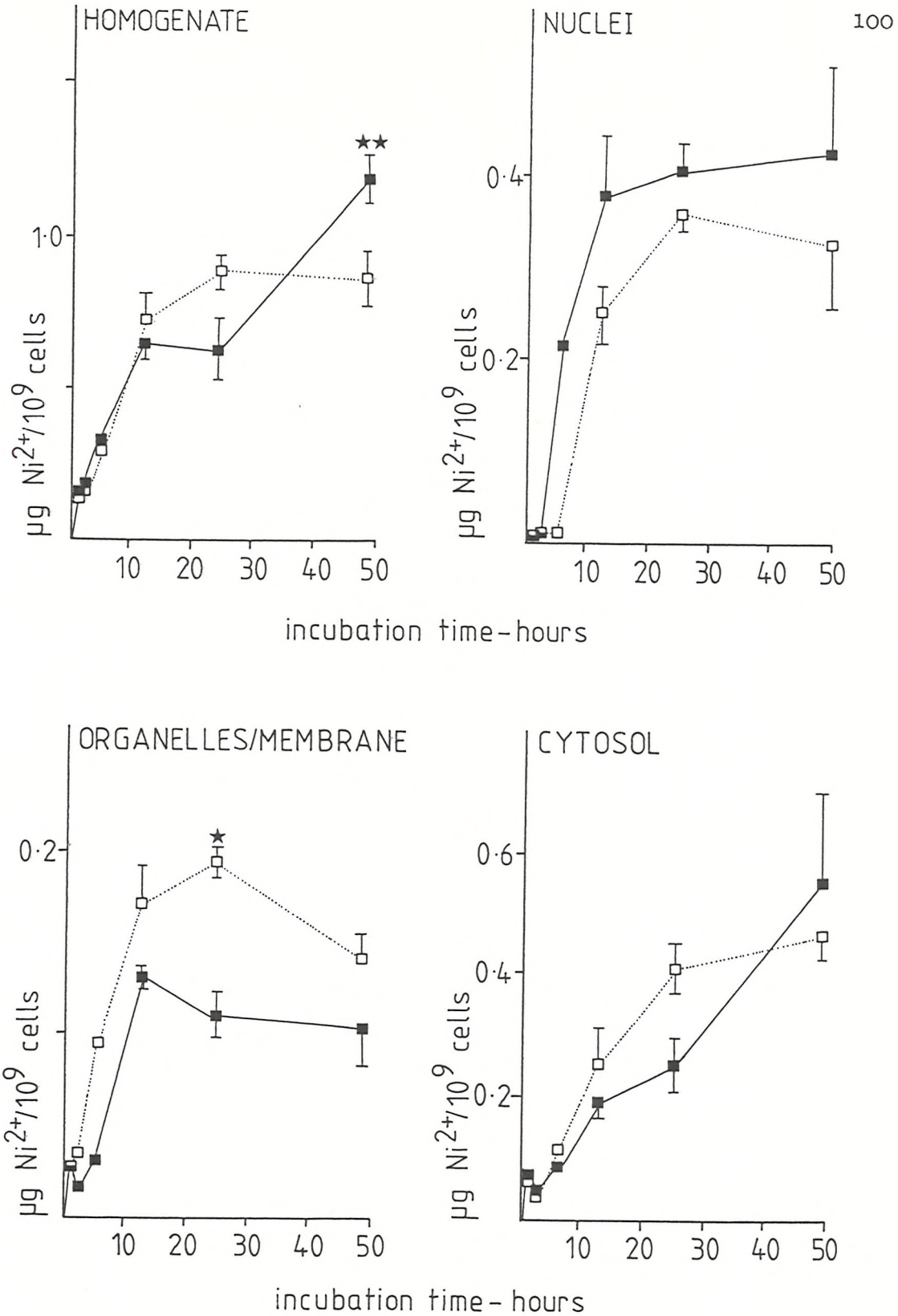


Figure 6.4. Uptake and subcellular distribution of Ni^{2+} in the presence and absence of cadmium ions. □ 1μM nickel sulphate only, ■ 1μM nickel sulphate + 0.1μM cadmium acetate. Points are means of three replicates, bars represent ± standard errors. Significant differences between treatments are denoted by stars, star code as in figure 5.1.

to ionic weight differences can be compensated for;

$$\text{Number ions/cell} = \frac{\text{Accumulation}}{10^{9} \times \text{i.w.}} \times \text{Avogadros constant}$$

where: Accumulation is expressed in $\mu g/10^{9}$ cells
i.w. - ionic weight in ug
Avogadros constant = 6×10^{23}

Therefore at 48 hours there were:

$$\frac{95}{10^{9} \times (112.4 \times 10^{6})} \times (6 \times 10^{23})$$

$$= 5.07 \times 10^{8} \ Cd^{2+} \text{ ions per cell}$$

and:

$$\frac{0.9}{10^{9} \times (58.71 \times 10^{6})} \times (6 \times 10^{23})$$

$$= 9.2 \times 10^{6} \ Ni^{2+} \text{ ions per cell.}$$

Thus, Cd^{2+} is accumulated in cells to a level 55-fold greater than that to which Ni^{2+} is. This suggests that the mechanisms of uptake of these two metal ions are different.

Of the absorbed Ni^{2+}, approximately 40% bound to nuclei and a similar proportion to the cytosol fractions. The remaining 20% was found in association with the organelle/membrane pellet.

Figure 6.4 also compares the uptake and subcellular distribution of Ni^{2+} in the presence and absence of Cd^{2+}. Cellular uptake was similar irrespective of treatment up to 24 hours of incubation. Over the subsequent 24 hours the Ni^{2+} content of cells treated with both metals increased more rapidly than in cells treated with Ni^{2+} only. By 48 hours of incubation there was significantly more Ni^{2+} in cells treated with the two metal ions. Despite this difference in cellular uptake, nuclear binding of Ni^{2+} was unaffected by the presence of Cd^{2+}. Over the whole time course slightly more Ni^{2+} was found in the co-treated

cells, however, these differences were always within experimental variation. After 24 hours of incubtion more Ni^{2+} was found in the organelle/membrane pellets of cells treated with nickel alone than cells treated with Ni^{2+} and Cd^{2+}. At all other times differences in organelle/membrane binding were not significant. The cytosolic Ni^{2+} content was similar at all times whether cells were treated with Ni^{2+} alone or Ni^{2+} and Cd^{2+}.

6.5 The binding of Cd^{2+} to cytosol molecules

A series of G-75 elution profiles of Cd^{2+} (0.1μM) treated muntjac cell cytosol fractions are plotted in figure 6.5. The results are expressed as relative cadmium content, this is the raw data standardized on the bases of cell number and the ratio of labelled to unlabelled Cd^{2+} with which the cells were treated. Three Cd^{2+}-binding peaks can be seen with Ve/Vo ratios of 1, 2 and 2.7. The first two peaks can be tentatively identified as high molecular weight proteins (Ve/Vo = 1) and metallothionein (Ve/Vo = 2) (Cain and Holt, 1979). A Cd^{2+}-binding peak at Ve/Vo = 2.7 has not been commonly reported in the literature. Some experimental work attempting to characterize the molecule(s) eluting at this volume is described in Chapter 7.

The major Cd^{2+}-binding species in muntjac was that eluting at Ve/Vo = 2.7 (figures 6.5, 6.6 and 6.7). Note that the involvement of the Ve/Vo = 2 peak (putative metallothionein) is minimal, particularly in cells treated with 1μM Cd^{2+}. In this respect muntjac cells are different to other cultured cells and the rodent liver (see section 2.2). By summing the relative cadmium content of each fraction in the three peaks the Cd^{2+} binding to the different peaks can be quantified. Such data is presented in table 6.1. Up to 12 hours of incubation, the high molecular weight proteins (Ve/Vo = 1) bound an increasing percentage of the total cytosolic Cd^{2+}. After 48 hours this proportion decreased to 1%. The putative metallothionein (Ve/Vo = 2) also showed a tendency to bind an increasing proportion of the cytosolic Cd^{2+} followed by a reduction in its Cd^{2+} binding capacity. In contrast, the Ve/Vo = 2.7 peak initially bound 4.3% of the cytosolic Cd^{2+}, by 5 hours this dropped to 3.5%. From 5 to 48

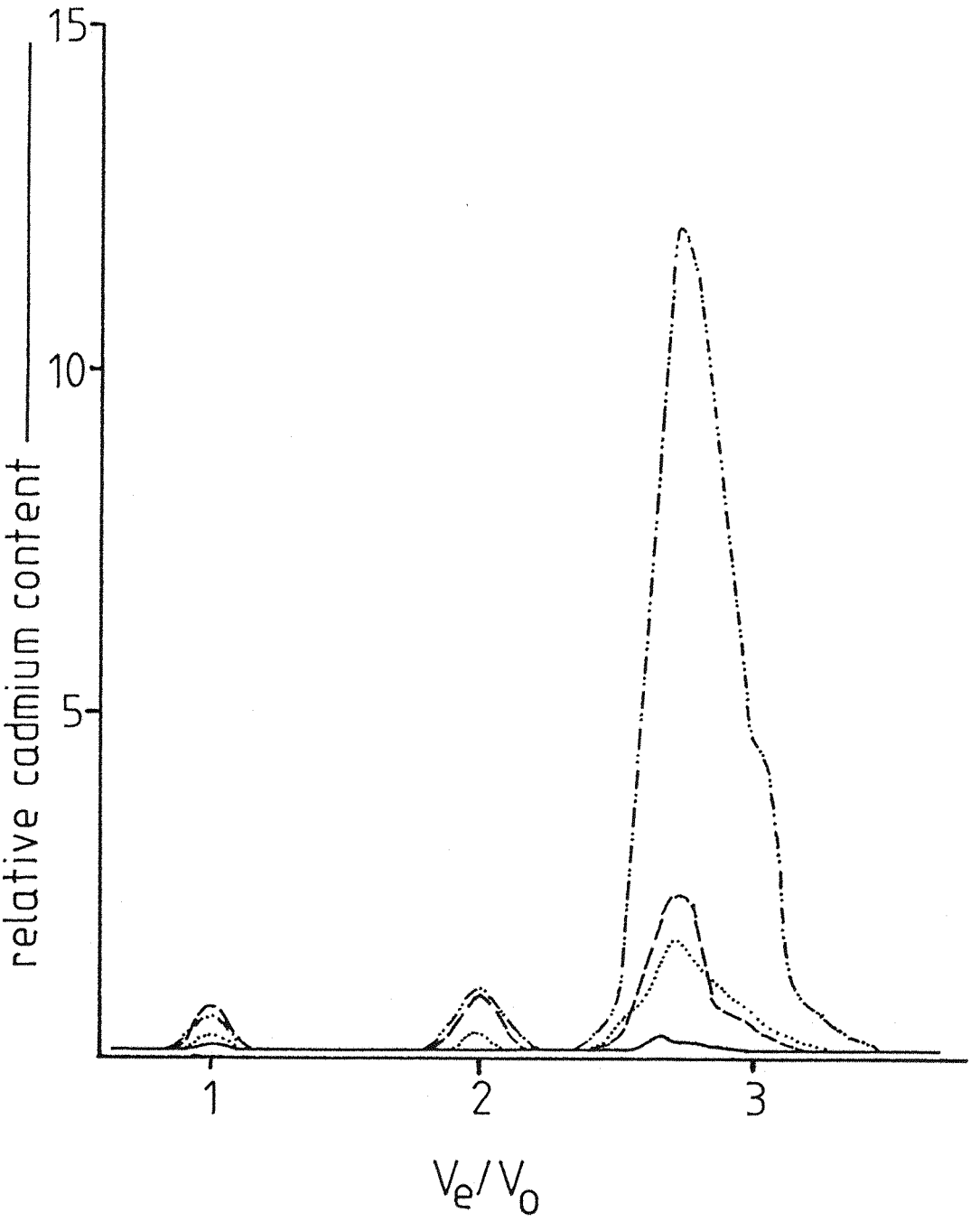


Figure 6.5. G - 75 gel filtration chromatography profile of cadmium binding to cytosol molecules.
Cells incubated in 0.1μM Cd^{2+} for: ——— 1 hour, ... 12 hours, — — — 24 hours, —·—·— 48 hours.

TABLE 6.1 NOTE:

As the counting efficiency of the gamma counter used for ^{109}Cd determinations was not established, the absolute Cd content of each peak could not be calculated. Therefore, results are expressed as relative amount Cd per peak. This value refers to the counts per minute per peak standardized on the basis of cell number in the original sample and the ratio of radioactive to non-radioactive Cd used in the experiment multiplied by a constant such that 7000 standardized counts per minute is equivalent to a relative amount of 1.

Slight discrepancies between the relative amounts and their corresponding percentages could arise from rounding up errors in the initial calculations of relative amounts.

hours there was a steady rise in the proportion of Cd^{2+} bound to this fraction. This increase may be due to 'de novo' synthesis of the Ve/Vo = 2.7 molecule(s).

TABLE 6.1 Relative amounts and percentages of Cd^{2+} binding to cytosol molecules. Cells incubated in 0.1μM Cd^{2+} for 1 - 48 hours. Averages of two determinations, percentages are relative to total cellular Cd^{2+}

Time hours \ Peak (Ve/Vo)	1		2		2.7	
	rel. amt.	%	rel. amt.	%	rel. amt.	%
1	0.2	3.8	0.09	2.7	2.72	43.7
2	0.18	5	NOT	DETECTED	1.89	40.3
5	1.00	8.4	0.55	5.8	4.03	35.4
12	2.03	11.9	1.18	12.5	9.19	55.8
24	1.99	7.8	1.82	6.8	11.11	52.3
48	0.81	1	4.46	6.3	66.62	75.5

6.6 The effects of EMS and Ni^{2+} on Cd^{2+} binding to cytosol molecules

Tables 6.2 and 6.3 show the relative amounts and percentages of Cd^{2+} bound to cytosol components of cells treated with 0.1μM Cd^{2+} and 1μM Ni^{2+} or 100μM EMS. The most noticable difference between the cytosol binding patterns of cells treated with Cd^{2+} plus another compound and with Cd^{2+} alone is the smaller amount of binding to the Ve/Vo = 2.7 peak. Refering back to figures 6.1 and 6.2, it can be seen that this is not due to a reduction in the total amount of the metal accumulated in the cytosol fractions. Binding to other cytosol peaks is not increased markedly either. This suggests that co-treatment with EMS or Ni^{2+} tends to increase the proportion of free Cd^{2+} present in the cytosol. A casual observation that it took longer for residual radioactivity to be removed from columns through which cytosols from Cd/EMS or Cd/Ni treated cells had been passed lends support to this idea.

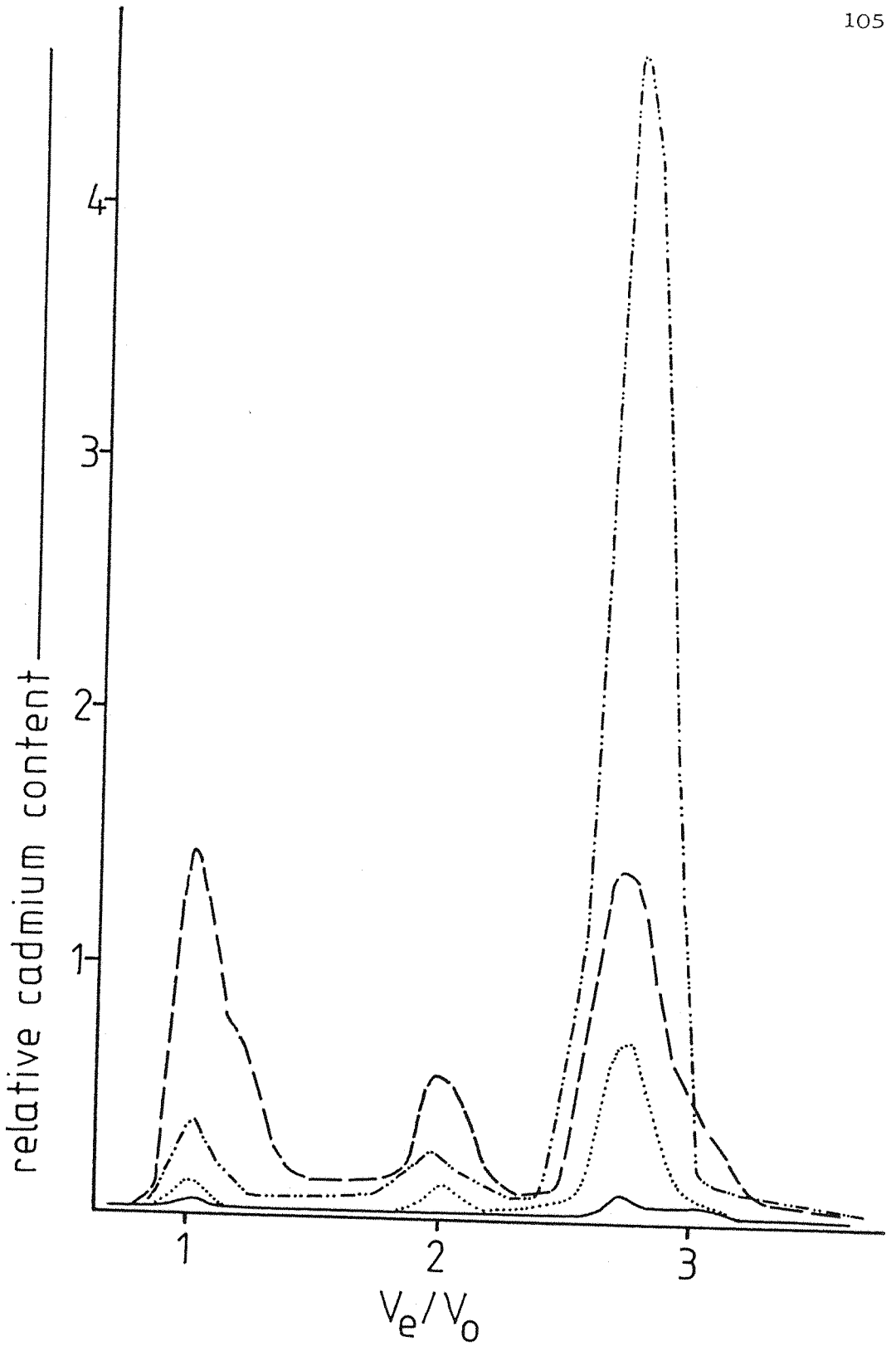


Figure 6.6. G-75 gel filtration chromatography profile of cadmium binding to cytosol molecules.
Cells incubated in 0.1μM Cd^{2+} and 1μM Ni^{2+} for:
——— 1 hour, ... 12 hours, — — — 24 hours,
—·—·— 48 hours.

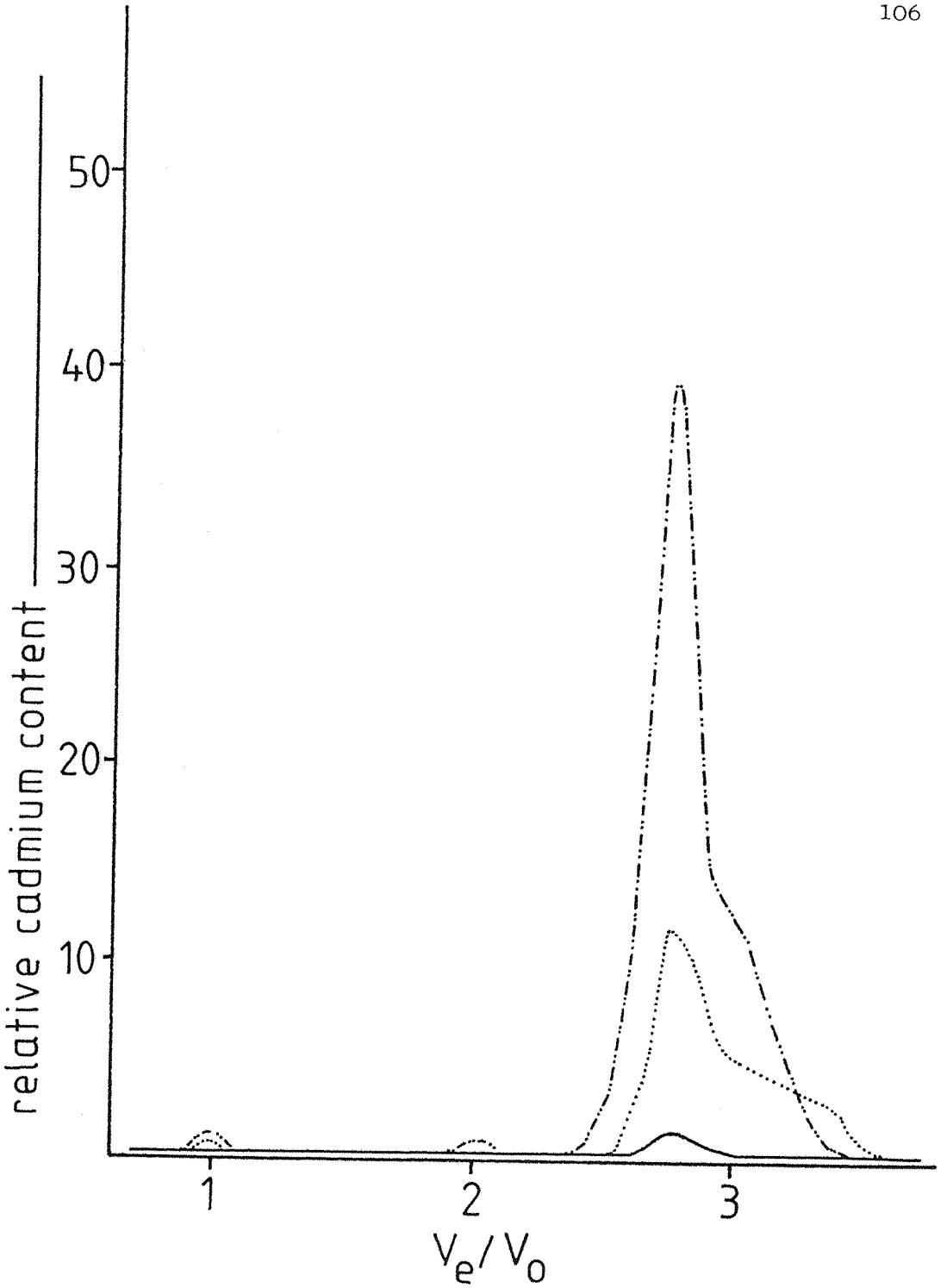


Figure 6.7. G - 75 gel filtration chromatography profile of cadmium binding to cytosol molecules.
Cells incubated in 1μM Cd^{2+} for: ——— 2 hours, ····· 24 hours, – – – – 48 hours.

TABLES 6.2 and 6.3 NOTE:

As the counting efficiency of the gamma counter used for ^{109}Cd determinations was not established, the absolute Cd content of each peak could not be calculated. Therefore, results are expressed as relative amount Cd per peak. This value refers to the counts per minute per peak standardized on the basis of cell number in the original sample and the ratio of radioactive to non-radioactive Cd used in the experiment multiplied by a constant such that 7000 standardized counts per minute is equivalent to a relative amount of 1.

Slight discrepancies between the relative amounts and their corresponding percentages could arise from rounding up errors in the initial calculations of relative amounts.

Cd^{2+} binding to the putative metallothionein (Ve/Vo = 2) was not affected to a great extent by the presence of either the alkylating agent or the other metal ion. Alteration of Cd^{2+} binding to high molecular weight proteins was minimal also.

TABLE 6.2 Relative amounts and percentages of Cd^{2+} bound to cytosol molecules, cells incubated in 0.1μM Cd^{2+} + 100μM EMS for 1-48 hours. Averages of two determinations, percentages are relative to total cellular Cd^{2+}.

TIME hours \ PEAK (Ve/Vo)	1		2		2.7	
	rel. amt.	%	rel. amt.	%	rel. amt.	%
1	0.17	5	0.08	2.5	1.89	40.2
2	0.16	9.9	0.08	4.3	0.44	23.9
5	0.24	3.3	0.39	6.3	1.36	30.0
12	0.46	5.2	0.92	10.9	4.37	52.3
24	1.9	9.2	1.25	6.05	10.92	53
48	1.41	6.5	0.59	3.8	23.42	69.5

TABLE 6.3 Relative amounts and percentages of Cd^{2+} bound to cytosol molecules, cells incubated in 0.1μM Cd^{2+} + 1μM Ni^{2+} for 1 - 48 hours. Averages of two determinations, percentages are relative to total cellular Cd^{2+}.

TIME hours \ PEAK (Ve/Vo)	1		2		2.7	
	rel. amt.	%	rel. amt.	%	rel. amt.	%
1	0.17	6.8	0.08	3.4	0.9	34.4
2	0.92	22.9	0.65	11.6	1.76	26
5	0.6	6.6	0.49	6.3	3.2	41.8
12	0.47	6.5	0.6	8.4	3.3	55.5
24	5.75	17.5	2.7	8.3	7.63	30.5
48	1.22	4.1	1.72	6.1	17.5	58.5

6.7 Discussion

Cadmium uptake into muntjac cells was rapid, the intracellular Cd^{2+} concentration reached 0.5ug/10^9 cells by 48 hours of incubation in 0.1µM Cd^{2+}. Although there were signs of the rate of uptake decreasing after 24 hours, this cannot be due to depletion of Cd^{2+} available for uptake in the treatment medium. The average number of cells per culture dish used in these experiments was 10^6. By 24 hours 10^6 cells would have removed .0005ug Cd^{2+} from the culture medium. The medium contained 0.1uM Cd^{2+} i.e. 10^{-7} x 112.4 x 10^{-3} = 0.011µg Cd^{2+}/ml (concentration x ionic weight x conversion factor for g/l to µg/ml). 2.5 ml of medium was used in each dish, therefore 0.028 ug Cd^{2+} were available for uptake. So only 1/56th of the total Cd^{2+} available in the treatment medium was accumulated in the cells after 24 hours, leaving ample remaining for further uptake.

The pattern of Cd^{2+} uptake in muntjac cells treated with 0.1µM Cd^{2+} was similar to that seen in CHO cells (Hildebrand et al., 1979). However, the time course of Cd^{2+} uptake into muntjac cells exposed to 1µM Cd^{2+} was markedly different. Some possible explanations for this observation were mentioned in Section 6.2.

Most Cd^{2+} that entered the muntjac cell was located in the cytosol fraction. Experiments using other cell culture systems and whole animals have had similar results (see Section 2.2). Although there was a tendency for the percentage of total cellular Cd^{2+} in cytosol fractions to increase with time, the trend was not as striking as found in the rat liver (see Yoshikawa and Suzuki, 1976, for example). Despite the finding that the proportion of total cellular Cd^{2+} bound to nuclei dropped throughout the incubation period, the absolute concentration continued to increase.

Therefore, Cd^{2+} does enter the muntjac cell and reach the nucleus (cellular target for chromosome damage). There is no 'lag' period during which Cd^{2+} was not detectable in the cell or any of the subcellular fractions. By 48 hours the nuclei of cells incubated in 0.1µM Cd^{2+} and

1μM Cd^{2+} had accumulated 0.1μg $Cd^{2+}/10^9$ nuclei and 28μg $Cd^{2+}/10^9$ nuclei respectively. These accumulations represent:

$$\frac{0.1}{10^9 \times (112.4 \times 10^6)} \times (6 \times 10^{23})$$

$= 5.3 \times 10^5$ Cd^{2+} ions bound per nucleus in cells incubated in 0.1uM Cd^{2+}

and:

$$\frac{28}{10^9 \times (112.4 \times 10^6)} \times (6 \times 10^{23})$$

$= 1.5 \times 10^8$ Cd^{2+} ions bound per nucleus in cells incubated in 1uM Cd^{2+}.

Given that the muntjac nucleus contains 9.9pg DNA (Rees and Bootman, 1982) and the average molecular weight of a deoxyribonucleotide is 360g per mole, the nucleus contains approximately:

$$\frac{9.9 \times (6 \times 10^{23})}{360 \times 10^{12}} = 1.65 \times 10^{10} \text{ nucleotides}$$

or 8.25×10^9 nucleotide pairs. Thus, the nuclei of cells incubated in 0.1μM Cd^{2+} contain 1 Cd^{2+} ion bound per 1557 base pairs and those of cells treated with 1μM Cd^{2+}, 1 Cd^{2+} ion bound per 55 base pairs. Considering these high ratios, it is surprising that chromosomal damage is not induced by Cd^{2+}. It must be borne in mind however, that the nucleus contains considerable amounts of protein to which Cd^{2+} could also bind. Also it is possible that the metal binds to sites in the nucleus which are not involved in SCE or chromosomal aberration formation.

The Ni^{2+} uptake experiments showed this metal to be readily taken up by muntjac cells, although at a slower rate than cadmium ions. Ni^{2+} bound to cytosol, nuclear and organelle/membrane fractions (in a decreasing order of magnitude). Ni injected into mice and rats binds mainly to mitochondrial and microsomal fractions of myocardium (Mathur et al., 1979) and liver (Herlant-Peers et al., 1983). However, Heath and Webb (1967) found most intracellular nickel bound to the nuclei of rhabdomyosarcomas induced by the metal. So the intracellular distribution of Ni^{2+} appears to depend upon the cell type being investigated.

No consistently significant changes in the cellular uptake and subcellular distribution were caused by co-treatment of cells with Cd^{2+} plus Ni^{2+} or EMS. Combined treatment with the two metals significantly altered Cd^{2+} uptake at only one time point. More importantly, neither Ni^{2+} or the alkylating agent significantly increased or decreased the extent of Cd^{2+} binding to nuclei. Therefore, it is unlikely that alteration in Cd^{2+} uptake or nuclear binding accounts for the interactions between Cd^{2+} and EMS or Cd^{2+} and Ni^{2+} in chromosome assays. Nonetheless, the Cd^{2+} binding to organelle/membrane and cytosol fractions may be of importance in determining the genetic damage caused by the 'other mutagens'. Cd^{2+} binding to these fractions tended to be greater in cells treated with 1μM Cd^{2+} and the other compounds than in cells treated with 1μM Cd^{2+} alone. On the other hand, cells treated with 0.1μM Cd^{2+} alone tended to accumulate more Cd^{2+} in cytosol and organelle/membrane fractions than did co-treated cells. So, again, there is no consistent trend evident.

The extent of nuclear binding of Ni^{2+} was not affected by the presence of Cd^{2+}. However, at 48 hours, significantly more Ni^{2+} was found in homogenates of cells treated with Ni^{2+} and Cd^{2+}. More generally, differences in uptake and intracellular distribution of Ni^{2+} in cells treated with the one or both ions were not consistent throughout the time course of the experiments. Hence, it would seem unlikely that Cd^{2+} induced alteration to Ni^{2+} uptake and subcellular distribution can account for the changes seen in chromosome damage assays. Although it is tempting to speculate, it cannot be said that Cd^{2+} does not alter the uptake or subcellular distribution of any of the other mutagens.

The distribution of Cd^{2+} among cytosol molecules of muntjac cells is different to that found in any other cell type. A molecule (or molecules) which bind a large, and with time, increasing proportion of the cytosolic Cd^{2+} was identified. It does not elute from G-75 gel filtration columns at the same volume as rat liver metallothionein (compare figures 6.5 - 6.7 with figure 3.6). Small amounts of Cd^{2+} did elute at a volume close to that one would expect to find metallothionein (Ve/Vo = 2), but, compared to the lower molecular weight comoponent (Ve/Vo

= 2.7) its involvement in Cd^{2+} binding was minimal. Further investigations into the nature of the Ve/Vo = 2.7 component are described in Chapter 7. It is worth mentioning at this point that metallothionein is not the only Cd^{2+}-binding molecule identified in mammalian cytosols. Recently, Waalkes et al (1984) identified a Cd^{2+} binding protein, distinct from metallothionein in a number of properties, in rat testes.

The major Cd^{2+}-binding component in the cytosols of cells treated with Cd^{2+} plus another compound was also the Ve/Vo = 2.7 molecule(s). Although, the extent of Cd^{2+} binding to this peak was considerably less in co-treated cells than in cells exposed to the cation alone (but total cytosol Cd^{2+} levels were similar). Therefore, there must be an increased amount of free Cd^{2+} in the cytosols of co-treated cells. It is not clear whether this effect is important in the interactions between Cd^{2+} and the other mutagens described in Chapter 5. If it is, it can only be influencing chromosomal aberration. Co-treatment of cells with Ni^{2+} and Cd^{2+} led to synergistic yields of chromosomal aberrations and SCEs; Cd^{2+}/EMS treatment gave rise to an increased frequency of chromosomal aberrations but antagonized the formation of SCEs. Thus, the potentiation of chromosomal aberration formation could be due to the increased levels of free Cd^{2+} in co-treated cells. However, this data can be interpreted differently. It could be argued that the drop in Cd^{2+} binding to the Ve/Vo = 2.7 component was due to displacement of the Cd^{2+} by EMS or Ni^{2+} (depending on treatment). As cells continuously exposed to Cd^{2+} alone showed no increase in SCEs or chromosomal aberrations, free Cd^{2+} is probably not involved in either type of nuclear lesion. So cells maybe protected from EMS or Ni^{2+} induced damage by being bound to the Ve/Vo = 2.7 substance. Further experiments are required to resolve the role of this low molecular weight molecule in Cd^{2+}/other mutagen interactions.

6.8 Summary

1. Cd^{2+} can readily enter muntjac cells. The time course of uptake is concentration dependent.

2. Most Cd^{2+} that enters cells binds in the cytosol but significant amounts of the cation are found in other subcellular fractions.
3. Ni^{2+} can enter muntjac cells, although at a slower rate than Cd^{2+}. Binding to all subcellular fractions occurs.
4. Muntjac cells are different from other cells in that most cytosolic Cd^{2+} binds to a low molecular weight component (Ve/Vo = 2.7) not a metallothionein-like protein.
5. Alterations to the uptake and subcellular distribution of Cd^{2+} by EMS and Ni^{2+} are small and probably do not account for the interactions seen in genotoxicity assays.
6. Cd^{2+} induced alterations to Ni^{2+} uptake and subcellular distribution are small. Again, these differences probably do not explain the interactions between the metals in chromosome assays.
7. Co-treatment of cells with Cd^{2+} and Ni^{2+} or Cd^{2+} and EMS appears to increase the level of free Cd^{2+} in the cytosol. The significance of this observation in explaining the interactions in genotoxicity assays is unclear.

Chapter 7 Cadmium Resistant Cells: their characterization and responses to other mutagens

Virtually all the published work involving Cd^{2+} resistant cells has focused on two points (i) the role of metallothionein in Cd^{2+} detoxification and Cd^{2+} resistance and (ii) the mechanisms of genetic regulation of Cd^{2+} resistance. Some more recent work has concerned the relative cytotoxicity of cancer chemotherapeutic drugs to wild type and Cd^{2+} resistant cells (or, more corectly, cells with an enhanced metallothionein induction capacity). The Cd^{2+} resistant variant of muntjac produced in this study has been put to somewhat different use. As pointed out in Chapter 1, Cd^{2+} resistant cells may be considered the 'in vitro' counterpart of the cells of an individual who has been occupationally exposed to cadmium over a long period. For this purpose it is not desirable to have cells resistant to extremely high concentrations of the cation which would be much greater than those found in exposed persons. To aid the interpretation of the effects of other mutagens on Cd^{2+} resistant muntjac cells, experiments comparing them to their normal counterparts were conducted. The first part of this chapter concerns the production and characterization of the Cd^{2+} resistant cells while the second details the effects of other mutagens on these cells.

7.1 Characterization of Cd^{2+} resistant cells

Normal muntjac cells, when exposed to 5μM Cd^{2+} were unable to survive beyond 14 days (see figure 4.1). By observation under an inverted phase contrast microscope, it could be seen that cells would cease dividing after 4-5 days in this concentration of the metal ion. In contrast, the Cd^{2+} resistant cells (CR5) produced by the method detailed in section 3.4.1. were able to proliferate, apparently indefinitely, in 5μM Cd^{2+}. Thus, normal and CR5 cells differ in their susceptibility to the cytotoxic action of Cd^{2+}.

7.1.1 General Observations

During the course of producing the resistant cells, several casual unquantified observations were made concerning the nature of Cd^{2+} toxicity and the CR5 cells themselves. Most of these observations were made by examination of cell cultures under an inverted phase contrast microscope.

When the Cd^{2+} concentration in culture medium was increased, say, from 1μM to 2μM, following a period of 2-3 days a phase of cell death would occur. Cell division appeared to stop about 1 day after placing cells in the higher metal concentration. A week or more would pass before division resumed. Not all cells would become active at the same time, mitosis would start in a few isolated areas in the culture vessel. Of the cells that remained quiescent some became (or were) very large and multinucleate. As large cells were always present in cultures of CR5 cells, even after subculturing for several months, they were probably continually produced. The large cells seen soon after increasing the Cd^{2+} concentration did not die off as did many of the other quiescent cells of more normal morphology. Death of the quiescent cells appeared to occur in waves. Each wave following a few days after a change of medium and addition of fresh Cd^{2+}, however, the waves of cell death did not follow every change of medium.

The doubling time of cells that had recently begun to divide in a previously growth inhibitory Cd^{2+} concentration seemed longer than normal. The cell cycle time of the CR5 cell line finally produced was similar to that of normal muntjac cells. This observation is based on the fact that in SCE assays a high proportion of differentially stained (second round) metaphases could be obtained after culturing CR5 cells in BUdR for 48 hours. The same time was required for a high yield of differentially stained metaphases of normal cells.

7.1.2 Persistence of Cd^{2+} resistance

The viability of CR5 cells, once established, was close to that of normal muntjac cells growing in the absence of Cd^{2+}, i.e. 95 - 100% as determined by trypan blue dye exclusion. If CR5 cells were grown in the absence of Cd^{2+} for two weeks then returned to 5μMCd^{2+}, their growth and viability was unaffected. Population doubling time remained approximately 24 hours and viability was 95-100%. On return of CR5 cells grown in the absence of the cation for one month to 5μMCd^{2+} some cells would not survive. Most cells would continue to proliferate as normal but 2-5% would round up and detach from the culture vessel surface. So, Cd^{2+} resistance is maintained in some cells for one month. This represents enough time for 28 cell cycles, thus, each cell would have given rise to 2.7×10^8 (2^{28}) progeny. Any intracellular molecules, the production of which depended on the presence of Cd^{2+}, would be very greatly diluted in this time.

7.1.3 Cd^{2+} uptake and subcellular distribution in CR5 cells

In figure 7.1 the results of an experiment following Cd^{2+} uptake and subcellular distribution in normal and CR5 cells are plotted. Prior to incubation in radioactive Cd^{2+}, the CR5 cells were grown in the absence of Cd^{2+} for a week. This would have diluted the intracellular Cd^{2+} content of the cells 128-fold due to the completion of seven cell cycles. So, the uptake of Cd^{2+} into CR5 cells with a much diminished cellular concentration of the cation was being followed. Both cell types were incubated in a total Cd^{2+} concentration of 1μM (1μCi/ml $^{109}CdCl_2$ + 0.99μM cadmium acetate). The use of this metal ion concentration allowed direct comparison of the cell types without significant interference by cytotoxic effects.

The uptake of Cd^{2+} into whole cells (homogenate graph in figure 7.1) is greater and more rapid in CR5 cells. By 48 hours of incubation CR5 cells contained approximately 4 times the amount of Cd^{2+} accumulated in normal cells. Despite this enhanced cellular uptake, the amounts of

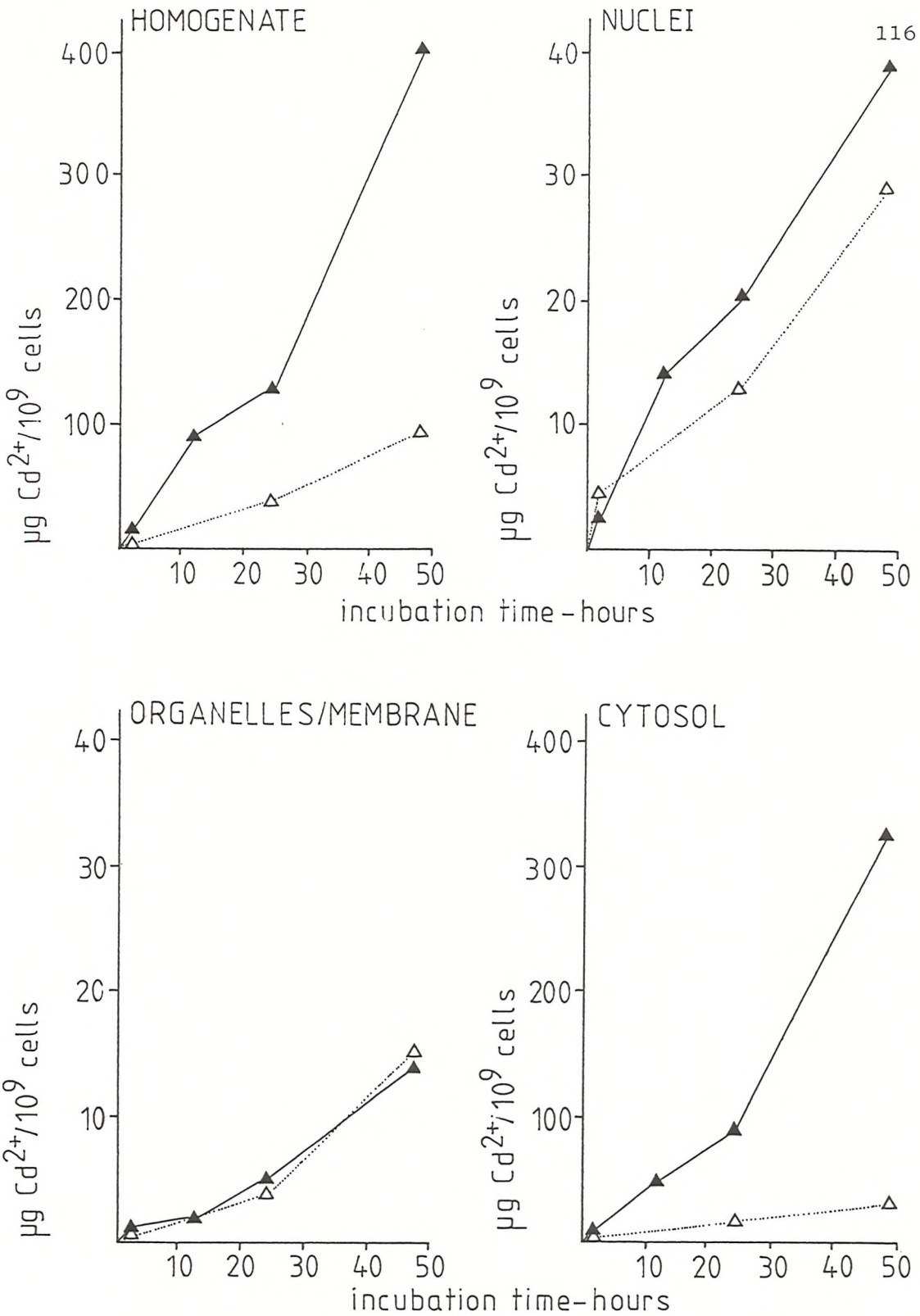


Figure 7.1. Uptake and subcellular distribution of 1μM Cd^{2+} in normal △·····△ and CR5 ▲——▲ muntjac cells. Each point represents the mean of two replicates.

the cation bound to nuclear and especially organelle/membrane fractions is similar in both cell types. The extent of binding to organelle/membrane fractions is virtually identical in normal and CR5 cells. After about 5 hours of incubation the nuclei of CR5 cells accumulated slightly more Cd^{2+} than their normal counterparts. The difference is approximately 10μgCd^{2+} per 10^9 cells, or 1.3 times the amount in normal cell nuclei. By comparison to the increased cellular accumulation of the metal ion, the excess nuclear binding is minimal. the bulk of the Cd^{2+} that entered the CR5 cell was found in association with cytosol fractions.

The results plotted in figure 7.1 expressed in terms of percentage total cellular Cd^{2+} bound to each fraction can be seen in table 7.1. In the resistant cell line there is a clear tendency for the proportion of Cd^{2+} bound to nuclear and, to some extent, organelle/membrane fractions to decrease with time. The proportion of the metal bound in the cytosol increased with time, the largest percentage increase occuring between 24 hours and 48 hours. The cytosol fractions of normal cells did not show a similar trend, in fact, the reverse was seen.

Table 7.1 Percentage of total cellular Cd^{2+} bound to subcellular fractions of normal and CR5 muntjac cells. Cells incubated in 1uMCd^{2+} for 1-48 hours

TIME (hours)	CELL TYPE	% BOUND TO:		
		NUCLEI	ORGANELLES/MEMBRANE	CYTOSOL
2	N	31.5	6.7	61.7
	CR5	17.3	4.2	76.5
12	N			
	CR5	20.1	2.6	77.4
24	N	28.5	12	59.8
	CR5	17	4.76	77.6
48	N	32	21.2	45.5
	CR5	10	3.7	86.2

Gel filtration chromatography was carried out in order to determine the nature of Cd^{2+} binding to cytosol components. Cytosol fractions from the above experiments were saved and run through G-75 columns by the method described in section 3.8.1. Results are shown in figure 7.2. As was found in normal cells (see preceeding chapter), the major Cd^{2+} binding molecule(s) eluted at a Ve/Vo ratio of 2.7. However, unlike in their normal counterparts, no other peaks of Cd^{2+} binding were detected in CR5 cells. The resistant cells also differed from normal cells in that after 24 hours of incubation in 1μM Cd^{2+} there was a rapid increase in the percentage of Cd^{2+} bound to the Ve/Vo = 2.7 peak (see figure 7.3).

7.1.3.1 Characterization of the Ve/Vo = 2.7 Cd^{2+}-binding molecule(s)

The major Cd^{2+}-binding molecule(s) of the cytosols of both normal and CR5 cells eluted from G-75 gel filtration columns as a single peak around Ve/Vo = 2.7. As this elution volume is close to the total column volume, prediction of its molecular weight from a calibration curve (figure 3.6b) would be unreliable. It certainly does not behave as does rat liver metallothionein (figure 3.6c).

Further analysis of the Ve/Vo = 2.7 Cd^{2+}-binding peak involved anion exchange chromatography. This technique separates molecules on the basis of charge rather than size (as in gel filtration). The method used is described in section 3.8.2. Figure 7.4 shows a typical anion exchange elution profile of pooled Ve/Vo = 2.7 peak fractions obtained from several G-75 column runs. Essentially all the radioactivity (i.e. Cd) was found in association with one peak eluting around fraction 100. Ve/Vo = 2.7 peaks from normal and CR5 cells behaved similarly on anion exchange columns. The Cd^{2+} binding peak did not elute until the gradient had reached a fairly ionic strength implying the molecule has strong negative charge. Also, as only one peak was observed it is most likely that a single molecule is involved.

Taken together, evidence from gel filtration and anion exchange chromatography suggests that the major Cd^{2+} binding component of muntjac

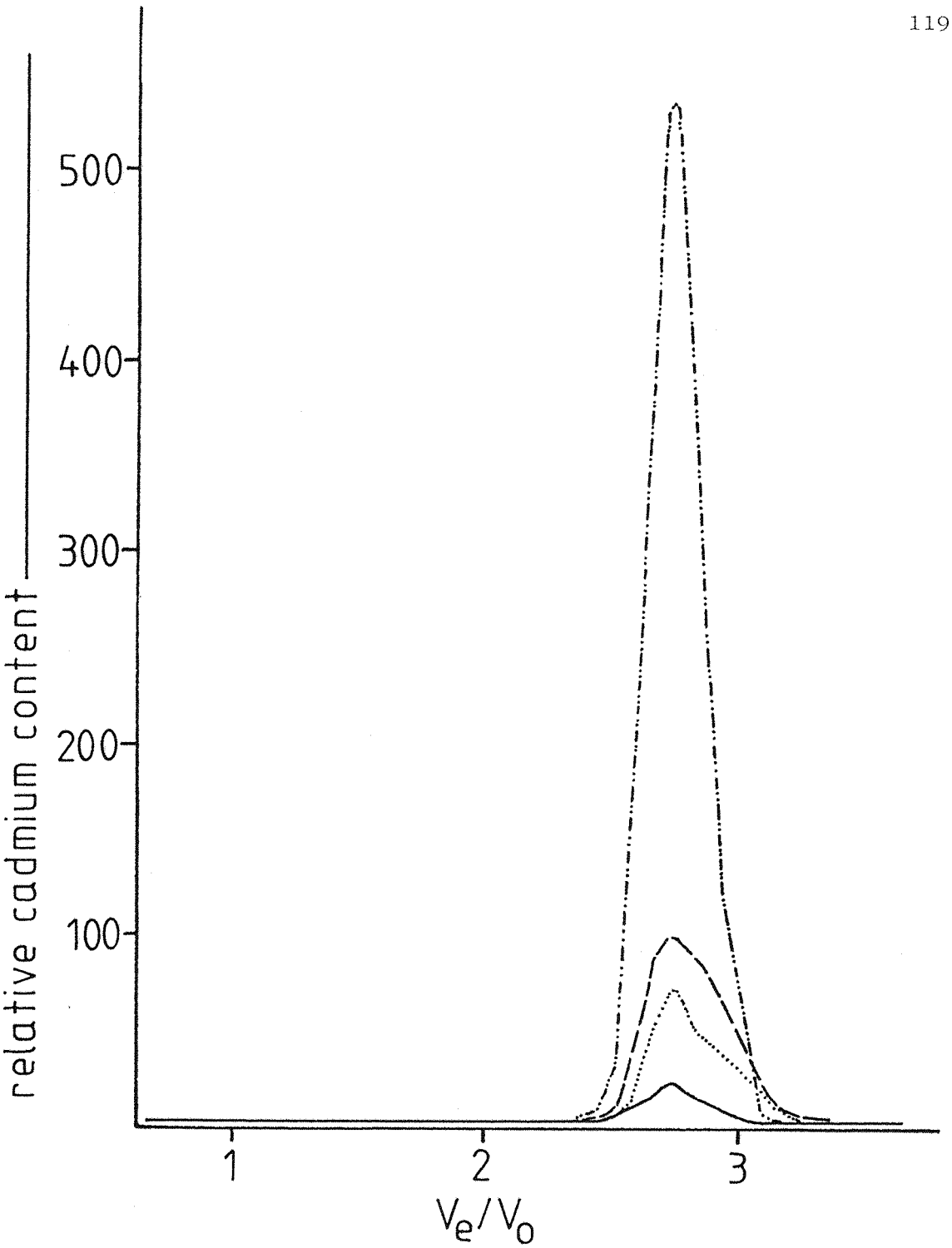


Figure 7.2. G - 75 gel filtration profile of Cd^{2+} binding to cytosol components of CR5 cells incubated in 1μM cadmium acetate for: ——— 2 hours, ····· 12 hours, – – – 24 hours, –·–·– 48 hours. Note lack of binding to components other than at $V_e/V_o = 2.7$.

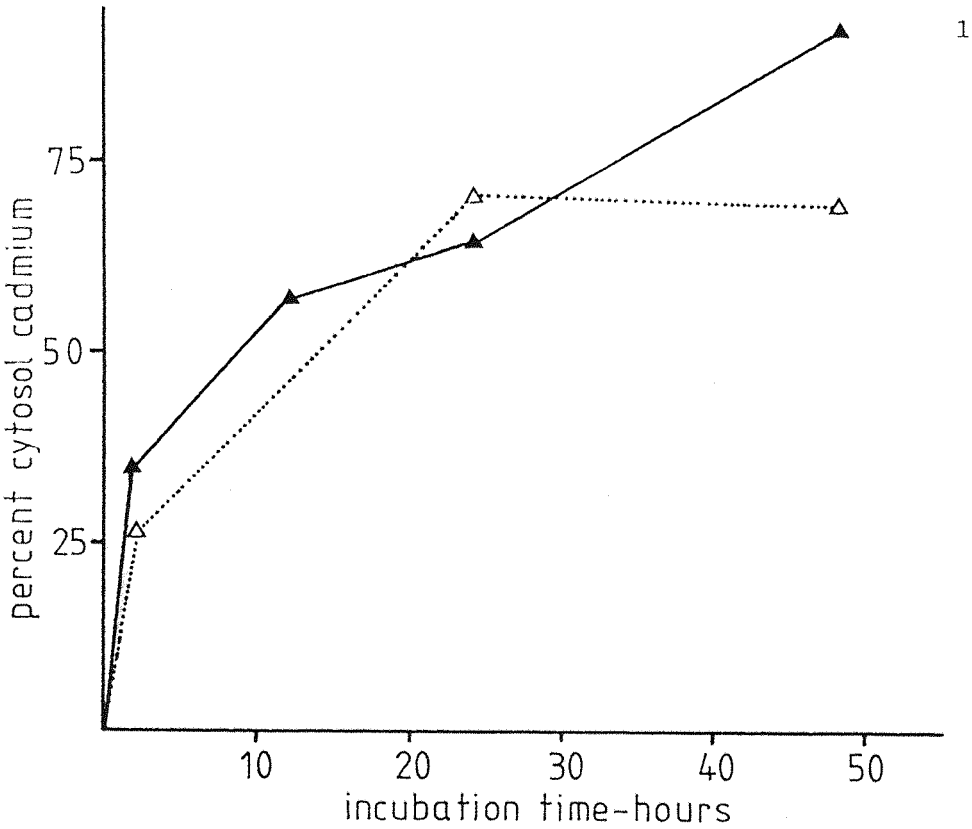


Figure 7.3. Percentage of total cytosol Cd^{2+} bound to G - 75 $V_e/V_o = 2.7$ peak. Cells incubated in 1μM Cd^{2+}. △·····△ normal and ▲——▲ CR5 muntjac cells.

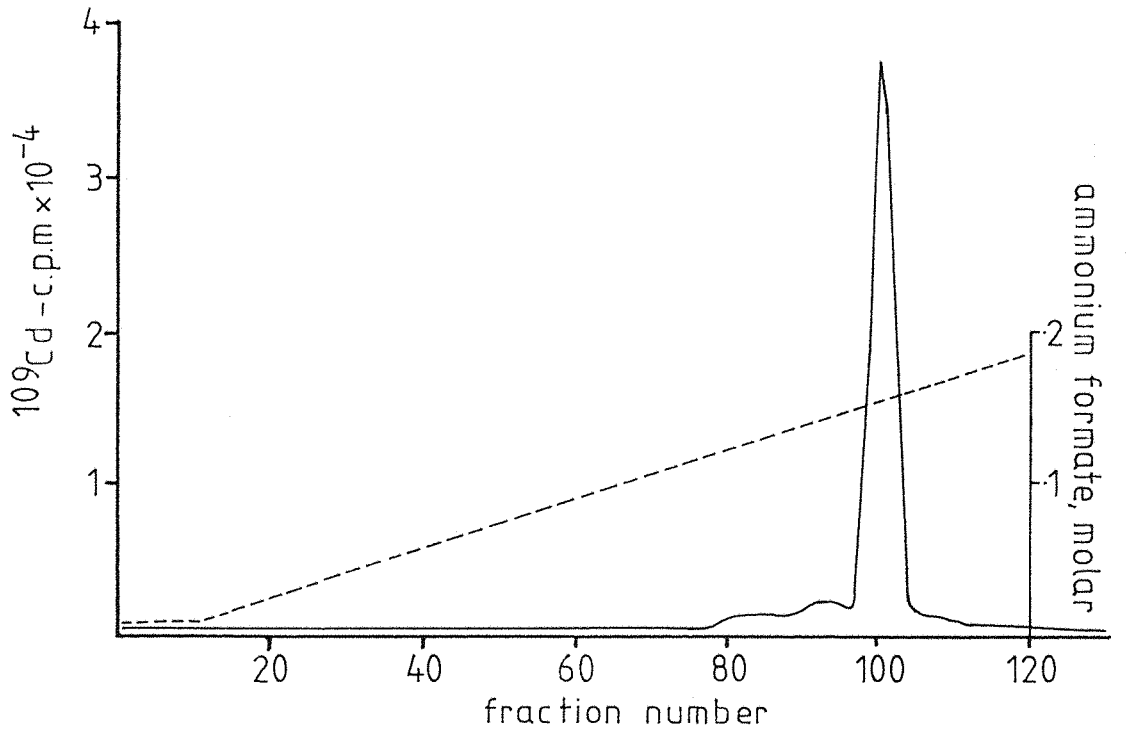


Figure 7.4. Behaviour of $V_e/V_o = 2.7$ peak on DEAE A - 25 columns.

cytosol is a single low molecular weight molecule with a strong negative charge.

Attempts to separate the G-75 Ve/Vo = 2 peaks on DEAE columns were unsucessful, probably due to the small amount of material involved. The relatively low levels of radioactivity associated with the peaks was such that any enrichment of activity in the fractions from anion exchange columns was small in comparison to background radiation.

The u.v. absorbtion spectrum of the G-75 Ve/Vo = 2.7 Cd^{2+} binding molecule is shown in figure 7.5A. Absorbance is high at 254nm and low at 280nm. The low A_{280} suggests few, if any aromatic groups are present. Absorbance at 254nm is indicative of a Cd-sulphydryl bond (Kagi and Vallee, 1961). The absorbance spectrum of the Cd^{2+} binding molecule is in many respects similar to that of a mixture of Cd^{2+} and glutathione (1:4), compare figure 7.5A and 7.5B. Both have a peak of absorbance at 254nm and, when acidified, absorbance at wavelengths less than 250 is increased. The spectra are different in that some reduction the absorbance at 254nm was observed when the Cd^{2+}/glutathione solution was acidified, no such alteration was observed following acidification of the Cd^{2+}-binding molecule.

7.1.4 Ultrastructural changes in CR5 cells

To complement the above biochemical work, an investigation of differences between normal and CR5 muntjac cells at the ultrastructural level was carried out. Untreated and Cd^{2+} treated normal cells and CR5 cells were fixed at intervals after addition of fresh culture medium and examined in the electron microscope. I am grateful to Dr. M.J. Ord for preparation and examination of the material.

Untreated normal muntjac cells had the following features (illustrated in figure 7.6):

1. Coated pit uptake at cell surface, especially noticable at 1 and 5 hours after addition of fresh medium. Coated vesicles were

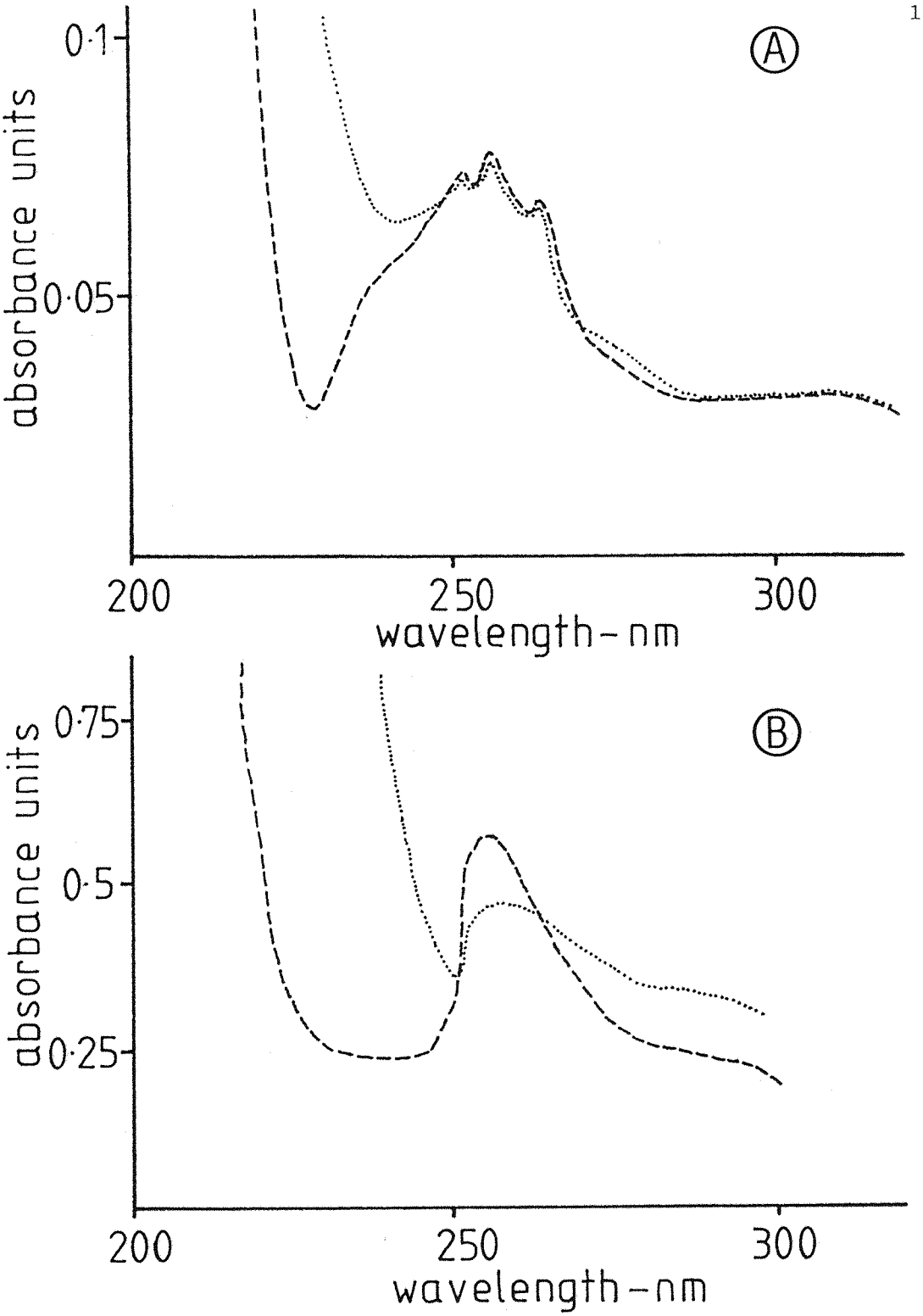


Figure 7.5. UV absorbance spectra of: Ⓐ muntjac G-75 $V_e/V_o = 2.7$ peak fractions, Ⓑ 0.3mM glutathione plus 0.075mM cadmium acetate in 10mM ammonium formate.
------pH = 7, ... pH = 1.

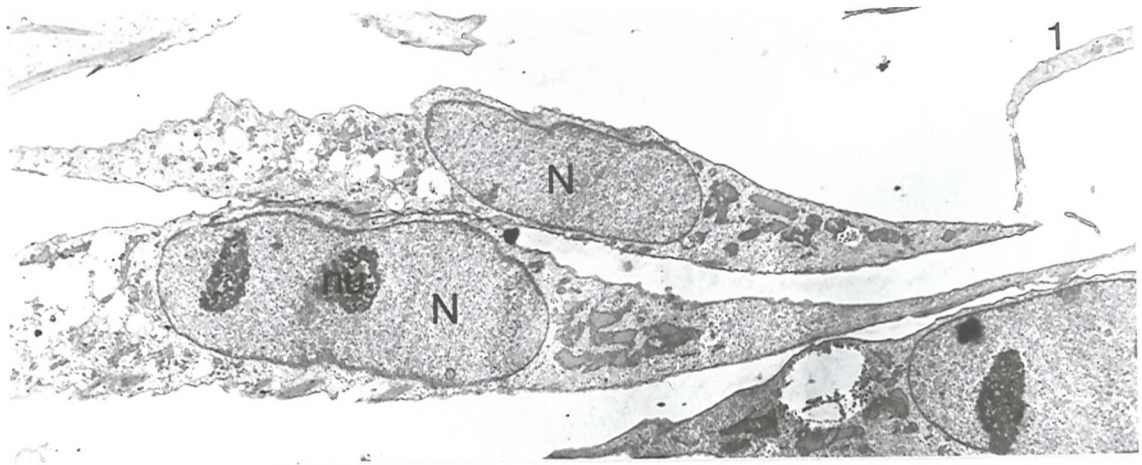
Figure 7.6.

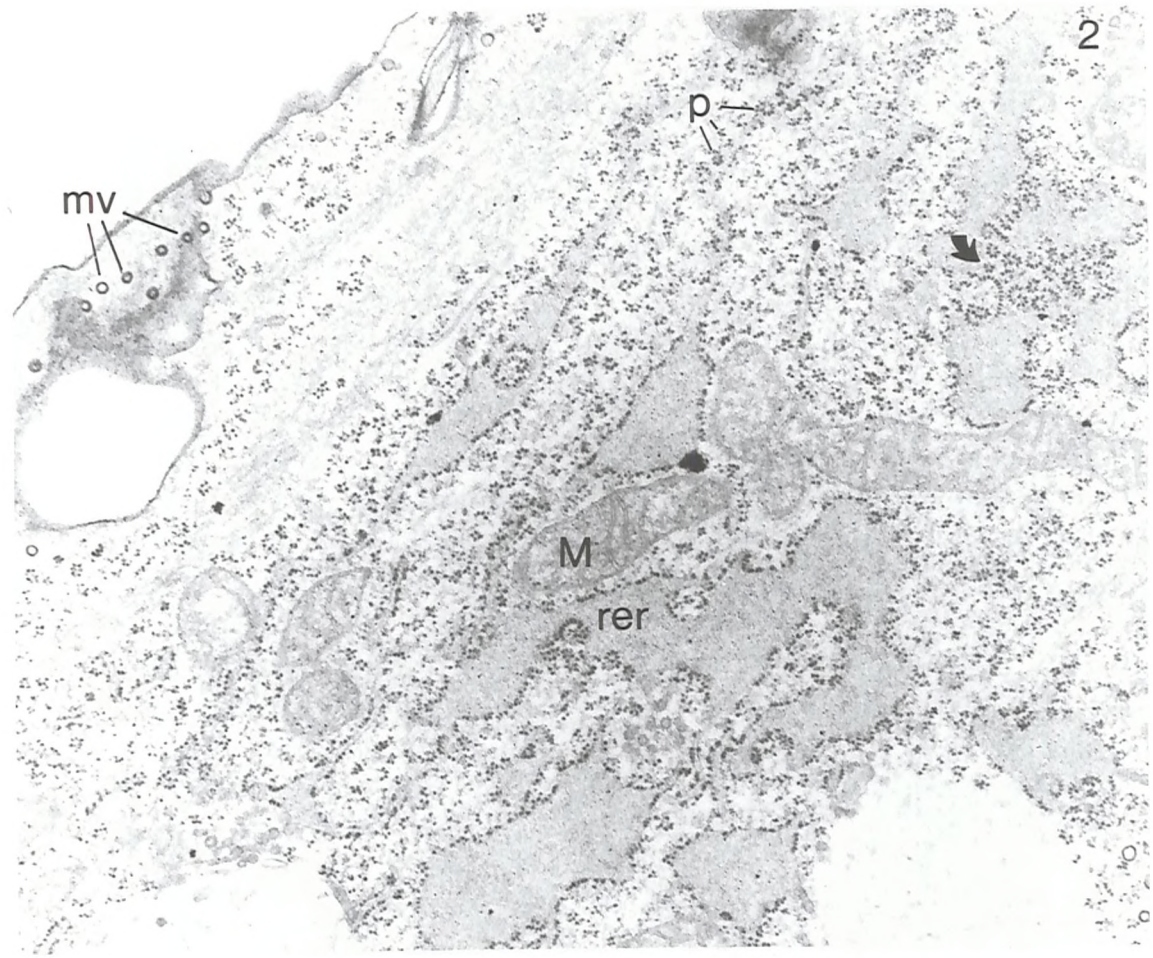
Ultrastructural features of normal muntjac cells.

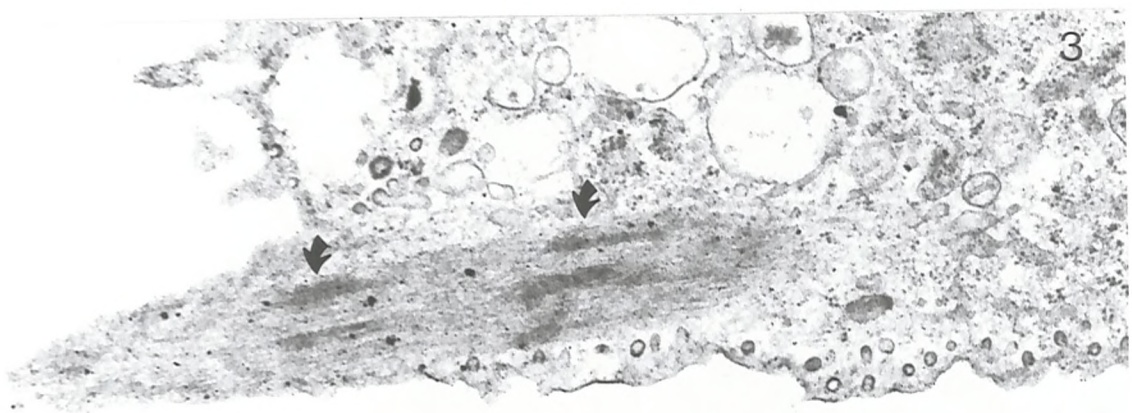
1. Two whole cells, 24 hours after addition of fresh culture medium. Note polarity of cells:- left hand end containing large uptake vacuoles and right hand end with rough endoplasmic reticulum. N - nucleus, nu - nucleolus.
   X 4,000.

2. Microvesicles (Mv), cluster type polysomes (p) and close associations of rough endoplasmic reticulum (rer) with conventional (light staining) mitochondria (M). Note rough endoplasmic reticulum has dilated lumen and ribosomes are sometimes arranged on its surface in 'squiggle' patterns (↘).
   X 28,000.

3. Pointed cell/substratum contact containing a large bundle of microfilaments with periodic dark staining regions (↓).
   X 28,000.


1
N
nu
N


2
p
mv
M
rer


3

seen within the cells, sometimes fusing with each other.

2. Large uptake vesicles - seen close to cell surface at 1 hour and more internally later. Only occasionally was any material seen inside these vesicles. These vesicles appeared to fuse with each other to form a large vacuole. At later time points, the membrane delimiting these vacuoles was less evident.
3. Cells often were polarized with most uptake activity occuring at one end of the cell.
4. Cell adhesion points were prominent and pointed in shape. Large bundles of microfilaments with periodic darker staining regions along their length ran from the adhesion point to the cell interior.
5. Mitochondria were of conventional form and frequently seen in close association with endoplasmic reticulum. These associations appeared more at 5 and 12 hours after changing culture medium.
6. Endoplasmic reticulum was predominantly rough and dilated (i.e. had a wide dark staining lumen). Ribosomes on the endoplasmic reticulum surface were sometimes arranged in random 'squiggle' patterns.
7. Few free, single ribosomes were present. Most were grouped as small cluster and helical form polysomes. Cluster form polysomes predominated.
8. Nuclei were fairly evenly staining, some darker condensed chromatin was seen towards the periphery. One or two nucleoli per nucleus were present, these were of 'fenestrated' morphology.

The general impression of normal cells was that they were metabolically active and showed no signs of damage or degeneration. By contrast Cd^{2+} treated normal cells (1μM Cd^{2+} for 1 - 24 hours or 5μM Cd^{2+} for 12 hours) showed several signs of cellular damage. Cd^{2+} treated normal cells differed from their untreated counterparts in the following ways (illustrated in figure 7.6A):

1. The large uptake vesicles frequently contained dark staining,

Figure 7.6A.

Ultrastructural features of cadmium treated normal muntjac cells.

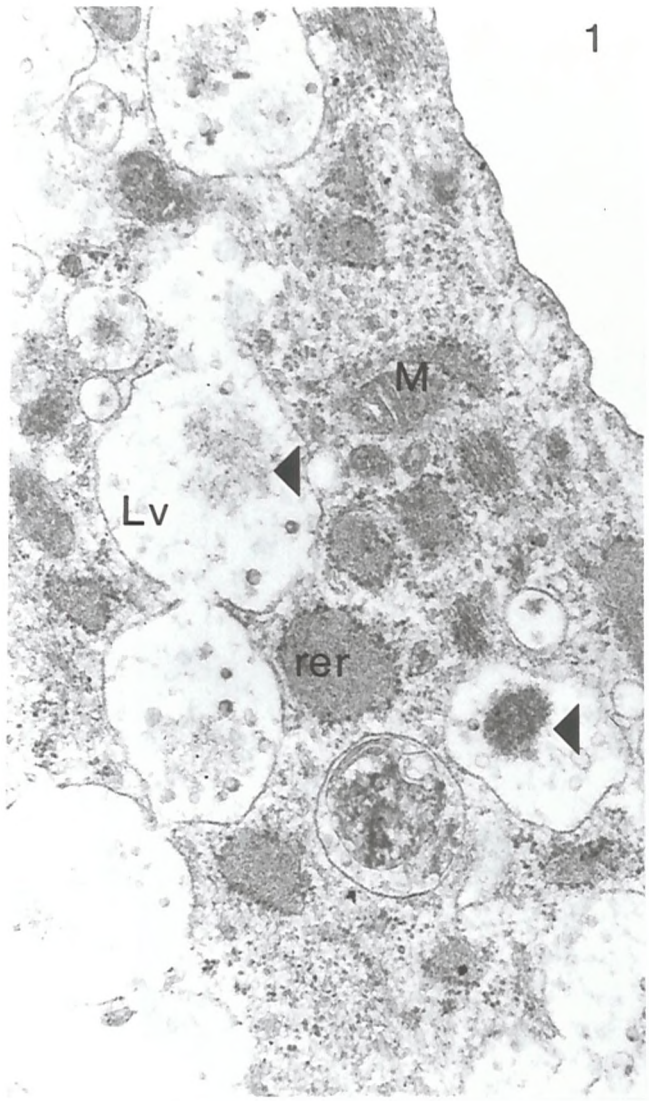
1. Cell treated with 1μM Cd^{2+} for 12 hours. Note large uptake vacuoles (Lv) with fuzzy inclusions (◀) and lack of close associations of mitochondria (M) and rough endoplasmic reticulum (rer).
   X 16,500.

2. Large vesicle with smooth membrane inclusion. Cell treated for 12 hours with 1μM Cd^{2+}.
   X 14,000.

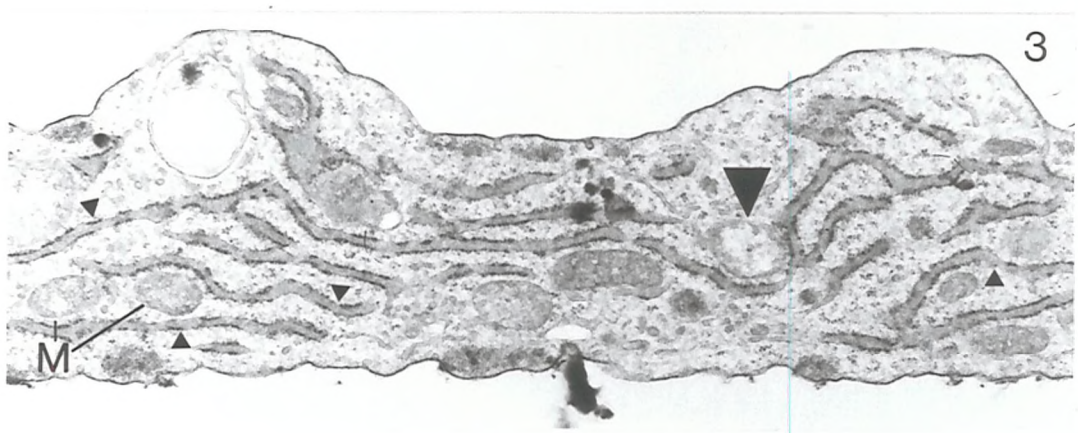
3. Stringy form rough endoplasmic reticulum (▲) in cell treated with 1μM Cd^{2+} for 24 hours. Also note the presence of conventional mitochondria (M) and at least one degenerating mitochondrion (▲).
   X 11,500.

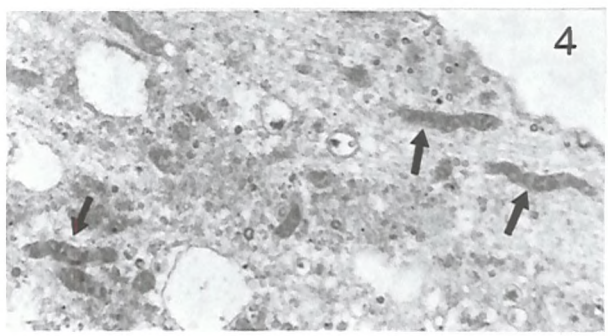
4. Condensed mitochondria (➡) in cell treated for 12 hours with 1μM Cd^{2+}.
   X 14,000.

5. Cell/substratum contacts (▲) in cell treated with 1μM Cd^{2+} for 12 hours. Note contacts are not as pointed as in untreated cells (figure 7.6).
   X 18,500.


1
M
Lv
rer


2


3
M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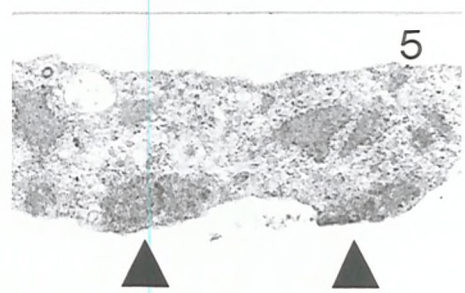

5

fuzzy particles or granular material. Occasionally a large amount of folded smooth membrane was seen in the vesicles of cells treated with 1μMCd^{2+} for 24 hours or 5μMCd^{2+} for 12 hours.

2. Cells contained a high proportion of smooth membrane vesicles of sizes betwen the large uptake vesicles and the small coated vesicles.
3. Cell attachment points were less pointed in longitudinal section. Flat longitudinal sections and circular cross sections were more common especially in cells treated with 5μMCd^{2+}.
4. Some mitochondria were of condensed form. A few 'ghost-like' forms were also seen, these are probably degrading mitochondria. All types (conventional, condensed and ghost-like) could be seen in one cell. In cells treated with 5μMCd^{2+} the number of mitochondria seemed to be reduced.
5. Less rough endoplasmic reticulum was present and associations with mitochondria were less evident. Often the endoplasmic reticulum had a narrower lumen, thus was of tubular rather than dilated morphology. Occasional areas of degranulated rough endoplasmic reticulum were seen.
6. Microtubules were much more prominent than in untreated cells.
7. The number of polysomes appeared to be reduced.

Overall, it appeared that Cd^{2+} treated cells were less organised and less active than untreated cells. Although there were differences between untreated normal cells and CR5 cells, the Cd^{2+} resistant cells seemed more active than Cd^{2+} treated control cells. The major differences between control and CR5 cells are as follows (see figure 7.7):

1. Large areas of loosely packed cytoplasm were present 1 hour after addition of fresh culture medium. These areas contained a number of small myelin figures (concentric rings of smooth membrane similar in morphology to the myelin sheath of nerve cells). Myelin figures were also observed scattered throughout the remaining cell cytoplasm. By 5 hours the size and number of myelin figures had increased. At 12 hours after addition of

Figure 7.7.

Ultrastructural features of cadmium resistant (CR5) muntjac cells.

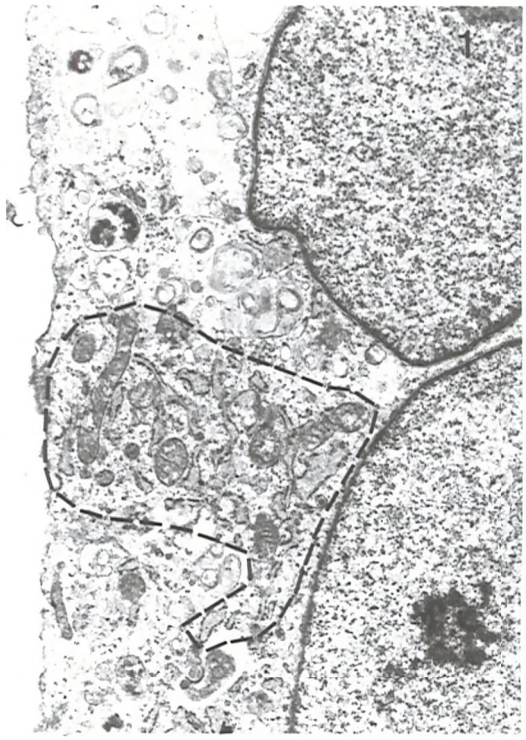
1. One hour after changing culture medium and addition of fresh Cd^{2+} (5μM). Note areas of loosly packed cytoplasm containing smooth membrane vesicles and small myelin figures surrounding an area of close mitochondria/ rough endoplasmic reticulum associations (within dotted line) as seen in normal cells.
   X 12,500.

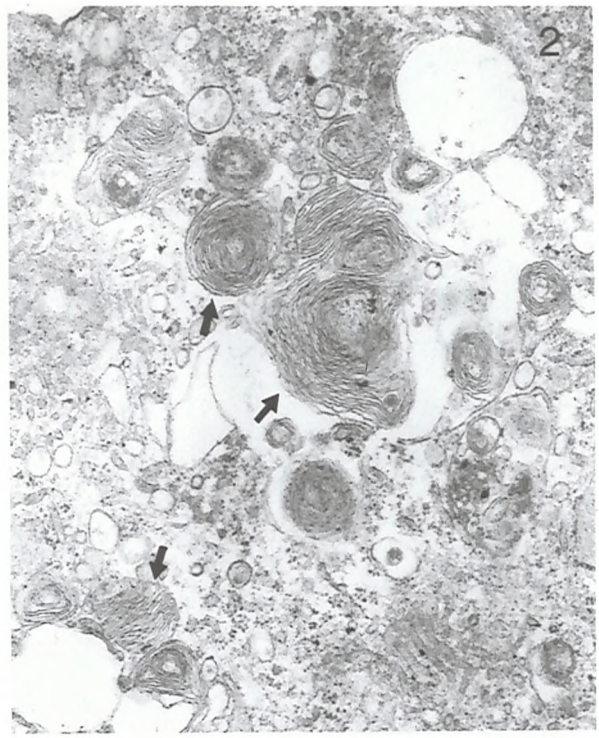
2. A cell 5 hours after changing culture medium and addition of fresh Cd^{2+} showing multi - lamellar myelin figures (➡) within the cytoplasm.
   X 26,000.

3. Five hours after addition of fresh Cd^{2+} and culture medium. Membrane bodies and myelin figures appear to be being gathered within a membrane (➡). Note conventional mitochondria (M) and microvesicles (mv) as seen in untreated normal cells.
   X 11,500.

4. Well defined bundles of myelin figures and membrane (➡); the remaining cytoplasm of this cell is normal. In right hand cell note loose bundle of myelin figures. 12 hours after medium change.
   X 5,000.

5. Twenty four hours after change of culture medium and addition of fresh Cd^{2+}. Note area of light cytoplasm (⬅) within otherwise fairly normal cytoplasm.
   X 6,500.


1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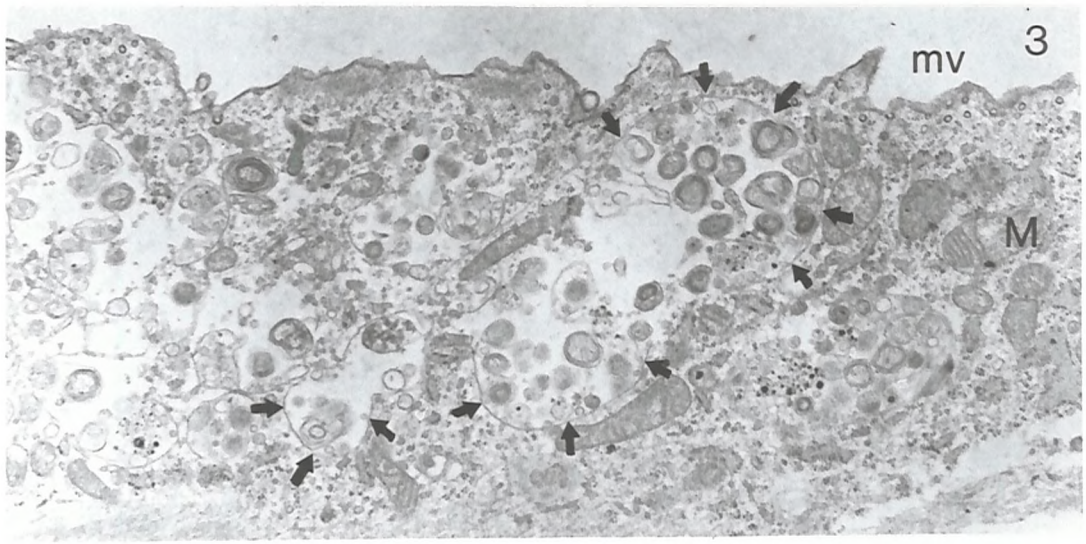
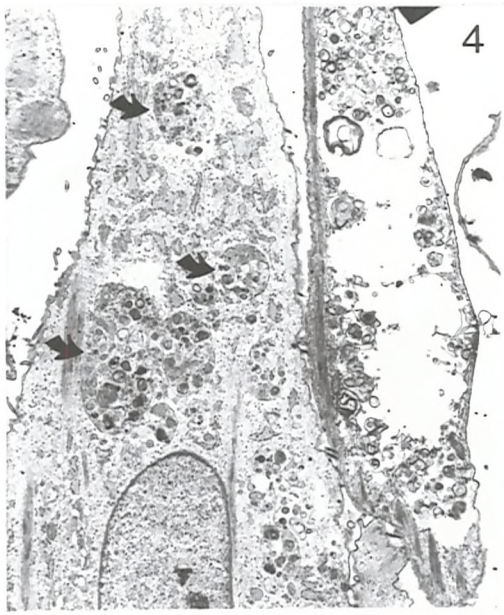
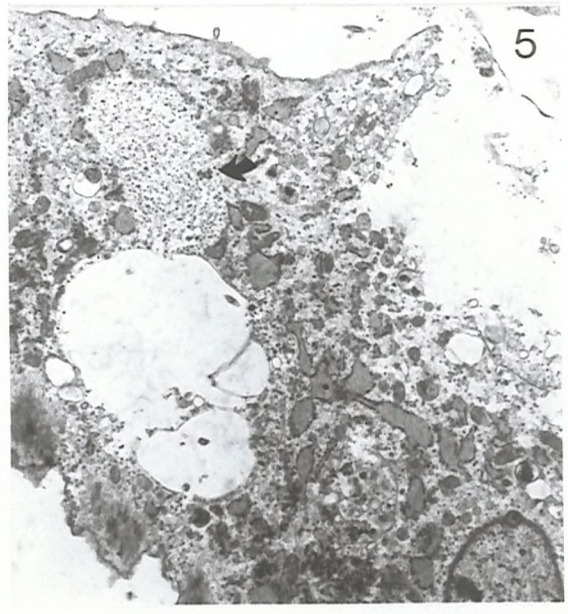

mv
3
M


4


5

fresh medium the myelin figures and some other cytoplasmic components tended to be gathered together into tightly packed areas which were frequently membrane bound. Sometimes these bundles of myelin figures lay close to the cell surface as if they were about to be expelled from the cell. A further 12 hours later the number of myelin figure bundles had decreased. Some cells contained large areas which stained lighter than the rest of the cytoplasm. These areas sometimes contained a few myelin figures but no other recognisable cytoplasmic organelles. This apparent sequence of myelin figure formation, gathering and expulsion or degradation was common to virtually all CR5 cells. Examples of cells at several different stages could be seen at most time points.

2. In areas apart from those containing myelin figures the cytoplasm appeared similar to that of untreated normal cells. No abnormal mitochondria were seen. Rough endoplasmic reticulum frequently had a dilated lumen and mitochondria/endoplasmic reticulum associations were common. Helical and cluster type polysomes were in evidence.

7.1.5 Time-lapse video analysis

The above ultrastructural investigation suggested a time course of myelin figure formation gathering and loss. It was not possible to determine if the final reduction in the number of myelin figures per cell was due to their intracellular degradation or expulsion from the cell. Experiments described in this section were designed to clarify this point. Time-lapse video recording allows the behaviour of living cells to be followed over extended periods. If CR5 cells expelled large bundles from their cytoplasm into the culture medium it should be detectable by analysis of time lapse video recordings. The equipment and for these experiments is described in section 3.4.2.4. Fresh culture medium was added to cells 18 - 24 hours prior to commencement of recording.

Analysis of recordings of normal muntjac cells revealed the following features of their behaviour:

1. Little movement of cells over the culture dish surface.
2. No visible expulsion of bundles of cytoplasm.
3. All attempted divisions were successful and normal (i.e. cell rounds up with chromosomes often becoming visible at the spindle equator, division into two equally sized daughter cells, daughter cells move away from each other and flatten. See figures 7.8 a-d and 7.9). No material was seen to be lost from the main cell body (bodies) during division.
4. 21% of the analysed cells divided. On average mitosis took 3 hours (from first signs of a cell rounding until the beginning of daughter cell flattening).

CR5 cells behaved differently from normal cells in the following ways:

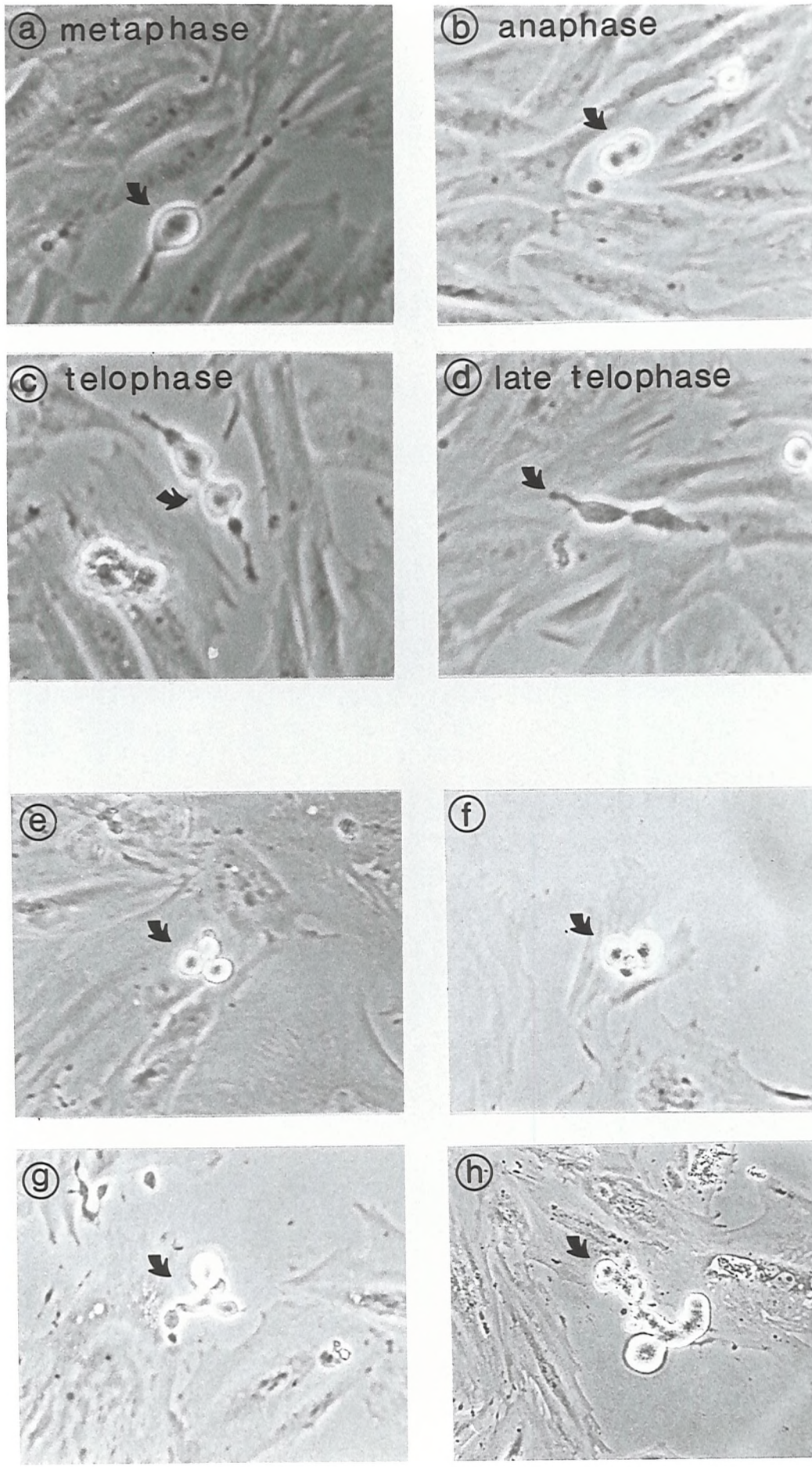
1. Some cells (approximately 5% of those analysed) extruded bundles of cytoplasm during interphase. Tracings made from video recordings of one such cell can be seen in figure 7.10.
2. A slightly lower proportion of cells (14%) attempted to divide.
3. Of the cells that attempted to divide 63% did so normally. Mitosis took an average of 2 hours 45 minutes.
4. 38% of attempted divisions were in some way abnormal. 4 types of abnormal division were seen:
   i) Cell rounds up and reaches a metaphase-like stage; when attempting to divide, the cell 'writhes' putting out and retracting blebs of various shapes and sizes. Cell shape is abnormal and rapidly changing during this phase. Eventually, after a variable length of time the cell ceases to move and sometimes detaches from the substratum. These cells did resettle during the course of recording. 8% of the total number of divisions were of this type.
   ii) Cell rounds up and goes through an abnormal and extended metaphase similar to that seen in type (i) divisions. Eventually the cell resettles as a single multinucleate cell. 10% of the total divisions were of this type. Tracings of this type of division are shown in figure 7.11.

Figure 7.8

Cell division in normal and Cd^{2+} - resistant muntjac cells. All photographs X200.

a - d) Stages of division in normal cells (arrowed cells).

e - h) Examples of CR5 cells undergoing abnormal division (arrowed cells).


ⓐ metaphase
ⓑ anaphase
ⓒ telophase
ⓓ late telophase
ⓔ
ⓕ
ⓖ
ⓗ

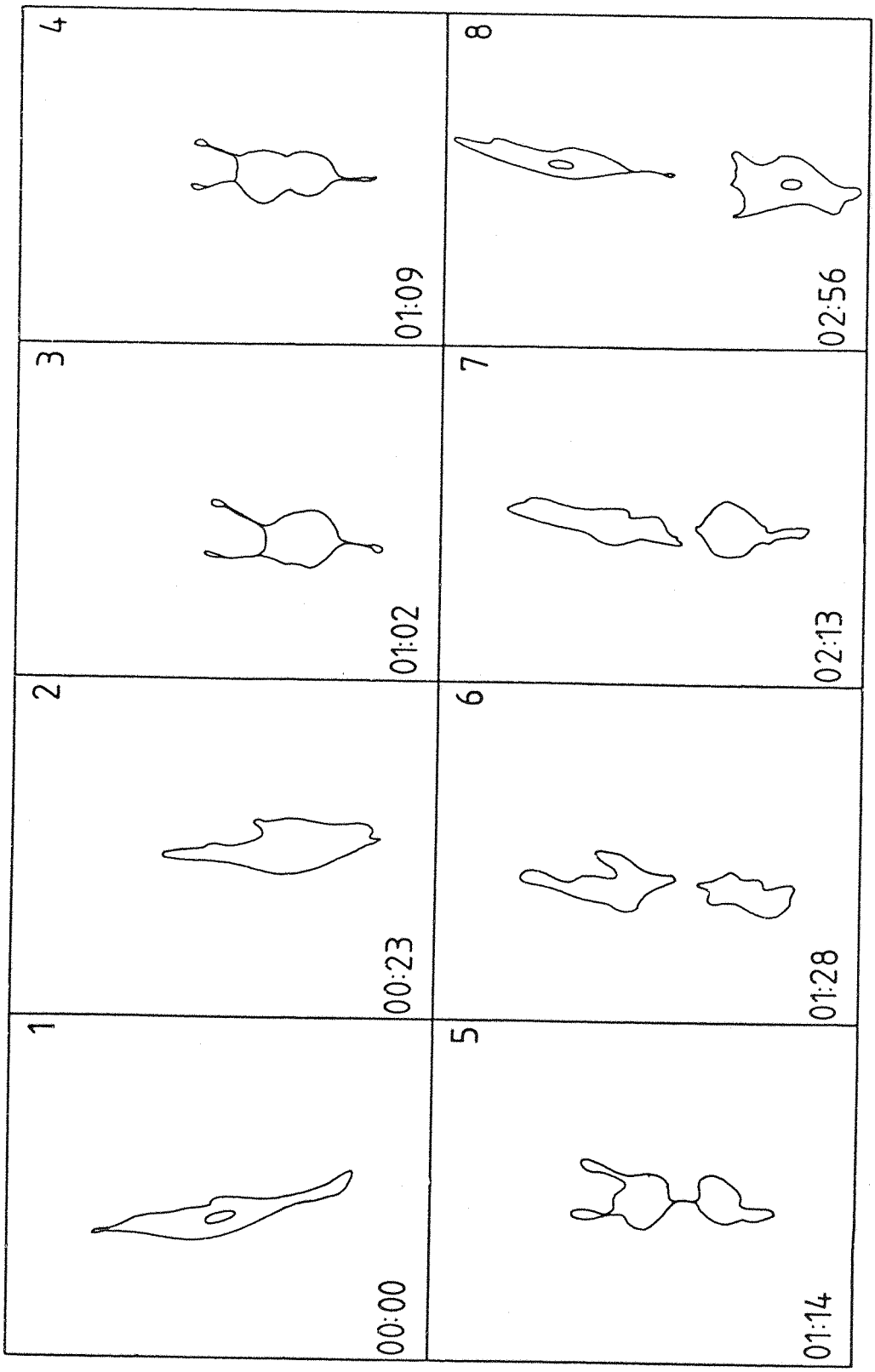


Figure 7.9. Time lapse video tracing: normal cell division.

Figure 7.10. Time lapse video tracing: interphase cell expelling a cytoplasmic bundle.

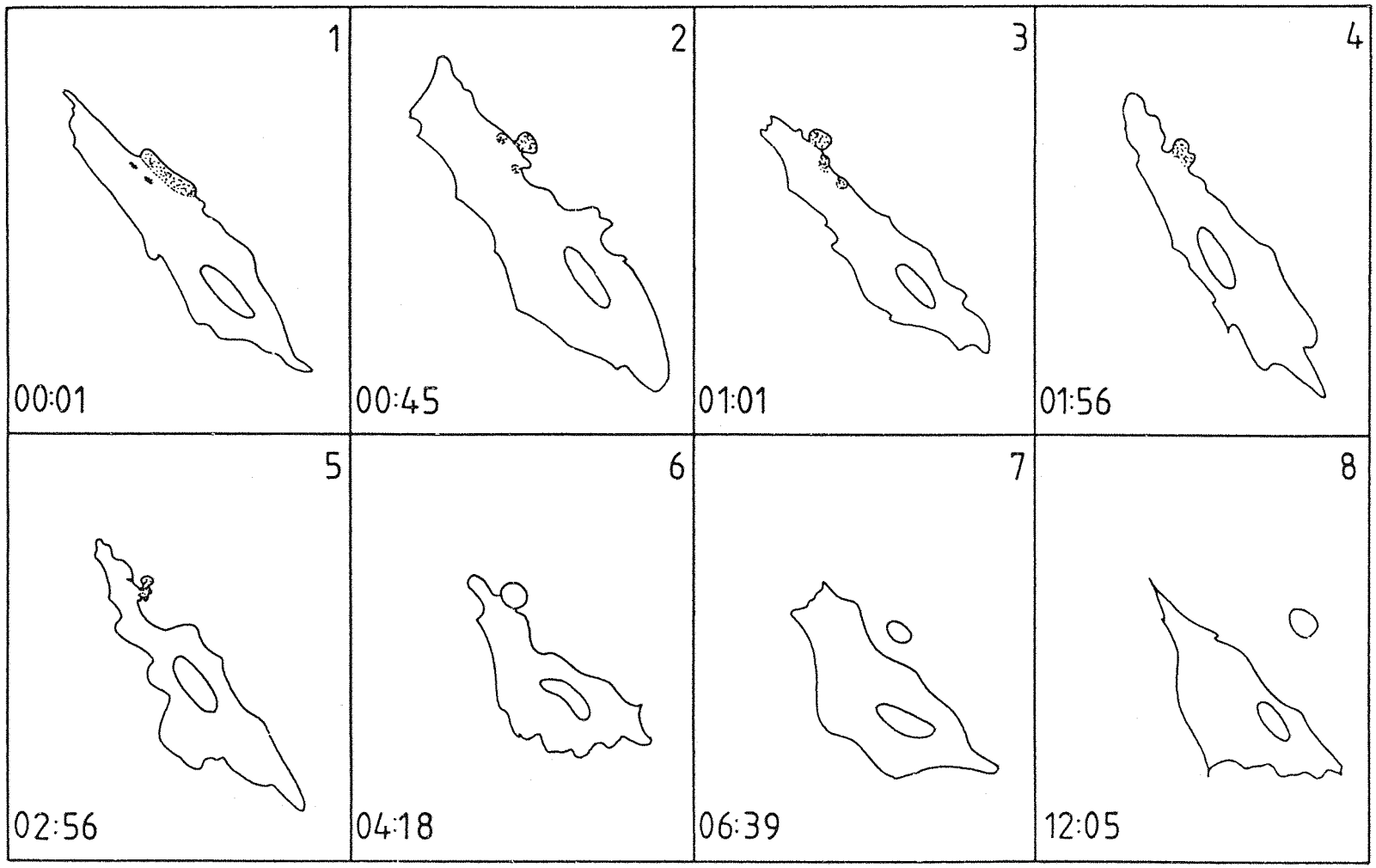
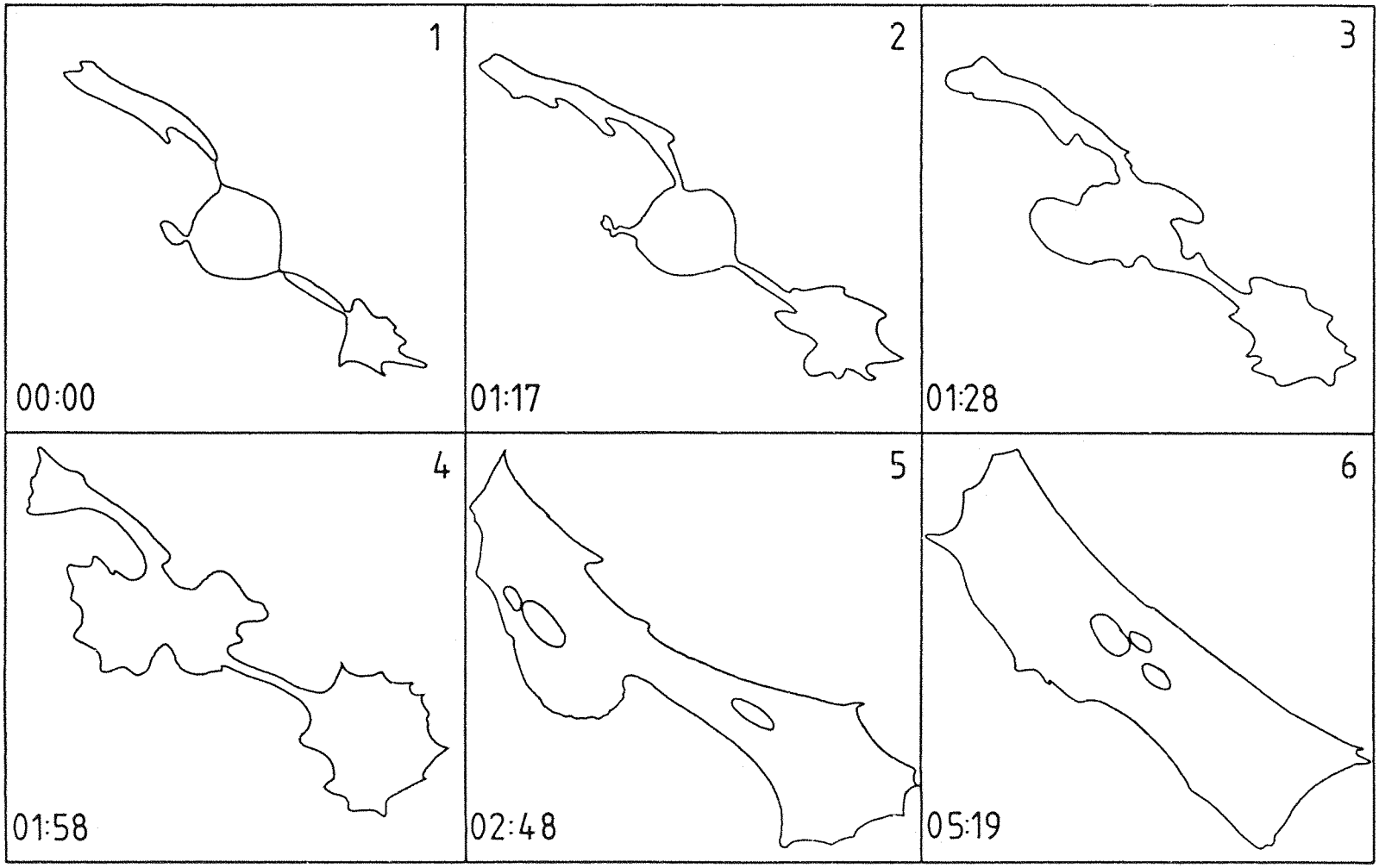


Figure 7.11. Time lapse video tracing: type (ii) abnormal division of CR5 cells.



iii) Cell rounds up and divides as normal but one daughter cell fails to settle after mitosis. See figure 7.12. 10% of the total divisions were of this type.

iv) Division is essentially normal but some bundles of cytoplasm are lost during division. See figure 7.13. 10% of the total divisions were of this type.

A summary of the time lapse video analysis is presented in table 7.2. In figure 7.8 e - h examples of abnormal metaphases are shown. These studies indicate that CR5 cells behave differently to normal muntjac cells in mitosis and interphase. Non-dividing cells appear to 'discard' bundles

Table 7.2 Summary of time lapse video analysis data

CELL TYPE	CELLS ANALYSED	CELLS ATTEMPTING DIVISION	NORMAL DIVISIONS	ABNORMAL DIVISIONS (i)	(ii)	(iii)	(iv)	CELLS EXPELLING VISIBLE CYTOPLASMIC BUNDLES
NORMAL	103	22	22	0	0	0	0	0
CR5	214	30	19	2	3	3	3	8

of cytoplasm into their surrounding culture medium. Such cells otherwise appear to be normal, they could go on to divide. As the content of these bundles was not determined it cannot be concluded that they account for the reduction in myelin figures in cells between 12 and 24 hours (see Section 7.1.4). Nonetheless the evidence is suggestive of this conclusion. Normal cells did not expel cytoplasmic bundles and, apart from the myelin figures, the cytoplasm of CR5 cells was similar to that of normal cells (see section 7.1.4). Thus, the myelin figure bundles are probably the structures seen being expelled from the CR5 cells.

Figure 7.12. Time lapse video tracing: type (iii) abnormal division of CR5 cells.

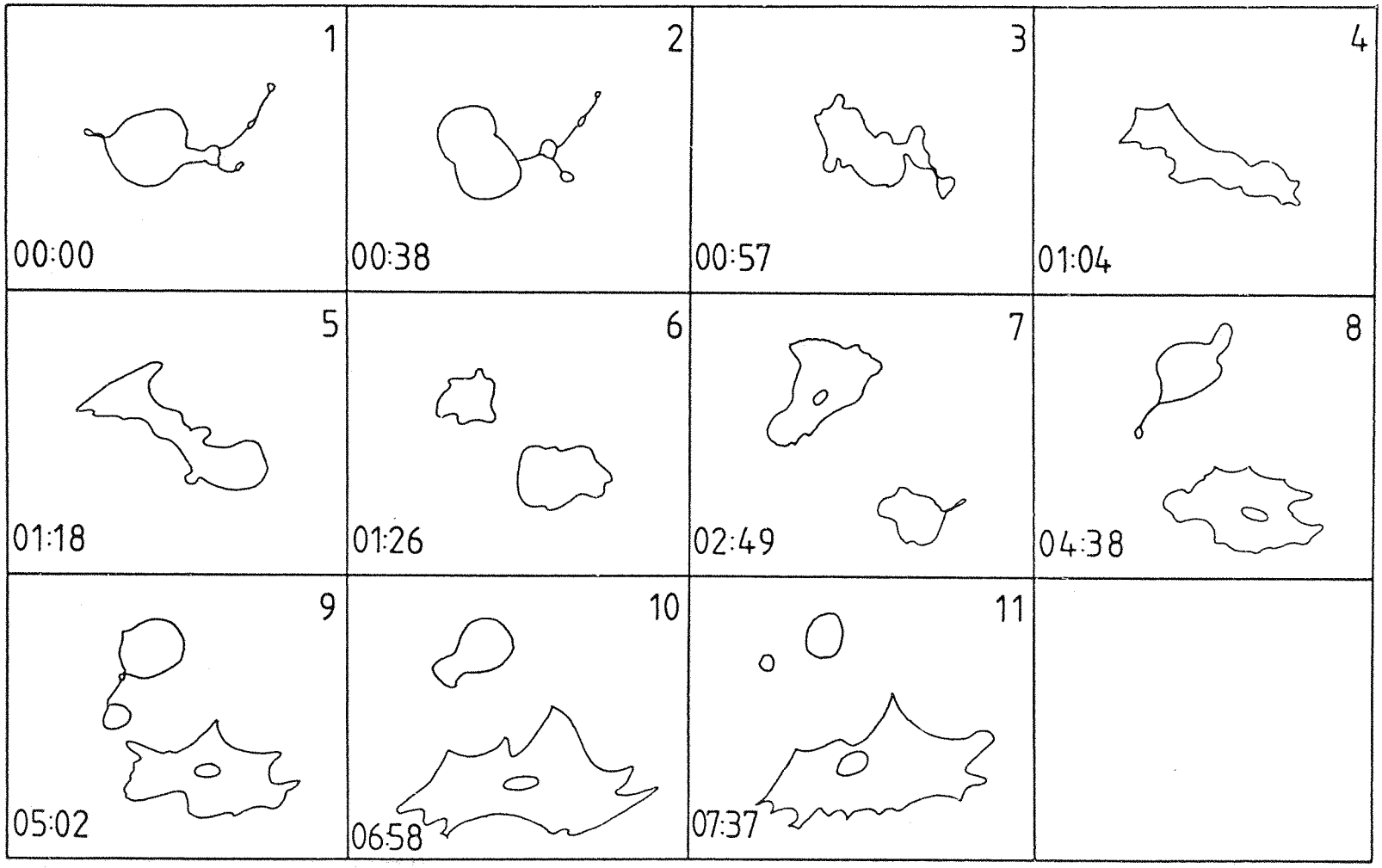
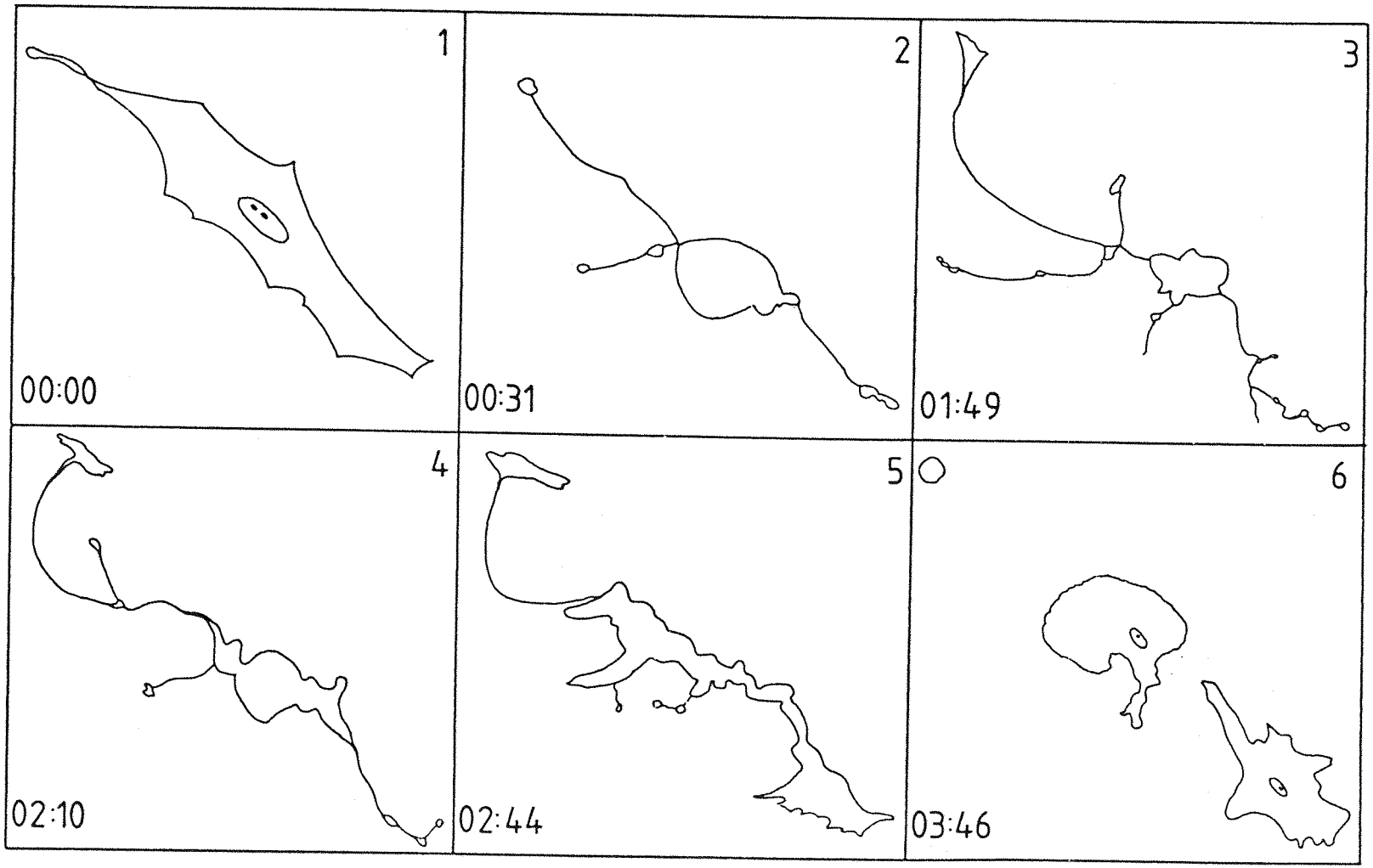


Figure 7.13. Time lapse video tracing: type (iv) abnormal division of CR5 cells.



7.1.6 Cytoskeletal studies

The abnormal divisions seen in some CR5 cells (see above) could be a reflection of more general cytoskeletal disturbances. The experimental work described in this section investigated this possibility.

The technique developed by Pena (1980), detailed in Chapter 3, provides a simple and rapid method for examining the cytoskeleton of cells in culture. Cytoskeletal structures are stained with coomassie blue after extraction of cells with the non-ionic detergent Triton X-100. Being a general protein stain, coomassie blue stains both microtubules and microfilaments. In the experiments described below, cytoskeletal preparations of normal and CR5 cells were made at 0.5, 0.75, 1, 1.5, 2, 3, 5, 8, 12 and 24 hours after plating onto grease free coverslips. In this way the settling behaviour and cytoskeletal development can be followed in the same preparations.

Figure 7.14 shows representative cytoskeletal preparations of normal (1, 3, 5) and CR5 (2, 4, 6) cells at 1, 5 and 24 hours after seeding.

The settling of normal cells could be classified into the following stages, times in brackets refer to when each stage was first observed.

1. Cells floats down onto glass after being in suspension. Soon after, a few slender extrusions from the cell body make an initial tight contact with the substratum. (30 minutes)
2. Further extension of slender processes, extending approximately half a cell diameter from the edge of the main body of the cell. A thin skirt of flattened cytoplasm extends around cell edge. (45 minutes)
3. Cytoplasmic skirt continues to extend outwards, either as a single sheet or long flattened processes. Outer edge of membrane appears ruffled. Cytoplasm extends either in all

Figure 7.14

Settling behaviour and cytoskeletal development in normal (1,3,5) and CR5 (2,4,6) muntjac cells.

1) Normal cells, 1 hour after plating.

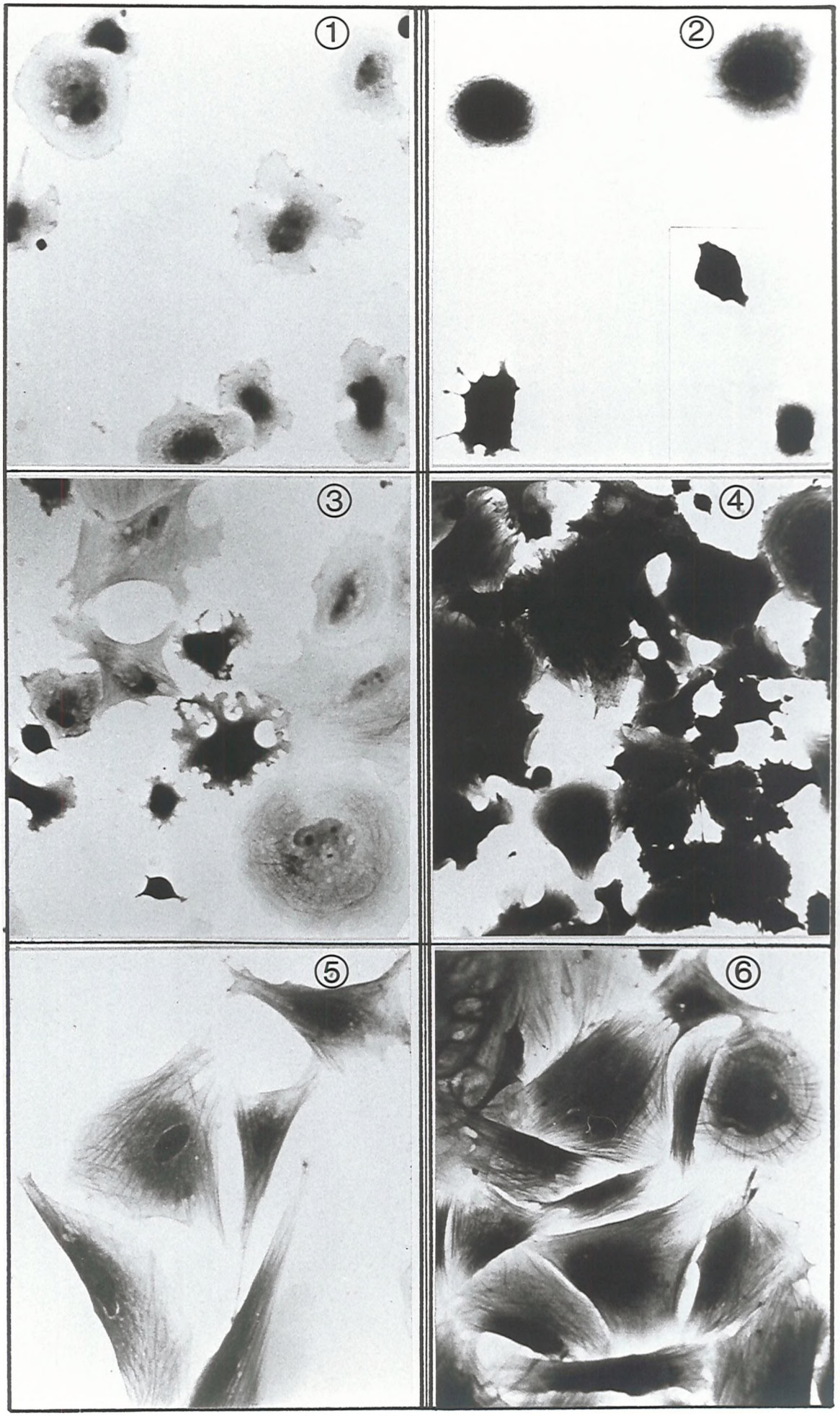
2) CR5 cells, 1 hour after plating.

3) Normal cells, 5 hours after plating.

4) CR5 cells, 5 hours after plating.

5) Normal cells, 24 hours after plating.

6) CR5 cells, 24 hours after plating.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④
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⑥

directions or mainly along the long nuclear axis. Few, if any cytoskeletal structures visible. (1 hour)

4. Cells continue to spread, in some cells cytoskeletal fibres are seen, usually arranged radially and concentrically. (3 hours).
5. Most cells are fully flattened and are beginning to take up a more elongate shape. All cells contain cytoskeletal elements. (5 hours)
6. From 5 hours onwards the cytoskeleton becomes a more dense array of fibres, most of which lie parallel to the long axis of the nucleus (and so usually the cell). Some fibres run parallel to the periphery of the cell.

The sequence of events in normal and CR5 cells was the same. However, there was a slowing of the whole process in the resistant cells, see figure 7.14.

So the cytoskeleton of normal and CR5 cells appears to be much the same. The small differences observed are not sufficient to account for the dramatic changes in cell division.

7.1.7 Transformed status of CR5 cells

The work of DiPaolo and Casto (1979) showed that Cd^{2+} could transform Syrian hamster embryo cells. As yet, no investigation of the 'transformed status' of the various Cd^{2+} resistant cell lines produced by other workers has been published. Transformation is not an 'all or nothing' event but a series of graded changes in cell phenotype, acquisition of immortality occuring prior to the ability to grow in semi-solid medium (Newbold et al., 1982). So, although normal muntjac cells are partially transformed (they are an immortal cell line), CR5 cells could differ from the parental cell line in quantitative assays of transformation. Two such assays have been employed in the present work, the ability to grow in soft agar and lectin agglutinability. The methods used are described in section 3.4.2.

In the soft agar growth assay 5×10^6 cells of each type were

screened. Cells were allowed to grow in the agar containing medium for 3 weeks. No normal muntjac cells formed colonies within the 3 week period. A total of 255 colonies were seen in the CR5 cell soft agar cultures, examples of colonies are shown in figure 7.15. Therefore the frequency of soft agar colonies was approximately 1 in 2×10^4 CR5 cells compared to none in normal muntjac.

Less dramatic differences were seen in the lectin agglutination assay. A summary of the results from three replicate assays is shown in table 7.3. CR5 cells did show a greater tendency to agglutinate in the presence of con A.

Table 7.3 Agglutination of normal and Cd^{2+} resistant muntjac cells by concanavalin A

	Con A concentration µg/ml					
	0		10		100	
CELL TYPE	CELLS PER CLUMP	CLUMPS/ 100 CELLS	CELLS PER CLUMP	CLUMPS/ 100 CELLS	CELLS PER CLUMP	CLUMPS/ 100 CELLS
NORMAL	0	100	2	73	5	28
CR5	0	100	4	32	13	10

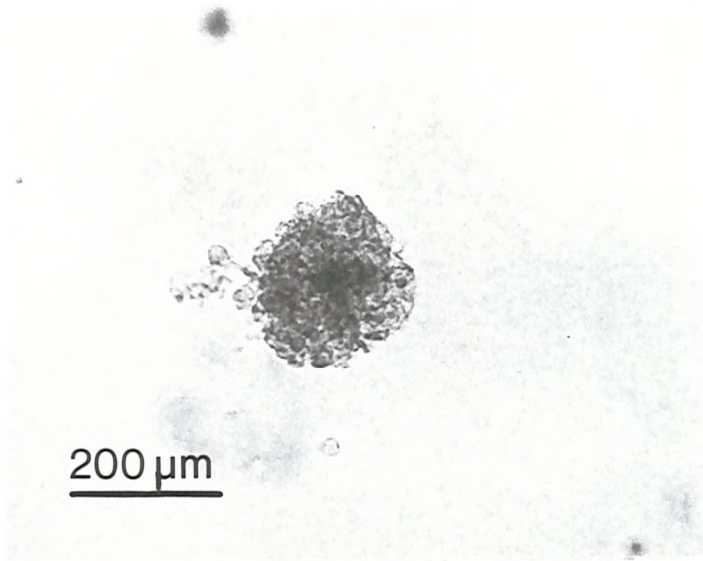
On the basis of the above results, it would appear that the Cd^{2+} resistant muntjac cells are more transformed than the parental cell line.

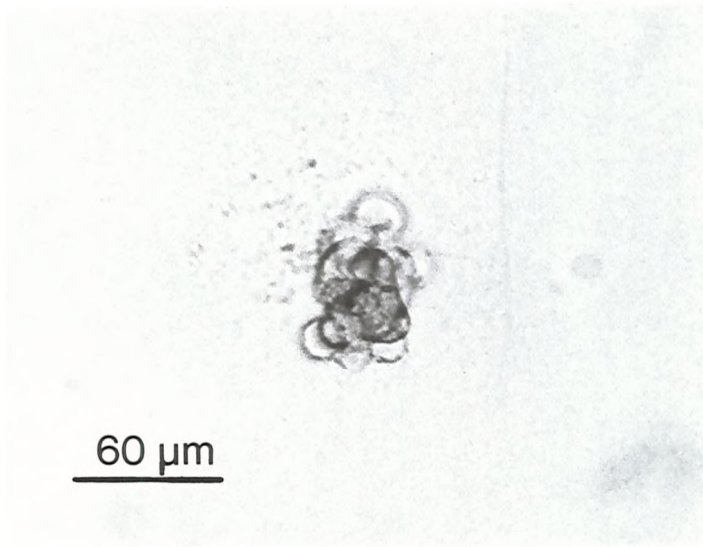
7.1.8 Discussion and summary of CR5 cell characteristics

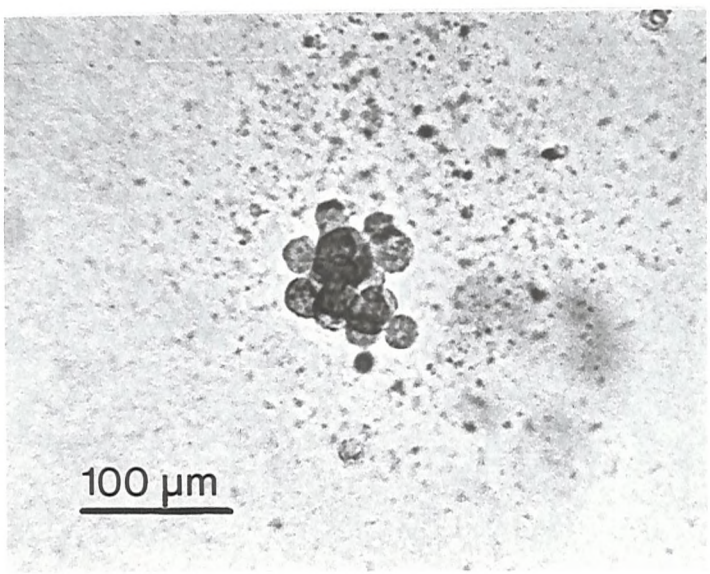
In most cells the switch to a Cd^{2+} resistant phenotype was stable for up to one month. This time represents a sufficient number of divisions, and so dilution of intracellular Cd^{2+} and cellular components, to eliminate the possibility that maintenance of the resistant phenotype requires the presence of the cation. Therefore, a basic change

Figure 7.15

Examples of colonies of CR5 cells growing in soft agar.


200 µm


60 µm


100 µm

in cellular physiology must have occurred.

The metabolism of Cd^{2+} in resistant cells is in many ways different to that in normal muntjac cells. Firstly, CR5 cells accumulated more Cd^{2+} more rapidly than normal muntjac cells. In this respect, CR5 cells differ from the Cd^{2+} resistant lines of CHO cells produced by Hildebrand et al. (1982) and Gick and McCarty (1983); these resistant cells took up less Cd^{2+} than the parental lines under the same conditions. In spite of the enhanced cellular uptake of the cation, the nuclei, cytoplasmic organelles and membranes of CR5 cells did not bind any more Cd^{2+} than their counterparts in normal cells. The cytosol binds the greatest amount of the metal ion in CR5 cells, apparently protecting other cellular components from Cd^{2+}. Within the cytosol, Cd^{2+} binds almost entirely to a single low molecular weight molecule. On the basis of the behaviour of this molecule on gel filtration and anion exchange columns and its u.v. absorbance spectrum, it can be concluded that it is not metallothionein. Virtually all the Cd^{2+} variants of other cell types show enhanced metallothionein production or Cd^{2+}-thionein binding (Rugstad and Norseth, 1978; Beach et al., 1981; Hildebrand et al., 1983; Gick and McCarty, 1983; Shworak et al., 1983). However, a Cd^{2+} resistant line of mouse thymoma cells has been produced which does not synthesize metallothionein (Beach et al, 1981). No other information is avialable concerning Cd^{2+} metabolism in this cell line.

The u.v. spectral analysis suggested that the major cytosolic Cd^{2+} binding molecule of muntjac might be glutathione. It is interesting to note that Katoh et al., (1984) found that glutathione was the major Cd^{2+} binding molecule in the livers of rats and humans not previously exposed to the metal. Comparison of the G-75 gel filtration elution patterns of the Cd^{2+} glutathione complex (published in Katoh et al, 1984) and the muntjac Cd^{2+} binding molecule (present data) highlights their similarity.

In parallel with the biochemical alterations there were ultrastructural differences between CR5 and normal muntjac cells. As far as can be ascertained, there are no published reports dealing with the ultrstructure of any Cd^{2+} resistant cell line. Normal muntjac cells treated with Cd^{2+} had many manifestations of cellular damage. The morphology of mitochondria and rough endoplasmic reticulum was altered in normal cells treated with Cd^{2+}. Cadmium-induced changes in these two organelle systems have been noted by other workers (Schlaepfer, 1971; Nishizumi, 1972; Stowe et al., 1972; Kawahara et al., 1975; Richardson and Spivey-Fox, 1975; Faeder et al., 1976; Hayes et al., 1976; Meiss et al., 1972). Reduction in rough endoplasmic reticulum/mitochondria associations does not, however, seem to have been noted previously. Proliferation of smooth membrane vesicles and loss of cytoplasmic organization are common features of Cd^{2+} toxicity in a number of species (Schlaepfer, 1971; Nishizumi, 1972, Stowe et al., 1972; Kawahara et al., 1975; Richardson and Spivey-Fox, 1975; Faeder et al., 1976; Hayes et al., 1976; Meiss et al., 1982). In contrast to Cd^{2+} treated normal muntjac cells, CR5 cells appeared to be more structurally organized and metabolically active. The major difference between normal and CR5 cells was the production, gathering and subsequent loss of large numbers of myelin figures in the resistant cells. Considering evidence from the ultrastructural and time lapse video studies together, it appeared likely that the bundles of myelin figures were expelled from the CR5 cells 12-24 hours after the addition of fresh culture medium and cadmium. Cd^{2+} treatment of whole organisms has been observed to cause proliferation of intracellular smooth membrane (Schlaepfer, 1971; Nishizumi, 1972; Stowe et al., 1972; Hayes et al., 1975; Meiss et al., 1982) and the formation of myelin figures (Gabbiani et al., 1974; Ord and Al-Atia, 1979). Therefore excess lipid production would appear to be a common feature of Cd^{2+} treatment this may be related to the metals ability to alter the structure (Suzuki and Matsushita, 1968) and possibly stability (Chvapil, 1973) of membranes.

The relationship between the biochemical and ultrastructural features of CR5 cells has not been determined. To establish whether the myelin figure bundles contain Cd^{2+} and/or the Cd^{2+} binding molecule would require autoradiographic and electron probe analysis.

Other features of the cadmium resistant muntjac cells include abnormal divisions and a more transformed phenotype than the parental cell line. Neither of these phenomena have been investigated in Cd^{2+} resistant lines of other cell types.

7.2 The Chromosomes of CR5 cells and the effects of other mutagens

Having established a number of the characteristics of CR5 muntjac cells, attention will now focus on their use in assays for chromosome damage. The effects of the panel of five DNA binding agents, previously used in combination with Cd^{2+} in normal muntjac cells have been investigated.

7.2.1 The chromosomes of CR5 cells

Metaphase preparations revealed that CR5 cells had very different chromosomes to those of normal muntjac cells. The number and morphology of the chromosomes were variable (see figure 7.16). Although a few chromosomes of normal morphology were seen, most appeared to consist of two or more whole or part chromosomes of normal form. The derivation of most of the CR5 cell chromosomes could be determined due to the readily identifiable morphology of the seven chromosomes of normal cells. No single type of 'compound chromosome' occurred with greater frequency than any other in the resistant cells. The frequency of chromosome and chromatid gaps and breaks was also elevated in CR5 cells. 12 chromatid gaps, 6 chromatid breaks, 19 chromosome gaps and 29 chromosome breaks were counted in a sample of 100 cells.

Variation in the number of chromosomes per cell was almost as great as the variation in chromosome morphology. Chromosome counts were based on the number of centromeres per cell. This measure gives the best indication of ploidy level as it takes into account the number of normal chromosomes which have contributed to each nucleus. In a sample of 100 metaphases, the number of chromosomes per nucleus ranged from 3 to 27, the modal number being 9.

Figure 7.16

Examples of CR5 cell metaphases. Control cells (i.e. grown in 5μM cadmium acetate only). Scale bars represent 10μm.

Note: ▶ dicentric chromosomes

▶ gaps and breaks

chromosomes of altered morphology

and frequent SCEs.


a


b


c

The frequency of SCEs per chromosome (total SCEs per metaphase divided by number of centromeres in the metaphase) was also greater in CR5 than normal cells. In a sample of 153 cells, drawn from four replicate cultures, a mean of 2.3 ± 0.22 (standard error) SCEs per chromosome were counted.

7.2.2 The effects of other mutagens on the chromosomes of CR5 cells

Due to the highly altered and variable nature of CR5 cell chromosomes, only the induction of SCEs by the other chemicals was determined.

Using the standard procedure (section 3.5.1), differentially stained metaphase spreads of CR5 cells grown in 5μM Cd^{2+} and treated with EMS, MMC, AO, H33258 or Ni^{2+} for 48 hours were produced. High yields of differentially stained metaphases were obtained in these experiments; thus, the cell cycle duration of CR5 cell was similar to that of normal muntjac cells. Results of these experiments are shown in table 7.4. Each mean is from a sample of 100 metaphases drawn from two replicate cultures.

Ni^{2+} and AO treatment did not significantly alter the baseline SCE frequency in CR5 cells despite their both being active inducers in normal cells. Thus, the resistant cells appear to be protected from the SCE-inducing effects of these two chemicals. However, the remaining mutagens were active in CR5 and well as in normal cells.

Given the significant difference in baseline SCE frequencies, comparison of the same treatments in the two cell types in terms of absolute SCE frequency is not the best indication of relative sensitivity. The increase in SCE frequency over baseline and the ratio of treated to baseline SCE frequency for each treatment in both types of cells are better indicators. These derived figures can be seen in table 2.5.

On the basis of the ratios of treated to baseline SCE frequencies, it would appear that the resistant cells are less sensitive than normal cells

to SCE induction by all the compounds tested. The same trend is seen when comparing increases over baseline frequencies. The one exception is H33258 where the difference between the values for normal and CR5 cells is minimal.

TABLE 7.4 SCE induction by DNA binding agents in CR5 cells

TREATMENT	MEAN SCEs PER CHROMOSOME ± SEM	SIGNIFICANT DIFFERENCE FROM	
		CONTROL CR5	NORMAL CELLS GIVEN SAME TREATMENT
-	2.2 ± 0.13	-	* * *
0.1mM EMS	3.35 ± 0.16	* * *	* * *
0.018μM MMC	4.04 ± 0.18	* * *	*
0.5μM AO	1.99 ± 0.11	NS	NS
5μM H33258	2.79 ± 0.11	* *	* * *
1μM $NiSO_4$	2.18 ± 0.18	NS	* *

TABLE 7.5 SCE induction by DNA damaging agents in normal and CR5 derived data

CELL TYPE / TREATMENT	INCREASE OVER BASELINE		RATIO TREATED: BASELINE SCE FREQUENCY	
	NORMAL	CR5	NORMAL	CR5
0.1mM EMS	1.23	1.1	2.29	1.49
0.018μM MMC	2.44	1.79	3.32	1.8
0.5μM AO	0.33	-0.26	1.31	0.88
5μM H33258	0.44	0.49	1.42	1.22
1μM $NiSO_4$	0.72	-0.07	1.69	0.96

7.2.3 Discussion

The finding of dramatic changes in chromosome morphology and number in CR5 cells serves to confirm the point made previously, that the Cd^{2+} resistant cells are more transformed than the parental cell line. In

general, highly transformed cell lines, HeLa for example, have a variable karyotype, distinct from that of the cells from which they originated. Of the four cadmium resistant cell lines described by Beach et al. (1981), two had increased and variable chromosome numbers. The karyotypes of the remaining two resistant cell lines were indistinguishable from those of the respective parental cell lines. The elevated SCE frequency in CR5 cells may also be indicative of transformation. SCE frequencies determined 'in vivo' are generally low in comparison to those of similar cells in culture (see, for example, Latt et al., 1978).

It will be recalled from Chapter 4 that continuous exposure of normal muntjac cells to Cd^{2+} did not induce SCEs or chromosomal aberrations. However, over a period of several months, it would appear that the cation can induce chromosomal rearrangements and SCEs. The length of time required for these changes to occur has not been established.

When considered in relation to their respective baseline SCE frequencies, normal cells were more sensitive to SCE induction by DNA binding agents than CR5 cells. It is possible that the low molecular weight Cd^{2+} binding molecule, present at elevated levels in CR5 cells, plays a role in the protection of these cells from DNA damage leading to SCE formation. The molecule may not be a specific Cd^{2+} binding agent, rather a molecule able to conjugate with, and detoxify, a range of xenobiotics. This seems all the more likely as the molecule is similar to glutathione, a small peptide known to be involved in the detoxification of many agents. It is also possible that the myelin figures observed in CR5 cells are involved in the metabolism of toxins and/or their expulsion from the cell.

Although interesting in its own right, the observation that Cd^{2+} resistant cells are less sensitive to SCE induction by DNA damaging agents than normal cells is of questionable practical significance. The absolute SCE frequency in the CR5 cells treated with all the agents, with the exception of AO, was greater than in normal cells given an identical treatment. Therefore, on encountering another genotoxin, a person

chronically exposed to Cd^{2+} may sustain a greater level of genetic damage than a non-exposed individual. However, it must be pointed out that the validity of CR5 muntjac cells as an 'in vitro' model of chronic Cd^{2+} exposure is uncertain. Cd^{2+} metabolism in the human has not been directly studied. Humans may react differently to long term exposure to the cation than muntjac cell cultures. Many other cell lines, including one derived from human skin (Rugstad and Norseth, 1975), appear to deal with Cd^{2+} differently than the muntjac cells used in the present work. If CR5 muntjac cells were a good model of environmental and occupational exposure of humans to Cd^{2+}, one would predict that the chromosomes of chronically exposed persons would be altered in some way. The available data, all of which concerns chromosomal aberration in Itai-Itai disease patients and industrially exposed persons (see Chapter 2), is variable. Thus it cannot be determined whether Cd^{2+} resistant muntjac cells are a good model of chronic Cd^{2+} exposure in humans.

Chapter 8. Mechanisms of interaction II: DNA repair

8.1 Rationale and experimental design

Throughout this project, SCE and chromosomal aberration induction have been taken as measures of genetic damage. The degree of genetic damage caused by a given treatment is influenced by several factors, such as: (i) extent of binding to DNA, (ii) type of DNA lesion formed, (iii) extent of repair of DNA lesions, (iv) rate and fidelity of DNA repair. Any, or all, of these factors could be involved in the effects of Cd^{2+} on the genetic damage induced by the other DNA damaging agents described in Chapter 5. In the case of Ni^{2+} and Cd^{2+} treatments, the extent of nuclear binding of either ion was unaffected by the presence of the other; yet, combined treatments synergistically enhanced the induction of SCEs and chromosomal aberrations. Therefore, factor (i) is an unlikely candidate for the mechanism of Cd^{2+}/Ni^{2+} interactions. Investigation of the involvement of factor (ii) would require complex chemical and biochemical analyses, whereas the role of DNA repair (factor iii) can be easily assessed. There are several known DNA repair systems: photoreactivation, excision repair, post-replication repair and various inducible repair pathways. Excision repair is probably the most important and accurate repair system of mammalian cells (Cleaver, 1977). The other major repair system of mammals, post replication repair, tends to be 'error prone' (Witkin and George, 1973) therefore it is one of the main processes by which wrong bases are incorporated into DNA. The measurement of unscheduled DNA synthesis (UDS) is one of the simplest and most reliable methods available for quantifying excision repair (Cleaver, 1977). This technique determines the amount of incorporation of radiolabelled nucleotides into DNA outside the normal period of replicative DNA synthesis (S phase). In the present experiments an autoradiographic method, involving grain counting over non-S phase nuclei, has been used to quantify UDS (see section 3.6 for details of method).

The extent of UDS induced by Cd^{2+}, the DNA binding agents and Cd^{2+}/DNA binding agent combinations was determined. Interactions between Cd^{2+} and the other DNA binding agents were defined by the

following 'interaction index':

$$\frac{AB}{A+B} > 1 \qquad \text{Synergistic interaction}$$

$$\frac{AB}{A+B} = 1 \qquad \text{Additive interaction}$$

$$\frac{AB}{A+B} < 1 \qquad \text{Antagonistic interaction}$$

where: A = UDS induced by DNA binding agent at concentration a
B = UDS induced by Cd^{2+} at concentration b
AB = UDS induced by combined treatment with the DNA binding agent and Cd^{2+} at concentrations a and b respectively.

UDS was also assessed in CR5 cells treated with each of the five DNA binding agents. Thus, the relative amounts of UDS induced by a given chemical in normal and CR5 muntjac cells could be compared.

8.2 Induction of UDS by Cd^{2+}

UDS, measured over the last two hours of a four hour treatment period, was induced by Cd^{2+} (see figure 8.1). The increases over controls at 1 and 10μM Cd^{2+} were statistically significant ($p < 0.001$).

Other studies of Cd^{2+}-induced UDS have conflicting, but mainly positive results. Cultured human fibroblasts treated with the cation were apparently unable to initiate UDS (Hollstein and McCann, 1979) but Cd^{2+} treated Petunia pollen did incorporate thymidine outside S phase (Jackson and Linskins, 1982). Chinese hamster V79 cells also appear to mend Cd^{2+}-induced DNA damage by an excision repair pathway (Ochi et al., 1983b).

As SCEs were not induced by Cd^{2+}, the lesions that lead to UDS and SCE must be different, or all the SCE-inducing lesions must be effectively

removed by excision repair.

8.3 Induction of UDS by combinations of Cd^{2+} and DNA binding agents

All the five DNA binding agents tested significantly increased the control level of UDS; AO at $p < 0.05$, EMS and MMC at $p < 0.01$, H33258 and Ni^{2+} at $p < 0.001$, see figure 8.1 B and C. By reference back to table 2.5, it can be seen that the positive effects of EMS MMC and Ni^{2+} confirm the results obtained by some other workers using different cell types.

The co-treatment of cells with EMS, MMC or AO and Cd^{2+} increased the level of DNA repair compared to the 'other mutagen alone' treatments. Treatment with Ni^{2+}/Cd^{2+} combinations led to a reduction in Ni^{2+} induced UDS. Co-treatment with H33258 and 0.1μMCd^{2+} decreased, while H33258 and 1μMCd^{2+} increased, the level of UDS induced by the DNA binding agent alone. As Cd^{2+} itself induced UDS, these changes caused by the cation cannot be directly interpreted as antagonistic, additive or synergistic. Thus, the data was converted into an interaction index, that is the quotient of the UDS induced by a combined treatment and the sum of that induced by the separate treatments (see section 8.1). The results expressed in terms of the natural logarithms of the interaction indices are shown in figure 8.2. The logarithmic transformation simplifies the figures one has to consider, an additive interaction is represented by zero, antagonistic and synergistic interactions by negative and positive numbers respectively. Examination of this transformed data reveals that combinations of either of the alkylating agents and Cd^{2+} gave rise to synergistic levels of UDS, while AO/Cd^{2+} treatments were additive and the remaining combinations antagonistic. In the cases of EMS/Cd^{2+}, MMC/Cd^{2+} and H33258/Cd^{2+} treatments, increased Cd^{2+} concentration did not increase the magnitude of the interactions.

The work of Samson and Schwartz (1980) and Laval and Laval (1984) has shown pretreatment of rodent cells with sub-toxic concentrations of the alkylating agent N-methyl-N-nitrosourea to protect cells from the

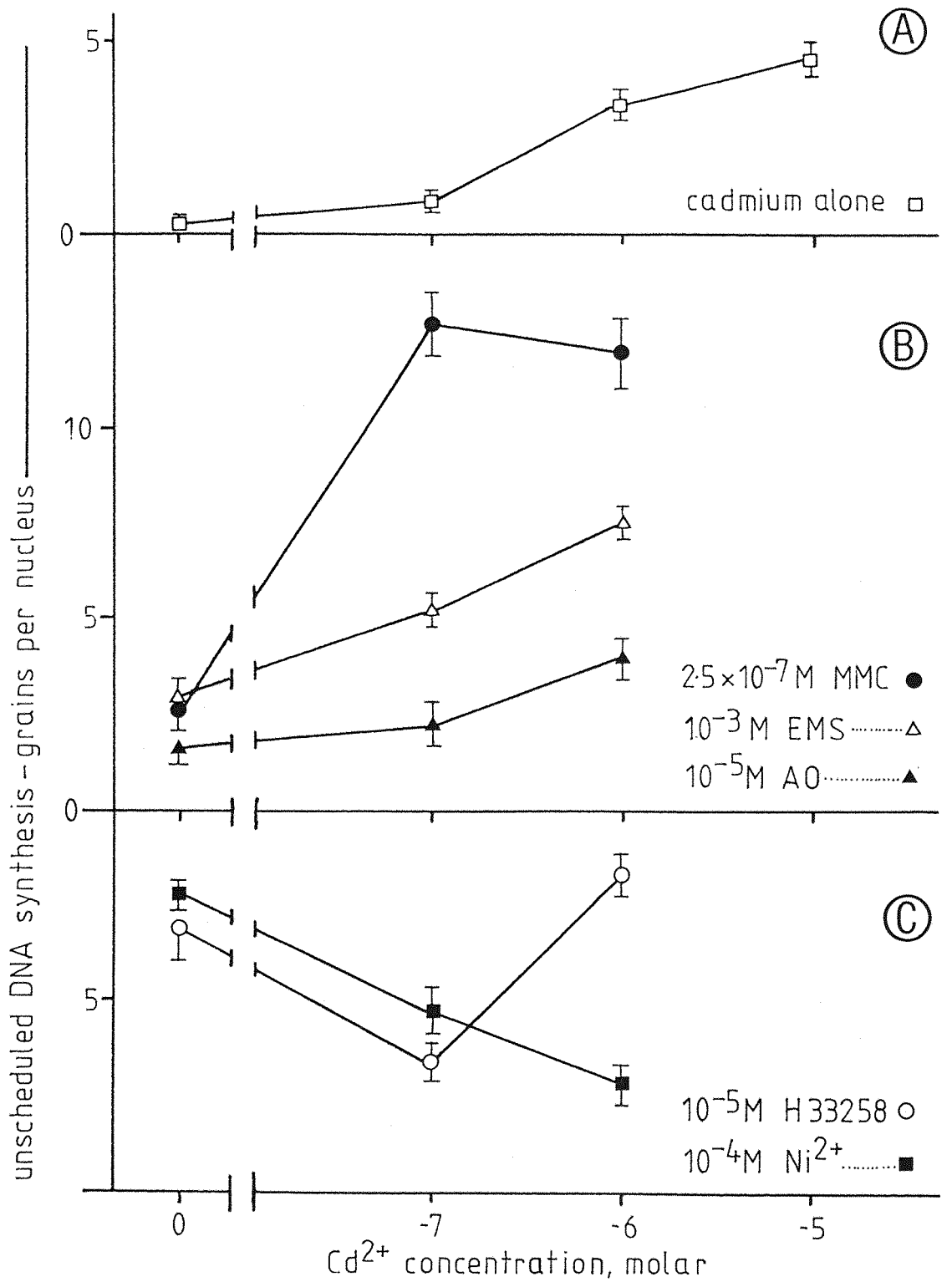


Figure 8.1. Induction of unscheduled DNA synthesis by: A cadmium acetate, B and C cadmium acetate in combination with other DNA binding agents.

Points represent means of grain counts over 100 non-S-phase nuclei drawn from 2 replicate cultures; bars represent ± standard errors. All treatments were of 4 hours duration, ^{3}H-thymidine being present for the last two hours.

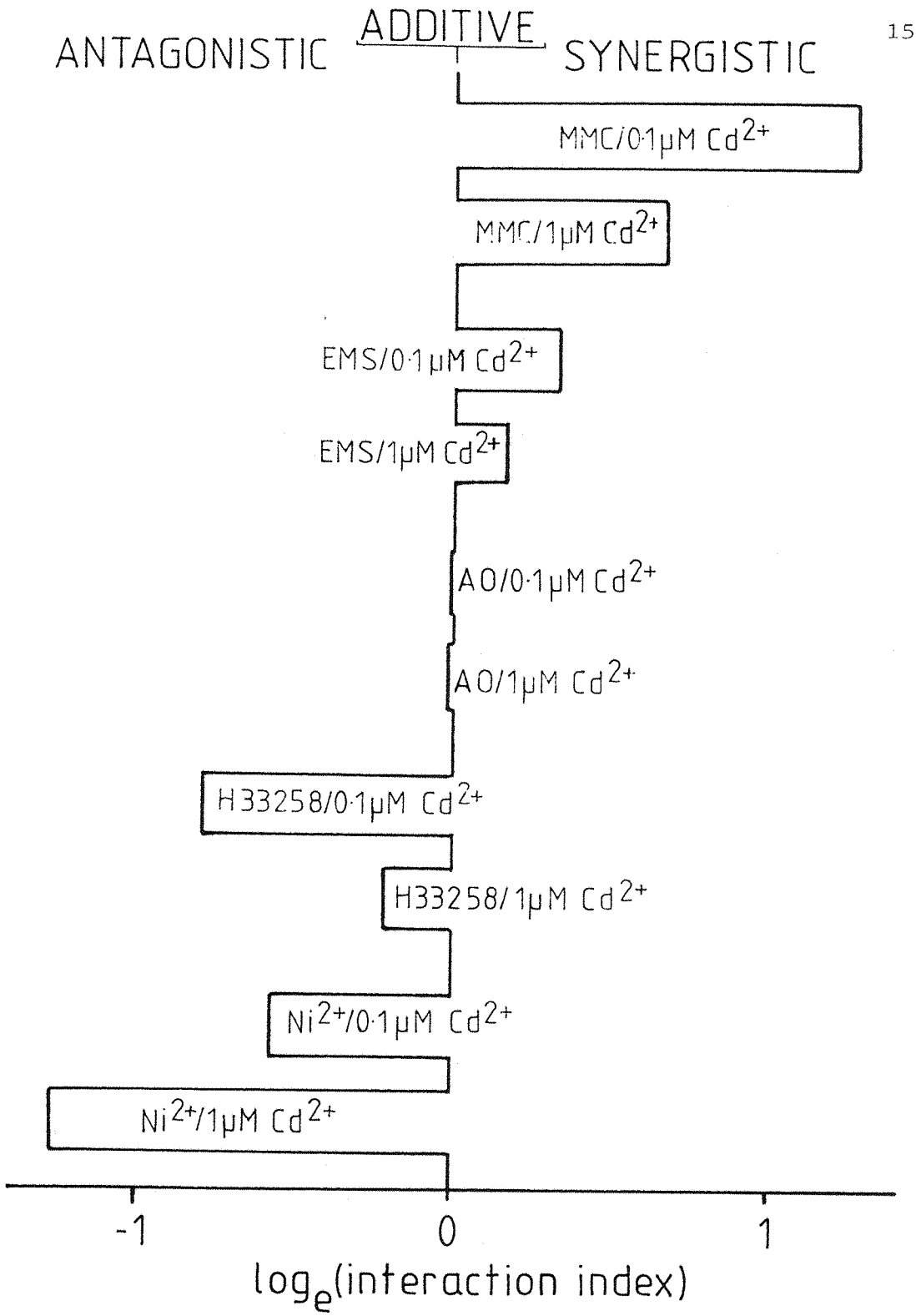


Figure 8.2. Unscheduled DNA synthesis data expressed in terms of natural logarithm of interaction index. Concentrations of compounds, unless specified, as in figure 8.1.

cytotoxic, SCE-inducing and mutagenic effects of a subsequent, higher dose of the compound. These phenomena are considered to involve inducible DNA repair systems. It is possible, therefore, that low Cd^{2+} concentrations induce a process able to repair alkylation damage by an excision repair-like pathway. That Cd^{2+} can induce the synthesis of proteins, other than metallothionein, in mammalian cells has been demonstrated by Levinson et al., (1980). The function of these proteins is unknown, although they do show some similarities to heat shock proteins. By the use of a range of compounds, Levinson and coworkers (1980) concluded that sulfhydryl-group reactivity was the property required to induce the synthesis of this set of proteins. Alkylating agents and the Cd^{2+} share this property.

If the above hypothesis is true, the putative inducible repair system must be specific for alkylation damage. Combinations of AO and Cd^{2+} induce very nearly additive levels of UDS; on the other hand, cells treated with Ni^{2+} and H33258 are less able to repair DNA damage in the presence of Cd^{2+}. There is no need to involve any special mechanism to explain the AO/Cd^{2+} interaction but the effects of Cd^{2+} on Ni^{2+} and H33258 induced UDS require further consideration. Cd^{2+} could be interfering directly with the enzymes responsible for the repair of Ni^{2+} and H33258 lesions. Alternatively, there may be steric interactions at the level of DNA binding, for example, repair enzymes may not be able to recognise a H33258 induced lesion when Cd^{2+} is bound in the vicinity of the lesion.

Whatever the reasons for the interactions, it is interesting to note the inverse correlation between UDS and SCE induction caused by combined treatments. Thus, the presence of Cd^{2+} reduces the induction of SCE by alkylating agents but enhances DNA repair induction; Ni^{2+} and H33258 induced SCE is increased by the presence of the cation while UDS is reduced. So, it would appear that there is some involvement of DNA repair in the Cd^{2+}/other mutagen interactions.

8.4 Induction of UDS in CR5 cells by DNA binding agents

The level of DNA repair in CR5 cells (incubated in 5μMCd^{2+}) was higher than in untreated normal cells. On average, CR5 cells had 6.7 ± 0.45 (mean ± standard error) grains over each non-S-phase nucleus. Normal cells treated with 5μMCd^{2+} would have approximately 8 grains per nucleus (figure obtained by interpolation of graph in figure 8.1). So, although CR5 cells appear to be actively involved in excision repair, they are not repairing to the same extent as normal cells treated with the same concentration of the cation.

Results of experiments investigating the induction of UDS in CR5 cells by the five DNA binding agents are shown in table 8.1. The treatment and labelling protocol for these assays was the same as that used for normal cells with the exception that 5μMCd^{2+} was present in the treatment medium at all times.

None of the DNA binding agent-induced alterations in the level of UDS in CR5 cells was significantly different from that in control CR5 cells. Absolute grain counts in DNA binding agent-treated CR5 cells were always lower than in normal cells given a similar treatment (compare table 8.1 and figure 8.1). Similarly the ratio of treated to control grain counts was less in the Cd^{2+} resistant cells than in the parental cell line (see table 8.1). Therefore, less DNA repair occured in CR5 cells treated with any of the five DNA binding agents than in the normal cells given the same treatment. This finding implies one of two conclusions:

i) The treatments cause a similar amount of DNA damage in the two cell types but CR5 cells are less able to repair the damage.
ii) Less DNA damage occurs in CR5 cells, therefore less repair is observed.

The former possibility can be ruled out as evidence presented in the previous chapter demonstrated that CR5 cells are less sensitive to chemically induced DNA damage (as assessed by SCE). Thus, it may be that the amount of mutagen reaching the nucleus of a CR5 cell is less than that

which would bind in the nucleus of a similarly treated normal cell.

Table 8.1 Induction of UDS by DNA binding agents in CR5 cells

TREATMENT	MEAN GRAINS PER NUCLEUS ± SEM	RATIO TREATED/ CONTROLS UDS IN CR5	RATIO TREATED/ CONTROL UDS IN NORMAL CELLS
-	3.37 ± 0.45		
1mM EMS	5.15 ± 1.17	1.53	9.19
.25μM MMC	4.35 ± 0.62	1.29	8.47
10μM AO	2.41 ± 0.32	0.72	4.78
10μM H33258	3.29 ± 0.47	0.98	21.72
100μM Ni^{2+}	4.81 ± 0.86	1.42	24.06

In discussing the results of the previous chapter, the possibility of the low molecular weight Cd^{2+} binding molecule being involved in the reduced sensitivity of CR5 cells to the other mutagens was mentioned. The experiments described in this chapter lend support to this idea. It has been shown that more efficient repair of DNA lesions (at least by an excision repair-type mechanism) is not likely to account for the reduction in sensitivity of CR5 cells to the five DNA binding agents.

8.5 Summary

1. Cadmium acetate can induce UDS in normal muntjac cells.
2. Cd^{2+} can antagonize the induction of UDS by some DNA binding agents and synergize that induced by some others.
3. There appears to be an inverse correlation between the induction of SCE and UDS by combinations of Cd^{2+} and other compounds. Therefore, suggesting a role for DNA repair in the mechanisms of interactions between Cd^{2+} and other mutagens.
4. Less DNA repair is induced in CR5 cells than in normal muntjac cells by an identical treatment.
5. No direct correlation between reduction in sensitivity of CR5 cells to DNA damage (SCE) and increased DNA repair was observed.

Therefore, a cytoplasmically-based mechanism, such as conjugation of genotoxins to the glutathione-like molecule, is more likely to be involved in the protection of CR5 cells from the effects of other genotoxins.

Chapter 9. General Discussion and Conclusions

9.1 The use of muntjac cells in toxicology

Few investigators have used muntjac cells for toxicological studies. The low and stable chromosome number of the muntjac cell line employed in the present work suits it to cytogenetic investigations. Metaphase analysis is rapid and could probably be automated by the use of image analysis or flow cytometric techniques. One possible drawback arises from the large size of the chromosomes which may impose structural limitations on the formation of chromosomal aberrations. Rees and Bootman (1982) found muntjac cells to be the least sensitive of six cell types to chromosomal aberration induction by mitomycin C. Similar structural limitations seem not to apply to SCE formation. Human lymphocytes, cultured in the same concentration of BUdR as was used in muntjac cell cultures, have a background SCE frequency of 6 - 7 per cell (M. J. Ord, personal communication), a very similar SCE frequency per cell was found in muntjac cells.

Experience gained in this project has shown the muntjac cell line to be adaptable to several techniques and easy to maintain in culture. Hence, muntjac cells are probably as useful as any other cell culture system for toxicological investigations. The major limitation is the paucity of background information available and the lack of extensive validation studies.

9.2 The genetic and cellular toxicity of Cd^{2+} in muntjac Cells

That Cd^{2+} is cytotoxic, does not induce SCEs and only induces chromosomal aberrations under certain conditions are not new findings. Rather they serve to confirm and extend many of the other investigations discussed in Section 2.4.3. Additionally, the cytogenetic data was required as background for later experiments.

As far as can be ascertained, the results presented in this study are the first demonstration of Cd^{2+} induced unscheduled DNA synthesis

in a mammalian system. UDS has been reported to occur in response to treatment with the cation in Petunia pollen (Jackson and Linskins, 1982) but not in cultured human fibroblasts (Hollestein and McCann, 1979) However, other techniques have successfully demonstrated increased excision repair in Cd^{2+} treated mammalian cells (Robinson et al., 1982; Ochi et al., 1983a).

Since Cd^{2+} was found to be effective in elevating the level of UDS in muntjac cells, it may be that all Cd^{2+}-induced lesions capable of causing SCEs or chromosomal aberrations are removed by excision repair. Alternatively, all three phenomena could be caused by independent mechanisms. The former conclusion more probably holds true because SCE-inducing lesions can be removed by excision repair. Evidence supporting this statement comes from two sources. Firstly, the hypersensitivity of cells derived from Xeroderma pigmentosum patients to SCE induction by UV light and alkylating agents (Xeroderma pigmentosum is a human disease caused by an autosomal recessive mutation and is associated with a predisposition to skin cancers). Xeroderma pigmentosum cells are defective in excision repair of DNA damage. The baseline SCE frequency in these cells is normal, but alkylating agents and UV irradiation induce far greater elevations in SCE frequency than equivalent doses of these mutagens would in normal cells (see review by Latt, 1981). Secondly, in cells of Allium cepa there is an inverse correlation between the time available for the repair of UV light-induced lesions and SCE frequency. This was demonstrated by Schwartzman and Gutierrez (1980) who followed SCE induction in cells exposed to UV light for 40 minute periods at varying times during the cell cycle. An increasing effectiveness of the treatment through G_1 and early S phase was noted. Peak SCE induction was obtained by irradiation in early S phase, a rapid drop in effectiveness followed until, by the end of S phase, irradiation did not elevate the SCE frequency observed in the subsequent metaphase. Taken together, this evidence implies that SCE formation depends upon the presence of lesions during S phase and that the SCE inducing lesions can be removed by excision repair. Therefore, SCEs can be interpreted as a measure of DNA damage. It is quite possible then, that the elevation of UDS by Cd^{2+} treatment of muntjac cells is, in effect, a reflection of the virtually complete removal of SCE-inducing lesions. Thus explaining

the lack of SCE induction by the cation in these cells.

Ultrastructural changes were also observed in Cd^{2+} treated normal muntjac cells. These changes, which were entirely cytoplasmic and indicative of reduced metabolic activity, were seen only in a proportion of the treated cells. This suggests that there is a natural heterogeneity in the cell population with regard to their susceptibility to Cd^{2+}. Following the culturing of cells for extended periods in the presence of increasing concentrations of the cation (up to 5 μM), a Cd^{2+} resistant population (denoted CR5) was obtained. Examination in the electron microscope suggested that the cytoplasm of these cells was regionalized. Some areas were morphologically similar to the cytoplasm of untreated normal cells while other areas appreared to be almost entirely devoted to the synthesis of membrane and myelin figures. With increasing time after the additon of fresh Cd^{2+}, these membranous areas became more compact and membrane bound. Eventually, the number of myelin figure bundles decreased. Although no direct evidence was produced, it seems likely that the myelin figure bundles were expelled from the cells during interphase. These novel observations may represent a mechanism for the detoxification or reduction in cellular burden of Cd^{2+}. As noted earlier, localization of Cd^{2+} in these cells by electron microscope autoradiography or electron probe analysis would help clarify this point. Nevertheless, the long term growth of cells in Cd^{2+} led to the development of a strain of muntjac cells able to tolerate concentrations of the metal that would kill all normal cells within a few days. This change in cell phenotype was stable over many generations when cells were cultured in the absence of Cd^{2+}; therefore it is likely that the change has a genetic, rather than epigenetic basis i.e. not involving a stable, inheritable change .

Analysis of CR5 cell metaphases revealed striking differences in chromosome morphology and number in comparison to the normal muntjac cell karyotype. In general, CR5 cell karyotypes were of greater and more variable ploidy, many of the chromosomes appeared to be derived from fusions of whole or part chromosomes of normal morphology. Thus di- and multicentric chromosomes were common. CR5 cells were also observed to have a higher frequency of SCE than normal cells. So, even

though exposure of normal cells to Cd^{2+} for 48 hours did not cause chromosomal changes, culturing in the presence of the cation for several months did lead to the induction of such effects. The time at which these changes occurred was not investigated. In addition to chromosomal changes, CR5 cell nuclei displayed a constitutively higher level of UDS. However, the level of UDS induced by treatment of normal cells with 5 μm Cd^{2+} was higher than that in CR5 cells. So the inverse relationship between excision repair and SCE induction, discussed in Chapter 8, may still hold.

Further evidence of the ability of Cd^{2+} to cause genetic changes comes from the cell transformation assays. CR5 cells more readily grew in semi-solid medium and were agglutinated by concanavalin A to a greater extent that the parental cells. As Cd^{2+} was found to transform Syrian hamster embryo cells (DiPaolo and Casto, 1979), it is not surprising to find chronic treatment with the cation further transforms muntjac cells. Even so, such observations have not been reported previously.

The extent of Cd^{2+} binding in the nuclei of CR5 cells was only marginally greater than in those of normal cells. The genetic changes in CR5 cells are not, then, due to a greater nuclear accumulation of the metal ion. However, there was a much greater cellular uptake of Cd^{2+} in CR5 cells. The greater proportion of this Cd^{2+} was located in the cytosol bound to a single molecule of low molecular weight. A greater relative cytosolic accumulation of Cd^{2+} seems to be a common feature of Cd^{2+} resistant varients of all cell types (Rugstad and Norseth, 1979; Hildebrand et al., 1982). In these other cell types virtually all cytosolic Cd^{2+} is bound to metallothionein. By contrast, in CR5 muntjac cells, the bulk of the cytosolic Cd^{2+} was found in association with a single molecule of lower molecular weight than metallothionein (as judged by G-75 column chromatography). This low molecular weight species bears some resemblance to glutathione; unequviocal identification would require amino acid analysis and/or detailed chemical analysis. The Cd^{2+} binding molecule was also found in the cytosol of Cd^{2+} treated normal muntjac cells. The proportion of cytosolic Cd^{2+} bound to the Cd^{2+} binding molecule was higher in CR5 than normal cells exposed to

equivalent concentrations of the metal ion. Therefore, CR5 cells more readily synthesize the molecule in response to Cd^{2+} or contain more of it constitutively. This Cd^{2+} binding molecule possibly plays a role in the detoxification of the cation; if it is indeed glutathione, this is all the more likely.

The relationship between the cytosolic low molecular weight Cd^{2+} binding molecule and the myelin figure bundles, observed in the ultrastructural study is not clear. Myelin figure formation was essentially absent from normal cells and the production of the Cd^{2+} binding molecule much lower. Thus both arise in response to long term Cd^{2+} treatment. Whether the two phenomena are part of a single Cd^{2+} detoxification mechanism or if either is involved in a cellular protection mechanism is not known. Unless they are very fragile, it would seem most probable that the myelin figures would have sedimented in the organelle/membrane pellets during subcellular fractionation. Only very minor differences were seen in the amount of Cd^{2+} bound to the organelle/membrane pellets of normal and CR5 cells. Hence, it is unlikely that the myelin figures are a major Cd^{2+} storage site. Also as the Cd^{2+} binding molecule was located in the cytosol it probably is not associated with the myelin figures. This argument would not hold if the myelin figures were extensively lysed during homogenization.

9.3 The effects of Cd^{2+} on the genotoxicity of other DNA binding agents

The primary aim of this project was to investigate whether and how Cd^{2+} might affect the action of other genotoxins. It has become clear in the course of the study that Cd^{2+} can influence the genotoxity of other compounds. The nature of the interaction can depend on the type of genotoxin, the scheduling of treatment and the endpoint of genotoxicity considered. As pointed out earlier, different endpoints of genotoxicity arise by different pathways and so may be differently affected by the presence of Cd^{2+}. SCE and chromosomal aberration induction correlate as well as most other short term genotoxicity assays with whole animal carcinogenicity data. So the two assays should give

as good an indication of the effects combinations of compounds may have on humans as any other test system. In all cases but one, the effect of Cd^{2+} on SCE and chromosomal aberration induction by another compound was similar. However, in most cases the SCE data was of greater statistical significance.

Considering the SCE data alone, the following points can be made:

(i) The effects of alkylating agents are reduced by concurrent or prior exposure to Cd^{2+}.
(ii) SCE induction by noncovalent DNA binding agents tends to be enhanced by Cd^{2+} treatment, only concurrent treatments lead to significant effects.
(iii) Concurrent exposure to Cd^{2+} and Ni^{2+} synergizes SCE formation while Cd^{2+} pretreatment antagonizes the effect of the other ion.

The trends noted in the chromosomal aberration results were similar to the above with the exception of EMS/Cd^{2+} combinations. A synergy between the two compounds was noted in the formation of chromosomal abberations.

What then, are the implications of these findings for the general population? Persons who have previously or are concurrently exposed to Cd^{2+} would apear to be protected, to some extent, from the carcinogenic effects of alkylating agents, but more susceptible to cancer induction by non convalent DNA binding agents. As Cd^{2+} pretreatment enhanced and co-treatment antagonized Ni^{2+}-induced SCE and chromosomal aberration, similar effects would probably occur in vivo.

As pointed out in the introduction, the five 'other genotoxins' used in the present study should be viewed as model compounds reflecting a range of chemical classes found among environmental carcinogens as a whole. Which human carcinogens do these compounds model? Well, many known or suspected environmental carcinogens form covalent adducts with DNA, these include pyrolizidine alkaloids, furocoumarins, aflatoxins and nitrosamines which occur in the human diet or can be formed in the body from dietary precursors. Many polycyclic hydrocarbons also

form covalent DNA adducts but, as the chemical species attached to DNA is usually very large, MMC and EMS probably do not represent particularly good models of these compounds. Some cosmetic products probably contain alkylating agents; certain suntan lotions contain psoralens which, when activated by sunlight, can crosslink complementary DNA strands. Several cancer chemotheraputic agents in current use, such as the nitrogen mustards and nitrosoureas are alkylating agents. Acridine compounds also have clinical uses in the treatment of malaria and other protozoan diseases, helminth infections and 'petit-mal' epilepsy. The anti-cancer drug, adriamycin is an intercalating agent. Most compounds with three or four adjacent cyclic goups in their structure are potential DNA intercalating agents, compounds with such structures are common among antibiotics and fungal toxins (see, for example, Glasby, 1979). Some acridine compounds have industrial uses as pigments (for textiles, leather, and cellulose), disinfectants and metal preservatives. Non-intercalative DNA binding agents are probably of lesser importance as environmental carcinogens than the previous two groups of compounds. Nonetheless, compounds able to bind to DNA in this fashion include anthramycin, netropsin and distamycin A (Krey, 1980). Compounds with structures similar to known non-intercalative DNA binding agents are not uncommon among antibiotics and fungal metabolites(Glasby, 1979). Some nickel compounds are human carcinogens, evidence available from *in vitro* cell transformation studies suggest that nickel (II) ions are the chemical species responsible for DNA binding and other intracellular damage (see Section 2.6.3). It would, however, be unwise to view Ni^{2+}, or any other individual metal, as typical of metal carcinogens. The wide variety of effects and mechanisms of action of this group of compounds makes generalization difficult.

When one considers the tremendous diversity of cellular effects attributed to Cd^{2+} (see Chapter 2), it becomes apparent that a single mechanism is unlikely to underlie the effects of Cd^{2+} on the genotoxicity of other compounds. The cation would disturb cellular metabolism in a number of ways, some with a great and others with a lesser influence on the genotoxicity of another chemical. Two major mechanisms by which Cd^{2+} could alter the genetic effects of other compounds have been investigated in this study. (i) cellular uptake and subcellular

distribution of Cd^{2+} and, in the case of Ni^{2+}, the other mutagen, (ii) DNA repair.

Only one consistent difference between the mutagen or Cd^{2+} alone treatments and the combined treatments was detected in the uptake and subcellular distribution work. The amount of Cd^{2+} bound to the low molecular weight Cd^{2+} binding molecule was less in co-treated cells than in cells treated with Cd^{2+} alone. As there was no increase in binding to other cytosol molecules, the free Cd^{2+} concentration was probably greater in co-treated cells. Considering the arguments put forward in the discussion of this data in Chapter Six and that Cd^{2+} co-treatment had opposite effects on Ni^{2+} and EMS induced SCE, it is unlikely that elevated cytosolic free Cd^{2+} has any effect on SCE induction. However, the effect of the cation on Ni^{2+} and EMS induced chromosomal aberration was similar. Therefore, the presence of high levels of free Cd^{2+} may be of importance in chromosomal aberration induction. At this point it is worth recalling the effect of a short Cd^{2+} treatment followed by a recovery period. The effect of these treatments was to induce chromosomal aberrations but not SCEs. If the temporal trend of increasing percentage cytosol Cd^{2+} binding to glutathione-like molecule was dependent on the continued presence of the cation, a short treatment would not lead to its production. Thus, there may be a high percentage of free Cd^{2+} in the cytosols of cells treated in this way. If this is true (it has not been investigated), the correlation between high cytosolic free Cd^{2+} content and chromosomal aberration induction would hold.

One other observation may be explicable in terms of uptake and subcellular distrubution, namely the opposite effects of co-treatment and pre-treatment with Cd^{2+} on Ni^{2+}-induced SCE and chromosomal aberration. If the low molecular weight Cd^{2+} binding molecule is not specific for Cd^{2+} (and if it is glutathione, it would not be), a proportion of a Ni^{2+} dose following a Cd^{2+} pretreatment may bind to the molecule. In this way other cellular targets may be protected. A similar mechanism could account for a reduction in toxicity of any molecule able to bind to the glutathione-like molecule after Cd^{2+} pretreatment. As explained

earlier (Section 6.6), alkylating agents may be able to bind to the glutathione-like molecule.

A more consistent correlation between the level of DNA repair in cells treated with Cd^{2+} and another genotoxin and chromosomal alteration (SCE in particular) has been found. Co-treatment with Cd^{2+} seemed to inhibit repair in H33258 and Ni^{2+} treated cells but synergize repair in cells exposed to alkylating agents. The inhibition of Ni^{2+} and H33258-induced repair could be due to the inactivation of repair enzymes due to the binding of Cd^{2+}. A restriction in the energy available for repair would have a similar effect;therefore, mitochondrial damage could be indirectly affecting DNA repair. If a reduction in energy supply were important in these interactions, then repair of alkylating agent induced damage should also be inhibited by Cd^{2+} co-treatment. However, this was not so, hence it is more likely that Cd^{2+} inactivated normal repair systems but induced a pathway for the repair of alkylating agent (and presumably Cd^{2+}) induced DNA damage.

9.4 The effects of DNA binding agents on Cd^{2+} resistant muntjac cells

The only information available in the published literature concerning the effect of other compounds on Cd^{2+} resistant cells deals with the cytotoxicity of alkylating agents used in cancer chemotherapy (Bakka *et al*, 1981; Tobey *et al*., 1982a, b, 1983; Endressen *et al*., 1983) and other metal ions (Evans *et al*., 1983). Most of this work involves the use of Cd^{2+} resistant CHO cells which, when grown in the absence of any extraneous metal, retain their ability to rapidly synthesize metallothionein in response to Cd^{2+} of Zn^{2+} treatment. Such cells grown in the absence of Zn^{2+} are more sensitive to killing by melphalan than the parental CHO cells. When metallothionein synthesis was induced by incubation in the presence of zinc ions for 9 hours then exposed to the alkylating agent, cytotoxicity was less than in non-induced cells. However, the relative protection seen in the various cell lines did not correlate with the extent of metallothionein synthesis. Also, parental cells, which synthesized very little metallothionein

in response to zinc, were protected by the pretreatment to the greatest extent. A better correlation was found between cellular glutathione content and alkylating agent resistance. A line of Cd^{2+} resistant CHO cells has been found to be sixfold more resistant to mercury ions and 1.2 - 2.2-fold more resistant to cobalt, zinc and copper ions than the parental cell line. Neither the extent of metal uptake or metallothionen protection showed a strong correlation with the increased tolerance of the Cd^{2+} resistant cells. These experiments demonstrate that the increased resistance of Cd^{2+} resistant CHO cells to other compounds is probably not due to metallothionein production, but conjugation to glutathione may be involved.

In the present study it was found that of the five compounds tested in CR5 muntjac cells, three (EMS, MMC and H33258) elevated the incidence of SCEs. The absolute SCE frequency in CR5 cells treated with all five of the other genotoxins, with the exception of AO, was higher than in normal cells given the same treatment. This suggests that persons continually exposed to high levels of Cd^{2+} would be more prone to tumor induction by several classes of chemical. As untreated CR5 cells displayed a higher frequency of SCE than untreated normal cells, the absolute SCE frequency induced by another compound tells nothing of the sensitivity of the cells to the other compounds *per se*. Comparison on the basis of the ratio of SCE frequency in treated cells to baseline SCE frequency showed CR5 cells to be less sensitive than the parental cells to SCE induction by all of the compounds tested. Also, the level of UDS in treated CR5 cells was less than in normal cells treated with the same chemical. Therefore, it is probable that less DNA damage (rather than less repair of a similar amount of damage) was caused by the compounds in CR5 cells. A mechanism similar to that suggested to account for some of the effects of Cd^{2+} pretreatment may be involved in the reduced sensitivity of CR5 cells to other genotoxins. As the CR5 cells contained a higher level of glutathione-like molecule, a greater proportion of the treatment chemical may bind to this species in the cytoplasm. Thus, the amount of compound available to react with nuclear components would be reduced. It is interesting to note the similarity of this mechanism to that suggested by Seagrave *et al* (1983) to account for the Zn^{2+} induced reduction in alkylating agent toxicity.

9.5 Conclusions

Cadmium (II) ions were found to be cytotoxic to muntjac cells, but SCEs and chromosomal aberrations were not induced by 48 hour continuous treatments with the metal ions. Chromosomal aberrations were only induced by a short Cd^{2+} treatment followed by a recovery period of 17 or 48 hours. The cytotoxicity of the cation could be overcome by long term culture in low concentrations of Cd^{2+}, subsequent challenge to 50 - 90% lethal doses and growth survivors. In this way a Cd^{2+} resistant variant of muntjac cells was produced which was able to grow in 5 μm Cd^{2+}. The mechanism of this resistance may involve the production of a low molecular weight, glutathione-like Cd^{2+} binding molecule and/or the production of extra lipid as shown by myelin figure formation.

The study of interactions between mutagens and environmental contaminants is still in its infancy. Data presented in this thesis demonstrated that Cd^{2+} is capable of altering the genotoxic effects of other DNA binding agents in normal muntjac cells. The nature and extent of the interactions revealed is dependent upon the DNA binding agent considered, the exposure protocol and, in some cases, the assay employed. Cd^{2+} resistant cells, which were used as a model of the cells of persons exposed to high levels of the cation over extended periods, seemed less sensitive than normal cells to SCE induction by DNA binding agents. However, as they had a higher baseline SCE frequency, the treated Cd^{2+} resistant cells had higher absolute SCE frequencies than normal cells exposed to the same chemical. So implying that persons occupationally exposed to the metal ion might be more likely to develop tumors than the population in general. These findings serve to highlight some of the shortcomings of testing single compounds in genotoxicity assays.

Given the wide variety of cellular effects attributed to Cd^{2+}. The basis of the Cd^{2+}/genotoxin interactions is probably complex, involving several biochemical and cellular processes. Experimental work has suggested an involvement of DNA repair systems in some types of interaction and binding of genotoxins to a cytosolic glutathione-like molecule in others.

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