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UNIVERSITY OF SOUTHAMPTON

STUDIES ON CADMIUM TOXICITY IN *AMOEBA PROTEUS*

Thesis submitted for the degree of Doctor of Philosophy by Ghada R. Al-Atia.

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UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF SCIENCE

BIOLOGY

Doctor of Philosophy

STUDIES ON CADMIUM TOXICITY IN *AMOEBA PROTEUS*

By Ghada R. Al-Atia.

In this thesis, cell survival, biochemical and electron microscopical techniques have been employed in the elucidation of the toxic effects of Cd^{+} on *A. proteus*. The toxicity demonstrated by lethality and ultrastructural changes was found to be time and concentration dependent.

Amoebae were shown to be capable of accumulating Cd^{+} to levels far greater than those of the surrounding media. Cd uptake was found to be of a biphasic pattern, i.e. initial rapid uptake - plateau - slow uptake - plateau. On return to Cd^{+} free medium, the Cd treated amoeba were shown to retain about 50% of the total Cd taken up. Within the subcellular fractions from Cd treated amoebae, a high proportion of Cd was always associated with the cytosol fraction, whereas, in the nuclear fraction there was an initial high proportion which then fell as the proportions in the microsomal and cytosol fractions continued to increase. Pretreatment of amoebae with a low dose of Cd induced a short-lived protection, of unknown nature, against a subsequent higher dose of Cd .

Gel filtration of the cytosol fractions demonstrated that Cd was associated with two major peaks of Cd-binding proteins, Peak I at > 45,000 MW and peak II at 10,000-12,000 MW. Cd treatment was shown to cause either a great increase in, or an induction of, the synthesis of peak II Cd-binding protein. Added cysteine increased the Cd incorporated into both peaks I and II but it caused a disproportionate increase in that incorporated into peak II. The parallels between peak II Cd-binding protein and the mammalian metallothioneins are discussed.

E.M. examination of Cd treated amoebae revealed extensive damage to the mitochondria, an increase of free detached ribosomes combined with an increase in the smooth membranes, and an increase of lipid found at particular doses.

Continuous culturing of amoebae in the presence of low concentrations of Cd yielded a population of 'Cd-adapted' amoebae. These amoebae demonstrated a recovery in ultrastructural morphology and percent survivals and a change in the pattern of Cd uptake from that of controls.

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Chapter One

General Introduction and Aims of the Research

In general when heavy metals are given to living systems at concentrations above the trace level, they are known to cause some form or another of metabolic disorder or toxicity. Heavy metals such as Fe, Cu, Zn, Co, and Se are examples of essential trace elements. Other heavy metals such as Cd, Hg, and Pb are known to accumulate in and be toxic to the living system. As yet there is no sufficient evidence to indicate any essential function for these elements in living organisms.

The toxic properties of soluble cadmium compounds have been known for a long time (Chizhikov, 1966). However, it is only recently that Cd^{++} has been recognized as a serious environmental contaminant. It is now known that continuous exposure to very low concentrations of Cd over long periods can produce chronic toxicity in man and other organisms.

1.1. Natural occurrence.

In nature cadmium occurs at an average concentration of 0.15-0.2 ppm in the earth's crust, in a fairly uniform distribution. The most common cadmium compounds naturally found in minerals are CdS, CdO, CdSe, and $CdCO_3$. Such minerals are usually found associated with zinc ores which often can contain other heavy metals, e.g. Pb, and Cu. Cd has also been produced as a byproduct from Pb and Cu industries. The proportion of Cd^{++} to Zn found in various ores ranges between 1:100 and 1:1000, (Fleischer et al, 1974, Nordberg 1974, Chizhikov 1966 and Fassett 1975).

The Cd concentration in soil ranges between 0.01 ppm to 7 ppm (Allaway 1968). This variation depends on the physical properties and composition of the soil such as pH, organic material, clay material, and levels of other cations e.g. Ca^{2+} Mg^{2+} Al^{3+}, and Zn^{2+} also on the degree of Cd pollution in the area. Cd mobility in the soil is greatly dependent on the pH value of the soil. In acid peat soil and others of low pH (below 5), it is likely that most of the existing Cd is in the free form Cd^{2+}. In alkaline soils (pH 8 and over) Cd is presumed to exist in complex forms, e.g. $CdCO_3$, $Cd_3(PO_4)_2$ or CdS, and will thus be less mobile (Babich and Stotzky 1978). Cd deposited near the soil surface can work its way down through the soil. Cd levels measured at different depths in smelter-contaminated soil were:-

Depth	Cd concentration
5 cm	44 ppm
10	32
20	6.9
30	1.4
40	0.4 (Fleischer et al 1974)

The adsorption of Cd by soil is also dependant upon the nature of the components. For example, it has been shown that Cd adsorption to different clay materials follows the sequence: vermiculite > illite > montmorillonite > kaolinite (Sweeton and Tamura 1975, cited by Babich and Stotzky 1978 ; Peterson and Alloway 1979).

In nonpolluted waters Cd concentration is usually less than 1 ppb. In rivers Cd is mainly found in sediments (clay and humus), and in suspended particles. The average concentration of Cd in sea water is 0.15 ppb. This low value is probably caused by the precepitation of Cd as insoluble compounds e.g. CdCO, CdS and $Cd_3(PO_4)_2$. In fresh water rivers having a low pH (4-5) a substantial proportion of the total Cd is probably present as free Cd^{2+}. In sea water (pH 8-9) Cd is mainly present as $CdCl_2$ and $Cd(OH)_2$. In mine waters Cd levels can reach up to 42 ppm. In sewage sludges (of 42 sites in England and Wales) Cd level ranged from less than 60 to 1500 ppm, (Fleischen et al 1974; Aylett 1979).

In the atmosphere Cd concentration is usually very low i.e. 0.4-26 ng/m^3. However, higher concentrations (2-~370 ng/m^3) have been measured in the atmosphere of large industrial areas.

Most naturally existing Cd is in an inorganic form. The known organic-Cd compounds, e.g. dimethyl-Cd and diethyl-Cd , are very unstable and are therefore not naturally available (Fleischer et al 1974, Babich and Stotzky 1978).

1.2. Sources of Cd pollution in the environment.

Industry and other activities of man are primarily responsible for the accumulation and alteration of Cd distribution in the environment. Table I gives some examples of industries which directly contribute to environmental Cd pollution.

Table I. Industrial sources of Cd pollution in the environment

Atmospheric pollution	Soil pollution	Water pollution
Mining & ore processing	Atmospheric deposition	Atmospheric deposition
Smelting	Mine water wastes	Mine water wastes
Pigment manufacture	Sewage sludge fertilizers	Sewage treatment effluents
Battery manufacture		
Electroplating	Fungicides and pesticides	Industrial effluents
Alloy manufacturing		
Fertilizer manufacturing		
Fossil fuel combustion		
Wear of automobile tyres		
Pesticides		
Steel works.		

Fleischer et al (1974), Fridberg et al (1971).

1.3. Some chemical properties.

The metal cadmium is a bluish silver-white, cadmium vapour is orange-yellow in colour. Cd falls into group IIB of the periodic table, with an atomic weight of 112.4, atomic number of 48, specific gravity 8.604 (cast Cd) and 8.690 (wrought Cd). At absolute zero Cd gravity is 9.25 g/c.c. There are 8 naturally existing Cd^{2+} isotopes which vary in their abundancy. Cd dissolves in Hg forming an amalgam. The solubility of Cd in Hg is 3.3% at $0^{o}C$. Cd in its compounds is usually present as a divalent cation Cd^{2+} and rarely as a trivalent cation Cd^{3+}. In its ionic form Cd^{2+} can enter several chemical reactions, e.g. incorporation into minerals, accumulation and complexing in biological systems, chelation reactions, and formation of soluble compounds or insoluble precipitants, (Aylett 1979).

$CdSO_4$ has an antiseptic property and is used in treatments of certain eye diseases. Cd-salicylate and Cd-acetate are used as external antiseptics. CdI_2, $CdBr_2$, and $CdCl_2$ are used in photography (films and flashes). (Fleischer et al, 1974 and Chizhikov 1966).

1.4. Biochemical aspects of Cd .

The toxic effects of Cd can be demonstrated in vivo and in vitro. In which Cd can alter the activity i.e. either activate, or inhibit, a large number of enzymes, in particular metal dependant enzymes, Table II. This is primarily due to cadmium's close association with essential cations, such as Zn^{2+}, Ca^{2+}, and Fe^{3+}, which form a functional and/or a structural part of many enzymes.

Table II. Enzymic activites affected by Cd (Vallee & Ulmer 1972).

a) Enhanced activities

Enzyme	Source
Acid phosphatase	Rat liver
Adenosine triphosphatase	Fowl RBC
Alanyl leucine dipeptidase	Bacterial
Arginase	Yeast
Carboxypeptidase A (esterase)	Bovine pancreas
Carboxypeptidase B (esterase)	Swine pancreas
Cholinesterase	Rat brain, spleen, heart, kidney
Glucose oxidase	Bacterial
Glucose 6-phosphate dehydrogenase	Mouse liver, and heart
Oxaloacetate decarboxylase	Bacterial
Prolidase	Swine kidney
Pyruvate dehydrogenase	Bacterial
Pyruvate decarboxylase	Wheat germ; yeast

Table II. (Cont.)

b) Inhibited activities

Enzyme	Source
Acid phosphatase	Canine liver
Adenosine triphospatase	Rat liver; rabbit muscle
Alcohol dehydrogenase	Equine liver
Alkaline phosphatase	Calf duodenum, E. coli, rat liver, kidney, and testis
Amylase	Bacterial
Carboxypeptidase A (peptidase)	Bovine pancreas
Carboxypeptidase B (peptidase)	Swine pancreas
Catalase	Chicken liver
Cytochrome oxidase	Rat liver mitochondria
Glycerol phosphate dehydrogenase	Rabbit muscle
Glycyl-glycine dipeptidase	Fish gut
Isocitrate lyase	Yeast
Phosphoribonuclease	Spinach leaves
Plasmin	Human serum
Proteinase	Bacterial
Prolidase	Swine kidney
Urease	Soybean

Between the common ligands, oxygen, nitrogen, and sulphur, Cd^{2+} binds most firmly to the sulphur ligand. Often the metal binding sites of metalloenzymes contain-SH ligand from which Cd^{2+} can displace most of the essential cations.

In the mitochondria of mammals and other organisms, Cd can uncouple the oxidation phosphorylation reactions. It does so by interfering with essential components of the enzymic reactions; for example by interfering with Ca which acts as a catalyst in these reactions. Cd can also affect the structural integrity of the mitochondria; for example Cd can displace Zn which is known to form a functional as well as an integral part of enzymes and membranes in the mitochondria. The uncoupling of the oxidation phosphorylation reactions by Cd is usually followed by swelling of the mitochondria and loss of respiratory control, and a temporary increase in respiration. The swelling of the mitochondria had been considered as a secondary effect of the uncoupling reaction (Fluharty & Sanadi, 1962, Jacobs et al, 1956; Diamond & Kench 1974).

1.4.1. Cd^{2+} interaction with some essential cations.

Calcium.

Cd^{2+} has an ionic radius similar to that of Ca^{2+} and thus can interfere with Ca metabolic functions. Several studies on a wide range of organisms have reported the interaction between Cd and Ca. The accumulated Cd level in the whole body and particularly in the haemolymph and gills of the crab Carcinus maenus was found to be

inversely related to the Ca level in the surrounding medium (Wright 1977c).

Rats maintained on low Ca diet accumulated 50% more Cd than rats which were maintained on normal Ca diet given the same dose of Cd (Friberg et al, 1971). Ando et al (1978), have reported that in Cd treated rats there was an increased fecal and urinary excretion of Ca , a higher Ca level in the blood, and a low gastrointestinal absorption of Ca . Also the Ca level and weight in bone decreased with time during exposure to Cd .

In vitro Cd was found to reduce the reactivity of the vascular smooth muscles. Adding Cd (0.5 mM) to isolated rabbit aorta, ventricles and atria, caused a decrease in the Ca content and an increase in the Cd content. Aortic contractions induced by K^+ or noradrenaline are attenuated by decreasing the Ca level. Low concentrations of Cd (0.02 and 0.1 mM) supplied in the bathing medium caused greater reduction in the tissue response to the K^+-induced contraction than that of the noradrenaline-induced contraction. However, Cd supplied at a concentration of 0.5 mM greatly reduced both the K^+ and the noradrenaline induced contractions. The addition of cysteine (1 mM) prevented or reduced the Cd effect (Toda et al 1975).

In brain tissues of mammals Ca is of importance in controlling membrane permeability and excitability. Peyton and Borowitz (1978), have reported that 0.5 h after mice were injected, i.p. with a low dose of Cd (1.1 mg/kg), the Ca level in the brain mitochondria

had increased while the Cd level in the brain tissue was low. 24 h after Cd administration there was an overall increase in Ca level in all the cellular fractions of the brain tissue and an elevation in the electroshock threshold. Between 0.5 h and 24 h from Cd administration the total Cd level in the brain tissue did not change, though the Cd value in the crude nuclear fraction was significantly higher at 0.5 h than it was at 24 h. It appears that low levels of Cd may have changed the membrane permeability to Ca. Berlin and Ullberg (1963), conducted an autoradiographic study on the fate of a single intravenous ^{109}Cd injection in mice. Whereas Cd appeared in many tissues in a matter of hours it was 16 days before an accumulation of Cd was detected in the brain tissue.

Zinc.

Zinc is an essential trace element for most organisms. It forms an integral and/or functional part of a large number of enzymes; such as some dehydrogenases, aldolases, peptidases, phosphatases, a transphosphorylase, an isomerase and aspartate transcarbamylase. It also has an important role in nucleic acid and protein syntheses and metabolisms (Vallee & Ulmer 1972). In the DNA molecule Zn^{2+} binds to the phosphate groups and aids in controlling the stability of the DNA structure. Zn^{2+} has also been found associated with the RNA of various cells, e.g. Euglena gracilis (Falchuk et al 1975; Eichhorn & Shin 1968; Slater et al 1971).

Zn deficiency in man and other mammals was found to cause growth retardation, hypogonadism, skin changes and slow healing of

wounds. Zn level was found to be low in cases of sickle cell anaemia and in some tumour tissues (Brewer et al 1976)*. Morphological changes due to Zn deficiency were noticed in the protozoan E. Gracilis (Falchuk et al 1975).

The close association of Cd and Zn in the environment is reflected in living systems. Cd^{2+} affinity for -SH groups is greater than that of Zn^{2+}; therefore Cd^{2+} is found capable of replacing Zn^{2+} in most Zn -associated proteins at such binding sites. The substitution of Zn^{2+} by Cd^{2+} in almost all enzymes results in alterations in their normal metabolic functions. In the enzyme molecule alkaline phosphatase of Escherichia coli the ratio of Zn^{2+}: enzyme is 4:1, two of these sites are believed to be structural and the other two functional. Cd^{2+} can substitute for Zn^{2+} in all 4 sites but the resulting Cd -enzyme (Cd -alkaline phosphatase) is not active (Lazdunski et al 1969). On the other hand, the substitution of Cd^{2+} for Zn^{2+} in horse liver alcohol dehydrogenase results in a more active enzyme than the original Zn -enzyme (Drum & Vallee 1970; Drum et al 1967).

Numerous studies in mammals have shown that treatment which Zn gives some protection against the toxicity of a subsequent Cd dose (Gunn et al 1961, Kar et al 1960 and Webb 1972b). The mechanism of this protection was explained by the production of Zn -thionein (7000 - 10,000 MW) in which Cd^{2+} can subsequently replace the Zn^{2+}. It has also been shown that the presence of Zn increases rather than decreases Cd uptake, but that the combined effect is less toxic (Gunn et al 1968 a, b & c).

* Cited by Prasad (1976).

Selenium

In mammals Se has been shown to be most protective against Cd toxicity, especially when given at a ratio of 2:1, Se to Cd . Se treatment produced a remarkable increase in Cd uptake by various tissues. For example, in Se -treated mice there was an increase of 150 - 250% in the Cd level of the testis, yet the toxic effect of Cd was greatly reduced (Gunn et al 1968b). In rats given a dose of ^{75}Se 4 weeks prior to ^{109}Cd treatment, gel separation of their testicular cytosol fraction showed that ^{109}Cd was eluted in a major peak of 34,000 MW, whereas ^{75}Se was eluted at a major peak of 140,000 MW. However, when the rats were given the ^{75}Se dose only 30 min prior to ^{109}Cd treatment, there was a marked increase in the Cd level of the cytosol fraction and both Cd and Se were eluted in one major peak of 110,000 MW. Though the Se protection mechanism against Cd is not yet fully understood, the shift in the Cd peak suggests a possibly protective mechanism can be induced by Se treatment (Chen et al 1974, 1975a and Prohaska et al 1977).

Iron

In mammals including man chronic Cd poisoning is known to give rise to hypochromic anaemia and microcytosis. In studies carried out on rabbits, Cd poisoning was found to increase the plasma volume with no increase in the erythrocyte number, accelerate the destruction of erythrocytes and causes a reduction in the size of newly formed erythrocytes. In spite of high levels of Cd in the bone marrow and other nearby tissues there was no evidence to suggest that Cd reduced the bone marrow activity with respect to

erythropoiesis. The Cd level in the bone was very low: this was also observed in human body of high Cd burden (Piscator 1976). In Cd -treated rabbits the microscopic examination of the femoral bone marrow did however reveal pronounced morphological changes, such as, degeneration of some connective tissue, hypercellularity, and abnormal fat cells. The hypochromic anaemia caused by Cd poisoning occurred without depletion of, but rather with an increase in the reserves of Fe^{3+} in the bone marrow. This suggests that Cd induced some form of inhibition in the transfer of Fe^{3+} to haemoglobin synthesizing cells. When Cd treated rabbits were given Fe , the bone marrow returned to a near normal appearance: i.e. there was a recovery from hypercellularity, the erythrocytes gained their normal size, the animal recovered from its hypochromic anaemia. However, other organs such as, liver, kidney, and spleen did not respond to Fe treatments (Berlin & Friberg 1960, Berlin et al 1961).

Other workers have reported that long exposures of rats to Cd affected the distribution of Fe^{3+} and other cations, such as, Mg^{2+}, Cu^{2+}, and Zn^{2+}. They found a loss of Fe^{3+} from both liver and kidney, but an increase in the Zn^{2+} level in liver, and the Cu^{2+} level in kidney (Stonard & Webb 1976, Brierley 1967). Irons and Smith (1976), reported that simultaneous administration of Cd and Cu resulted in redistribution of Cd^{2+} in the cytosol fraction of liver. In this case there was an apparent failure of metallothionein to sequester Cd^{2+}, possibly due to the aggregation of metallothionein in the presence of Cu^{2+}.

Copper.

Cu is an essential component of a number of metalloenzymes which are involved in biochemical and physiological functions in a wide range of organisms; e.g. cytochrome oxidase, tyrosinase, dopamine-β-hydroxylase erythrocuprein and caeruloplasmin in higher animals; ascorbic acid oxidase, plastocyanin, and ribulose diphosphate carboxylase in plants; and the oxygen carrier haemocyanin in molluscs and arthropods.

In vertebrates Cu is important in elastin and collagen formation preventing defects such as aneurisms and soft bones. One of the major roles of Cu is its involvement in Fe metabolism and haemoglobin formation. The Cu protein caeruloplasmin (ferroxidase activity) was shown to be directly associated with Fe metabolism in serum, being involved in the formation of FeIII-transferrin which in turn is required for the biosynthesis of haemoglobin and other Fe-utilizing systems. Heavy metals such as Zn and Cd can inhibit the ferroxidase activity of caeruloplasmin (Frieden 1971; Lee et al 1976). Syed et al (1979) have shown in an *in vivo* study on plaice (*Pleuronectes platessa*) that prolonged exposure to 2 ppm Cd produced a biphasic effect on caeruloplasmin activity. An initial increase in activity after 7 days exposure was followed by a fall, and by day 21 this activity was significantly below control values. A drop in the level of serum Cu paralleled the drop in caeruloplasmin activity. Liver Cu-enzymes showed similar biphasic changes in activity, although the Cu level in liver did not decrease. When Cd was added *in vitro* to these enzymes no inhibition was produced suggesting that Cd does not displace Cu in these enzymes and hence the observed *in vivo* effect on these enzymes seems to be indirect.

Cu deficiency causes a rapid fall in serum Cu level followed by a drop in serum Fe and erythrocyte Cu and eventually a dramatic reduction in the red cell volume. It also inhibits the release of Fe from the intestinal mucosa, liver parenchymal cells and the reticulo-endothelial system to the plasma (Frieden 1971).

1.4.2. Cd^{2+} interaction with vitamins.

In vertebrates treated with Cd the level of vitamin A was found to decrease in the serum and increase in the liver. Cd did not affect the rate of vitamin A absorption from the intestine (Sugawara & Sugawara 1978).

Vitamin D was shown to facilitate the absorption of Cd from the intestine, rachitic chicken treated with vitamin D has shown a marked increase in Cd uptake from that shown by untreated chicken (Friberg et al 1971).

Vitamin C was found to partially overcome the toxicity of Cd in the testis of young chicks by increasing the testicular alkaline phosphatase activity (Weber & Reid 1969, 1974). Its protective effect was also demonstrated in young coturnix (Fox et al 1971) and rats (Maji & Yoshida 1974).

1.4.3. Cd^{2+} interaction with chelating agents.

Engstrom and Nordberg (1978), reported in a short term study that when chelating agents such as ethylenediaminetetraacetic acid (EDTA), nitrilotri-acetic acid (NTA), or sodiumtripoly-phosphate $Na_5P_3O_{10}$ (STPP), were injected into mice subcutaneously together with Cd^{2+}, the level of Cd taken up by the liver decreased but the toxicity of Cd was markedly increased. In a long term study, when these combinations were given orally, no changes in Cd toxicity was noticed.

The presence of EDTA was found to increase Cd uptake and its subsequent concentration within the tissues of the mussel Mytilus edulis. This finding adds support to the hypothesis that Cd does not transport across the cell membrane as a simple hydrated ion but complexed to ligands on a carrier or on the cell membrane (Coombs 1979). In an in vitro experiment the complex Cd-EDTA was found to be incapable of penetrating to the inside of the cell, therefore, it was suggested that the Cd-EDTA complex can readily exchange the EDTA for a ligand to form a Cd-Ligand complex which can then penetrate the cell membrane. Hence, the contamination of aquatic systems with sewage effluents containing EDTA, used in detergents, can aid the uptake and accumulation of Cd by the aquatic animals, especially since EDTA is non-biodegradable. NTA however, is biodegradable therefore its use in detergents does not present the same problem (Coombs 1979).

The presence of EDTA in soil can increase the uptake of Cd by some plants. Therefore, it was recommended that chelators in agriculture should be used cautiously (Chaney & Hornick 1977).

1.4.4. Cd^{2+} interaction with cysteine.

Cysteine treatments were found to protect mammals against Cd^{2+} toxicity (Friberg et al 1971), even though added cysteine was found to increase the level of Cd^{2+} accumulated within the animal tissues (mainly liver and kidney) (Gunn et al 1968 a, b & c). Cysteine has also been shown to increase the level of Cd accumulated by tissue cells in culture - both mammalian and tumour cell lines. Here the added cysteine was found to be incorporated together with Cd into the Cd^{2+} -binding protein, metallothionein, (Hidalgo et al 1978, Rudd & Herschman 1978, 1979).

The protection of cysteine against Cd was also demonstrated in the bacterium Staphylococcus aureus (Tynecka & Zylinska 1974).

1.4.5. Cd^{2+} interaction with the nucleic acids.

Some heavy metals will bind to the DNA and RNA molecules of a wide variety of organisms (Sissoëff et al 1976). These metal ions are not bound at random to the DNA molecule; but are found at two particular sites on the DNA molecule: (Eichhorn & Shin 1968).

a) the phosphate residues forming the back bone of each DNA chain,

b) the bases which maintain the two DNA strands in register.

Ions such as Ca^{2+} and Mg^{2+} which bind exclusively to the phosphate groups are believed to increase the melting temperature of the DNA molecule and so increase its stability. Metal ions such as Ag^{2+} and Hg^{2+} which form co-ordination bonds exclusively with electron donor nucleotide bases decrease the melting temperature of the DNA

molecule and so reduce its stability. Metal ions capable of binding to both phosphate groups and bases can produce different effects on the DNA stability according to their relative affinities for these two sites. Examples of such ions are given according to their increasing affinity to base sites in relation to phosphate sites: $Co^{2+} = Ni^{2+} > Zn^{2+} > Mn^{2+} > Cd^{2+} > Pb^{2+} > Cu^{2+}$ (Eichhorn & Shin 1968, Izette et al 1971). Cd^{2+} has been shown to interact with RNA and DNA and most metalloenzymes (e.g. Zn -enzymes) which are associated with mitotic and meiotic mechanisms (Sissoëff et al 1976).

Cd was shown to have a biphasic effect (a depression followed by a rise) on the incorporation of thymidine into pulmonary DNA of rats. The same pattern was reflected in the level of cyclic AMP in the lung tissue. In the kidney there was a continuous depression of thymidine incorporation into the DNA as well as a reduction in the cyclic AMP at all times (Kascew et al 1976 a & b). Stoll et al (1976), have reported, a biphasic pattern of enhancement and inhibition of RNA synthesis by Cd in the rat liver which was dependant on dose and time and appeared to correlate with the hepatic concentration of the Cd . They have also shown in their vitro system that Cd at low concentration (10^{-8} M) increased the m RNA activity to incorporate labelled phenyl-alanine into microsomal protein; at higher concentration (10^{-6} M and over) Cd decreased the m RNA activity for this reaction. They have also shown that while Cd at 10^{-3} M did not significantly alter the DNA melting temperature, it did increase the U.V. absorbance at 260 nm which is suggesting a more complete unwinding of the DNA molecule.

Heath and Webb (1967), demonstrated the ability of Cd and other cations to induce tumour formation in mammals at the site of injection. The Cd content of the tumour tissue was considerably lower than that of the liver, kidney and spleen, and had a quite different intracellular distribution. 15% of the Cd of the tumour tissue was associated with the cytosol fraction; a small quantity was found in the mitochondria, with very little in the microsomes; most of the remaining Cd was associated with the nuclear fraction. The Cd content in the mixed nucleic acids obtained from the tumour tissue was 50-260 µg Cd/g. The Cd content of the DNA was 52 µg Cd/g. Other workers have demonstrated that a Cd dose of 0.17 to 0.34 mg given as a single subcutaneous injection to rats can produce pleomorphic sarcomas at the injection site. However, these doses were only found to be carcinogenic in tissues of mesenchymal mesodermal origins. No tumours were formed when the injections were given at ectodermal, endodermal or epithelial mesodermal sites, nor when Cd was administered orally (Gunn et al 1967, Haddow et al 1964, WHO 1977). In tissue cultures of mammalian cell lines, Cd has been shown to exert some cytogenetic effects such as, depression of mitotic activities, changes in the chromosomal structure inhibition in proliferation and the induction of strong stickness of the chromosomes (Rohr & Bauchinger 1976). These results all suggest that Cd could be a carcinogenic agent under certain conditions. However, such a conclusion is still much disputed, (Samarawickrama 1979).

1.4.6. Metallothionein.

Metallothioneins are proteins induced in mammalian tissues (e.g. liver and kidney) by certain metal ions such as Zn^{2+}, Cd^{2+}, Hg^{2+},

or Cu^{2+} (Piotrowski et al 1974). Different heavy metal ions have different affinities to metallothionein and they can substitute for each other in the protein molecule according to their affinities, e.g. $Hg^{2+} > Cd^{2+} > Zn^{2+}$.

Cd^{2+} is believed to be the most efficient of the metal ions in inducing the synthesis of a metalloprotein. Cd -thionein was first isolated by Margoshes and Vallee (1957) and characterised by Kagi and Vallee (1960, 1961). It had a MW of 6,600 - 11,000 and contained 5.9% Cd , 2.2% Zn , and Cu and Fe in far smaller proportions. It was devoid of aromatic amino acids but had a high cysteine residue content of 28% to 32%. The U.V. wavelength which gave the highest absorbance was 254 nm; this was shown to be due to the Cd -mercaptide bond. Since the first isolation and characterisation of Cd^{2+}-thionein in the late 1950's similar Cd -thioneins have been isolated from other mammalian tissues including those of man (Pulido et al 1966) and other vertebrates such as chicken (Weser et al 1973 a & b). Other metallothioneins have also been isolated e.g. Hg -thionein from fish (Bouquegneau et al 1975, also see Webb 1979a).

Cd -thionein separated from several mammalian tissues was found to consist of more than one variety of Cd-binding protein which have similar molecular sizes but were slightly different in their amino acid and metal composition; e.g. equine kidney cortex (Kojima et al 1976) rat liver (Shaikh & Lucis 1971), rabbit liver (Nordberg et al 1972), and mouse liver (Leber & Miya 1976). Differences have also been observed between the Cd -thionein obtained from different tissues of the same animal; e.g. liver or kidney (Webb & Daniel 1975 and Webb 1975; Webb 1979a).

1.4.6.1. The course of Cd-thionein production.

A number of workers have studied the course of Cd-thionein production in mammals, and in general a similar overall pattern has been demonstrated. In rats a single dose of Cd (below 1.5 mg/kg) injected subcutaneously results in the following sequence of events in the liver. During the 1-2 h following adminstration, the Cd^{2+} binds to high MW proteins in the liver cytosol fraction, as well as to other cellular components, e.g. nucleus and mitochondria; the level of metallothionein (Cd-thionein) increased rapidly; the Cd^{2+} is transferred from the high MW proteins to the newly produced low MW thionein- during this period Cd^{2+} may be removed from other cellular components, e.g. the nucleus and mitochondria, and transferred to the thionein of the cytosol fraction (Kapoor et al 1961; Bryan & Hidalgo 1976; Frazier & Puglese 1978; Tanaka et al 1974). A similar pattern of events has been observed in mice after a subcutaneous injection (Nordberg et al 1971 a). In rats, when the single dose injected subcutaneously was increased to over 1.5 mg/kg the synthesis of metallothionein was inhibited and the Cd remained bound to the high MW proteins of the cytosol fraction (Webb 1975). High Cd doses even when below the critical level at which metallothionein production is inhibited can produce a slower uptake and incorporation of Cd^{2+} by the rat liver and kidney (Shaikh & Smith 1976, 1977; Webb 1979 a & b; Squibb & Cousins 1974).

Though the general pattern of Cd^{2+} incorporation into various proteins and cellular components is similar in mammals, this pattern can vary between different tissues of the same animal e.g. liver or kidney. Also differences are evident in the findings of different workers regarding the length of time between the administration of

Cd and the production of Cd-thionein (lag phase), the time when maximum Cd^{2+} incorporation into thionein is reached and the half life of Cd-thionein in different mammalian tissues. Once Cd-thionein synthesis has been induced, further exposure to Cd during the period of Cd-thionein production will maintain this synthesis without the appearance of the lag phases, (Shaikh & Smith 1977; Cempel & Webb 1976; Webb 1979a). The production of Cd-thionein in response to Cd^{2+} has been reported in several mammalian tissues (Chen & Ganther 1975), also for some mammalian cell lines and some tumour cell lines in tissue culture (Rugstad& Norseth 1975, 1978; Rudd & Hershman 1978, 1979 and Hidalgo et al 1978). Several authors have shown that the mechanism involving the synthesis of metallothioneins also involves the de-novo synthesis of a short lived thionein mRNA. The metallothionein synthesis appears to be regulated by changes in the pool of translatable thionein mRNA (Squibb & Cousins 1977; Squibb et al 1977; Shapiro et al 1978; Andersen & Weser 1978).

1.4.6.2. The function of metallothionein.

It has been shown in various mammals that a pretreatment with a low dose of Cd or Zn protects the animal against a subsequent normally lethal dose of Cd , or even against exposure to other cations such as Hg^{2+} (Leber & Miya 1976, Magos et al 1974 and Webb & Magos 1976). Several workers have attributed this protection to the production of metallothionein (Tarhaar et al 1965 and Friberg et al 1971). In rats the increased tolerance produced by a low dose of Cd^{2+} was maximal during 1 - 3 days after a pretreatment dose but it then decreased with time, despite the Cd-thionein synthesis and content being maintained high for longer than the 1 - 3 days period. Further the intravenous injection of Cd-thionein and in particular hepatic Cd-thionein has been shown to be 7 to 8 times more toxic than the equivalent dose of the ionic Cd^{2+} (Webb & Etienne 1977). A similar effect was also found in mice (Nordberg et al 1975). The administration

of Zn-thionein has been shown to protect against a lethal dose of Cd in fed rats, but has failed to do so in starved rats (Webb & Verschoyle 1976). Thus neither the presence of Cd-thionein nor of Zn-thionein is adequate to explain the induced protection (Webb 1979 c).

1.4.6.3. Cd-binding proteins of invertebrates.

The production of Cd-binding protein is not limited to vertebrates. Some invertebrates have also been shown to synthesize similar proteins. In the mussel Mytilus edulis, Noel-Lambot (1975) found that Cd^{2+} induced the synthesis of a Cd-binding protein which had similar properties to the Cd-thionein of mammals. In the blue-green alga Anacystis nidulans, Maclean et al (1972) observed the production of a Cd-binding protein in response to Cd^{2+} which was chromatographically similar to the Cd-thionein produced in mammals.

The presence of Cd-binding proteins of low MW, containing -SH groups in a wide variety of invertebrates has been claimed by several authors; e.g. the crabs Scylla serrata, Cancer magister, the shrimp Acetes sibogae, the chiton Cryptochiton stelleri (Olafson et al 1979 a & b); the limpets Patella vulgata, and Patella intermedia (Howard & Nickless 1977) and the periwinkle Littorina littorea (Noel-Lambot et al 1978).

Coombs (1979) has listed the relative distribution of Cd within the different tissues of various aquatic organisms (invertebrates and vertebrates), he also gives the subcellular distribution of Cd (as a percentage of the total whole tissue) in the kidney homogenate of the mussel Mytilus edulis to be 2% in the nuclear fraction, 14% in the mitochondria, 4% in the microsomes and 80% in the cytosol fraction, and for the whole soft tissue homogenate of the oyster Crassostrea gigas to be 41%, 11%, 3% and 45% respectively.

Webb (1975), has suggested that the production of metallothioneins can be both organ and species specific. Maclean et al (1972), have suggested that the production of metallothioneins could be a matter of "evolutionary antiquity". What ever the case may be, cadmium has been found to accumulate in and the Cd-binding proteins to be synthesized in a wide variety of organisms. The capability for metalloprotein production cannot yet be limited to any particular group of organisms or cell types.

1.5. Toxicity of Cd in man.

In man, as in other mammals the liver and kidney are the main

organs in which Cd is accumulated. Acute Cd poisoning, or the ingestion of 10 mg of Cd , can cause the following symptoms: vomiting, servere gastrointestinal irritation resembling that of food poisoning, diarrhoea, headache, metallic taste, muscular aches, salivation, abdominal pain, loss of weight, rapid pulse, brown urine. A concentration of 5 mg/m^3 of Cd in air if inhaled over a period of 8 h is an estimate of a lethal dose for man. 1 mg/m^3 of Cd in air inhaled over 8 h can give the following symptoms: metallic taste dry mouth, pain in chest, dyspnoea, cough with frothy or haemorrhagic sputum and muscular weakness. In chronic Cd poisoning loss of smell sensation and yellow teeth can be observed in addition to some of the above indicated symptoms. A suggested treatment against an ingested toxic dose of Cd is : the taking by mouth of milk, eggs, or sodium sulphate (30 g/250 ml of warm water); also an early gastric lavage (Cooper 1974). Chronic Cd poisoning may be responsible for some cases of hypertension, since one form of hypertension is usually found to be associated with the accumulation of Cd in kidney (Schroeder & Vinton 1962), Schroeder 1967). The data available on the induction of hypertension by Cd is greatly influenced by different strain susceptability and Na^+ uptake (Friberg 1977), but in rats it has been shown that Cd accumulated in the kidney can cause constriction of (thickening of walls and narrowing of lumen) small and medium renal arteries, and mild dilation of the large renal arteries (Fowler et al 1975). As there are no reports of hypertension in Cd exposed workers, this matter requires further evaluation.

When the body is exposed to low doses of Cd most of the Cd is retained in the kidney and very little is found to be excreted in

the urine. Bearing in mind that the quantity of Cd excreted is dependant on age and subject to high individual variability the finding of a high level of Cd in the urine could indicate either:

a) Recent exposure to a high dose of Cd resulting in a high body burden.

b) Renal dysfunction i.e. either in its own right or more probably as a result of Cd^{2+} poisoning.

The kidney can accumulate 100-300 µg Cd/g wet weight of tissue before any symptoms of renal dysfunction appear. Renal dysfunction is first indicated by protein urea, followed by amino acid urea, then the appearance of glucose, phosphate and other cations in urine. The latter can result in resorption of minerals from bones and could also cause kidney stones, a common feature in Cd -exposed workers.

The bone disease Itai Itai, was first recognized in Japan and then in some French and British Cd exposed workers. Reports suggested that it is caused by Cd poisoning. The occurence of this disease is enhanced by poor diet particularly diets with low protein, Ca^{2+} and vitamin B contents. Itai Itai disease is especially prevalent in women over 45 years of age who have had several pregnancies. The pathological symptoms of this disease are dominated by pain in the back and legs, multiple bone fractures, skeletal deformation resulting in a marked decrease of body height (Friberg et al 1971; Lauwerys 1979; Piscator 1976).

1.6. Summary of the major aspects of Cd toxicity in vertebrates.

Most of the studies on the toxic effects of Cd have been carried out on mammals, but, similar effects have been observed in other vertebrates. About 75% of the administered Cd dose is

retained in the body, mainly in liver and kidney, with approximately one third of this being in the kidney. Considerable accumulation of Cd also takes place in pancreas, salivery glands and spleen. Usually only small quantities of Cd are found in bone tissue, muscles and the nervous system. The reproductive organs, especially the testes have been shown to be highly sensitive to Cd. Administered Cd first appears in the blood, mainly the RBC, where most of the Cd is found bound to high MW protein but within 1 h Cd leaves the blood and accumulates in the different tissues i.e. a high Cd level in the blood could indicate very recent exposures (Nordberg et al 1971b). Thereafter, the major organs in which Cd level was found to increase are-first in the liver (1-2 h after administration of Cd). If no further Cd is administered the Cd level in the liver decreases while that in the kidney increases. In the various organs most of the Cd accumulated in the form of Cd-thionein. As described for man, further accumulation of Cd in the kidney can result in renal dysfunction. In chronic cases of Cd poisoning, morphological damages and enzymatic changes can be observed in most organs but in particular the liver and kidney (Feader et al 1977, Fowler et al 1975, Friberg et al 1971, and Nishizumi 1972).

1.7. Could Cd be an essential trace element?

In general Cd has been found to be a non-essential and toxic element to the living system, but Schwarz and Spallholz (1976, 1977) have reported the unusual finding that Cd at extremly low concentrations can improve the growth and general condition of rats. The list of

essential trace elements is still increasing and several more have been added recently e.g. tin, vanadium, silicon, and nickel. The evidence in favour of Cd is still very slight and at the moment Cd must be regarded as non-essential.

1.8. Toxicity of Cd in the invertebrates.

The literature available on Cd toxicity in invertebrates is primarily concerned with the uptake, accumulation, and distribution of Cd among the different tissues of the animals. Throughout it has been shown that these organisms can accumulate Cd to much higher levels than that of their environment. Examples of animals used for such studies are the shore crab Carcinus maenas (Wright a, b 1976), some species of marine crustacea and mollusca (Mullin & Riley 1956, Coughtrey & Martin, 1976, 1977, and James 1977) and the earthworm (Van Hook 1974, also see Coombs 1979).

Noel-Lambot (1975), has studied the distribution of Cd within homogenates of the mussel Mytilus edulis. Cd^{2+} was found principally bound to a low MW protein of high cysteine content which was synthesized in response to the Cd^{2+}. Thus a similarity to mammalian metallothionein and its production was established. Bubel (1976) has shown that Cd causes some cellular and ultrastructural damage in the gills of Jaera nordmani (Rathke) (Crustacea, Isopoda). He found some swollen and extensively damaged mitochondria together with apparently normal ones.

In the damaged mitochondria, the matrix was less dense, diffuse and flocculated, and that near disintegration, it became more electon-transparent.

1.9. Effect of Cd on plants.

Various plant tissues were found capable of accumulating Cd to much higher levels than those of the soil solutions. For example, corn seedlings grown in 0.1 ppm Cd^{2+} containing solution accumulated 90 ppm Cd^{2+} in their leaves; turnip plants grown in 1.0 ppm Cd^{2+} containing solution accumulated 469 ppm Cd^{2+} in their leaves (Page et al 1972).

Autoradiographic studies have shown that Cd is accumulated mainly in the roots, the vascular tissues of the stem and the leaves. The minimum level of Cd is found in the seeds and grains. The sub-cellular distribution of Cd within plant cells has indicated that Cd^{2+} is associated with a wide variety of macromolecules and also with the cell wall fragments. In soybeans 75% of the total Cd was found to be associated with the soluble protein fraction (Casterline & Yip 1975; and Peterson & Alloway 1979). Plants grown in Cd polluted areas such as industrial and metal smelting areas have been shown to contain much higher levels of Cd than those grown in unpolluted areas. In general plants must accumulate a considerable concentration of Cd before showing any visible injury. Injuries due to Cd toxicity in plants are mainly identified as retardation in growth, red-brown colouration of the leaf margins and veins, root damage, wilting and a decrease in photosynthesis (Page et al 1972; Alloway 1968; Babich & Stotzky 1978).

A major source of Cd accumulation in plants and food stuff is the use of phosphate fertilizers, sewage sludge, and pesticides, all of which can contain Cd in the treatment of agricultural soils. The chemical form of Cd available in the soil, water, fertilisers and pesticides has a great importance in determining the degree of Cd accumulation in plants; e.g. the relatively high solubility of $CdCl_2$ over that of CdO has accounted for the higher uptake of Cd by plants exposed to the chloride form of Cd^{2+}. Other soil properties such as pH, temperature, clay and mineral composition, Ca^{2+} content, aeration, and organic matter can influence Cd uptake by plants (Lagerwerff & Brower 1972; Lagerwerff 1971; Peterson & Alloway 1979).

1.10. Effect of Cd on microorganisms and fungi.

Microorganisms form an integral part in most food chains. Their presence in a wide variety of habitats, e.g. atmosphere, fresh and marine waters, biological matter and soil, enables them to play an important role in the circulation and redistribution of inorganic elements in the environment. They have been shown to accumulate environmental pollutants such as Cd to high concentrations, thus bringing an access of such non-essential toxic elements into the food chains and consequently into higher organisms. It is clearly most important to investigate and understand the interactions that take place with toxic elements, e.g. interactions between microorganisms - toxic pollutants, microorganisms - microorganisms, and microorganisms - higher organisms.

1.10.1. Fungi.

Cd is widely used in fungicides. Various degrees of fungal adaptation (physiological and genetical) to Cd^{2+} and other heavy metal ions such as Cu^{2+}, Pb^{2+} and Zn^{2+} have been reported by several

workers (Ashida 1965). Cd tolerance in fungi is species specific. It occurs over a wide range of Cd concentrations from 10 to 1000 ppm. For example strain of Fusarium oxysporum grown in a 200 ppm Cd^{2+} containing medium accumulated up to 14,300 ppm Cd^{2+} in the mycelia; the same organism was also capable of accumulating other cations such as Cd^{2+}, Zn^{2+} and Pb^{2+} in concentrations much higher than those present in the medium (Hartman 1974, 1975 in Babich & Stotzky 1978). Cd toxicity varies according to the different fungal developmental stages, e.g. mycelial growth is more tolerant to Cd than fruiting body formation. Cd toxicity to fungi is also dependent on other factors; for example Cd toxicity is much higher in alkaline media. Clay materials in growth medium were also shown to inhibit Cd toxicity to fungi (Babich & Stotzky 1977, a, and c ; Cole et al 1968).

1.10.2. Yeast

In yeast the phenomenon of physiological adaptation to Cd is well established. When Saccharomyces ellipsoideus was successively subcultured in Cd containing medium the yeast showed an increased resistance to Cd . However, when the same batch of cells was further successively subcultured in Cd -free medium the yeast lost the tolerance it had gained and returned to its former susceptibility to Cd . A decrease in the levels of intracellular polyphosphates, total nitrogen, and RNA in some strains of yeast appears to reduce the organisms tolerance to Cd (Nakamura & Ashida 1959, Nakamura 1961 a and b).

Cd resistance is species specific in yeast. In some strains it can be genetically controlled (Nakamura 1963). Three distinctive categories of genetically controlled Cd resistant strains have been identified (Middlekauff et al 1956, 1957).

a) yeast which cannot grow in medium containing 50 ppm Cd^{2+}.

b) yeast capable of growing in medium containing up to 500 ppm Cd^{2+}.

c) yeast capable of readily producing mutations which enable them to grow in medium containing up to 250 ppm Cd^{2+}.

Lindegren and Lindegren (1973), has produced a respiratory deficient strain in which the mitochondria were grossly affected by Cd^{2+}.

1.10.3. Bacteria

Cd^{2+} tolerance in bacteria has also been shown to be species specific. In vitro some pseudomonas species have exhibited an unusual response to Cd^{2+}; at Cd^{2+} concentrations lower than 7 ppm the growth rate of the bacteria was reduced but it increased when the Cd^{2+} concentration was increased above 7 ppm; at 28 ppm Cd^{2+} the growth rate was equivalent to that of the control. But over 28 ppm Cd^{2+} and up to 70 ppm the growth rate was even greater than that of the controls (Williams & Wollum 1975). In the bacterium Staphylococcus faecalis, the growth rate was found to be stimulated by low levels of Cd^{2+} (5-10 ppm) In both these examples growth stimulation was attributed to low concentrations of Cd^{2+} at the cell surface inducing changes in the cell membrane permeability which enabled a better flow of nutrients to enter the cell which in turn was believed to result in increased metabolic activities, i.e. Cd was not considered a cellular requirement (Doyle et al 1975).

Ultrastructural changes and inhibition of growth were observed in Escherichia coli over early stages of exposure to Cd , but on further subculturing in Cd containing medium the cells returned to their normal morphology and growth rate. This early period of lag phase was described as an accomodation period. The Cd distribution in the accomodated cells was, 56% of Cd associated with the cell wall, 13% with the cell membrane, and 31% with the cytoplasm whereas, in the unaccomodated cells these values were 2%, 75% and 23% respectively. It was deduced from the change in distribution of Cd^{2+} that during the accomodation period the cells developed some repair mechanism as well as a method of preventing Cd^{2+} from entering the cell. Pretreatment with Zn prior to Cd treatment shortened the accomodation period (Mitra et al 1975).

Cd toxicity in bacteria was dependent on other physiological factors such as pH, composition of clay materials added to the medium and temperature (Babich & Stotzky 1977 a, b and c). For example Cd was found to exert greater toxicity on bacteria such as Actinomycetes and Bacillus cereus when the pH values were increased; the increase of clay materials was shown to reduce Cd toxicity; in gram negative bacteria the uptake of Cd was maximal at 37 °C and minimal at 4 °C. The addition of cysteine can reduce Cd toxicity in a large number of bacteria. It has been postulated that toxicity is due to Cd^{2+} inactivating -SH containing respiratory enzymes and so inhibiting the uptake of oxygen by the cell (Tynecka & Zylinska 1974).

Cd resistance can also be genetically controlled in bacteria. Some strains of Staphylococcus aureus carry Cd and possibly other heavy metal genetic resistance determinants on penicillinase plasmids (Novick & Roth 1968, Smith & Novick 1972 and Kondo et al 1974). This resistance to Cd was found to be mediated by changes in the cell membrane permeability to the cation and therefore a marked decrease of Cd uptake was observed in plasmid positive bacteria (Chopra 1971). A plasmid negative strain of S. aureus was found to take up Cd rapidly and retain about 40% of that Cd^{2+} firmly bound within the cell (Chopra 1975). Cd^{2+} was found capable of enhancing the antibacterial activity of some antibiotics such as erythromycin, streptomycin, and tetracycline (Ujiie 1959, cited by Babich & Stotzky 1978 ; Voss 1963).

1.10.4. Protozoa

The studies of Cd toxicity in protozoa have been primarily concerned with uptake, reduction in growth rate and lethality. In Tetrahymena pyriformis, Cd was found to be more toxic than most of the other heavy metals, the decreasing order of toxicity being $Cd^{2+} > Hg^{2+} > Co^{2+} > Zn^{2+} > Pb^{2+}$ on a weight basis (Carter & Cameron 1973). Sudo & Aiba (1974), have shown that Cd was most toxic to Colpidium campylum and Opercularia species strain MO, with the Opercularia showing a slightly better tolerance than the C. campylum. Vorticella microstoma can tolerate Cd better than either C. campylum or Opercularia but after 48 h in 0.8 ppm Cd^{2+} concentration V. microstoma showed a greatly reduced growth rate and cellular malformation. Cd^{2+} at concentration of 2-5 mM has been shown to cause an almost total loss of polyribosomal material in Paramecium

aurelia (Reisner et al 1975). Maclean et al (1972), using gel separation of extracts from the Cd or Zn treated flagellate Crithidia fasiculata, found the Cd^{2+} and Zn^{2+} associated with protein macromolecules.

1.11. Aims of the research.

At present the available literature on Cd toxicity in protozoa provides only heterogeneous and disconnected information. No comprehensive studies have been made in which the different aspects of toxicity (e.g. accumulation, morphology, ultrastructural damage, and lethality) could be compared and correlated in one organism. The aim of the present study is to provide just such an integrated picture of Cd toxicity using A. proteus. The work carried out is comprised of three main areas of investigation - living single cell studies, biochemical studies, and ultrastructural studies. The single cell studies provided data on the survival of amoebae subjected to a range of doses of Cd . The biochemical studies encompass the cellular uptake, retention, and distribution of Cd with time and the association of Cd with specific components of the cytoplasm. The ultrastructural studies show morphological changes in cell organelles which can be correlated with the levels of Cd administered, the Cd distribution within the cell and the relative toxicity of the dose.

1.12. A. proteus as an experimental organism.

The use of A. proteus as a model system in toxicology has been well argued by Ord (1979). The characteristics of amoeba

which were advantageous in the present study are listed:

1) The relatively large size of this free living organism facilitated handling and observation, and allowed the progress of individual cells to be readily monitored.

2) The ease of cloning single cells enabled accurate survival results to be obtained for all dose levels.

3) In toxicology the use of unicellular organism can eliminate many of the complications associated with multicellular systems. e.g. cell to cell interactions, variations in the reactions of different tissues to different toxicants, differences in the routes of toxicant administration, and the distribution throughout the body.

4) The natural habitats of the free living A. proteus being slow water streams and shaded fresh water ponds renders the organism in direct contact with environmental pollutants.

5) A. proteus has been used extensively in studies on carcinogens, mutagens, oxidation-phosphorylation uncouplers and other toxic substances. Thus to explore the effect of Cd in such a system could allow direct comparison to be made between Cd^{2+} effects and those caused by other toxic substances (Smith & Ord 1979, Ord 1979).

Although the use of A. proteus has many advantages in the study of toxic metals, as outlined above, there are also some difficulties (Daniels 1973). These are listed below with any precautions which could be taken in the present work to avoid misinterpretation of the results.

1. A. proteus cultures contain live food. The food Tetrahymena is known to process Cd^{2+} (Carter & Cameron 1973), and therefore it may contribute to any Cd^{2+} effects on or accumulation in A. proteus. As a precaution, care was taken to starve the amoebae for 24 h prior to experimentation to remove the large food vacuoles and to carefully wash all amoebae both before starvation and again before experimentation.

2. Our strain of A. proteus $P_{Da}X_{69}$, contain only one type of endosymbionts (E.M. observations and personal communication with M.J. Ord). The origin of these endosymbionts has not yet been established, but it is known that they contain DNA. These endosymbionts could not be eliminated (M.J. Ord, personal communication). It was realized that they could interfere with the overall Cd^{2+} effect and distribution within the amoeba. On cell fractionation the endosymbionts separated with the microsomal fraction; allowing upper estimates of their possible role in Cd^{2+} accumulation to be made (Section 4.4)

3. Small populations of synchronized amoebae can be obtained by picking up individual division spheres. In the present study large numbers of cells were required, e.g. 80,000 cells/batch, which could not be provided as division spheres. Therefore, unsynchronized cells were used throughout these experiments.

The relevance of the life history of A. proteus to its choice as an experimental organism for toxicology study is self evident. Its widely distributed natural habitat of slow water streams and

fresh water ponds brings it into direct contact with the many pollutants carried in surface waters or collected in sediments. The demonstrated ability of some protozoa to accumulate heavy metals to levels much higher than those of their surroundings may provide a significant route for the entry of heavy metals into food chains. The possible serious, though inestimable, environmental consequences of such pollutant concentration renders the mechanism of uptake and accumulation by protozoa worthy of investigation.

Chapter Two

Materials and Methods

2.1. Organism and culturing media.

The organism used in this study is Amoeba proteus strain $P_{Da}X_{69}$. The amoebae were grown and maintained in two types of culture; a) stock culture (wheat culture) and b) Tetrahymena fed culture.

The medium used in both cultures was a modified Chalkley's solution (MCS) (Chalkley 1930). A concentrated stock MCS was made up of the following components in 1000 c.c. of glass distilled water:

weight	component	final conc. in standard MCS
16g	NaCl	13.7×10^{-4} M
0.8g	$NaHCO_3$	0.48×10^{-4} M
0.4g	KCl	0.27×10^{-4} M
0.2g	$NaHPO_4.12H_2O$	0.28×10^{-5} M
0.2g	$CaHPO_4$	0.74×10^{-5} M
0.2g	$MgCl_2$	0.49×10^{-5} M

The MCS used (standard MCS) was prepared by diluting one ml of the concentrated stock MCS up to 200 ml using glass distilled water. The final concentrations of the different components of MCS are shown between the brackets above.

The pH was adjusted to 6.0 ± 0.1 with NaOH. All cultures and treatments were maintained at 20 ± 1 °C.

2.1.1. Stock (wheat) cultures.

The culture vessels used for stock cultures were either 75 mm diameter crystallising dishes or 25 x 8 x 5 cm plastic dishes. The latter were initially treated with concentrated H_2SO_4 in order to produce a hydrophilic surface to which amoebae can attach easily and treated dishes were rinsed with continuously changing water in a water bath for a minimum period of 24 h before use in culturing. The amoebae were grown in a culture comprised of untreated boiled wheat grains (5 to 10 grains/dish were boiled for 3 min in distilled water) and an added population of Colpidium sp. (ciliate), Chilomonas sp. (flagellate), the mould Dictyuchus sterile and bacteria, (Band 1978).

Subcultures were made at 5-8 week intervals by transferring one of the old wheat grains with an aliquot of balanced ciliate/flagellate/mould/bacteria suspension and amoebae plus 5 to 10 freshly boiled wheat grains to fresh MCS in a clean culturing vessel.

2.1.2. Tetrahymena fed cultures.

This culturing method was found to provide more uniform culturing conditions than those in stock (wheat) cultures where there is a much longer food chain. Therefore, the amoebae were maintained in tetrahymena fed culture for a minimum period of 2 weeks prior to any experiment, following the basic feeding method of Griffin (1960). The period of two weeks allowed the amoebae to readjust to the new food supply. Special care was taken not to have over crowded cultures, as the condition of amoebae from crowded cultures is very variable.

In Tetrahymena fed cultures, the MCS and the old food were tipped off and replaced daily. To ensure that all waste material and old food were washed away the tipping was repeated twice allowing amoebae to reattach between each tip. Fresh food was then added to the culture. The culturing vessels were changed 3 times a week.

2.1.3. Preparation of Tetrahymena.

Tetrahymena pyriformis, strain ST or W were grown in 50 ml of 2% proteose peptone in 100 ml medical flat bottle (autoclaved at 15 lb for 15 min), at $20^{\pm}1$ °C. During log phase the tetrahymena were harvested by washing twice in MCS using a sintered glass funnel (porosity 4). The tetrahymena suspended in MCS were then supplied to the amoebae at an approximate concentration of 15 tetrahymena/amoeba / day.

2.2. Treatment media.

These media were prepared by dissolving the required concentration of Cd^{2+} salt ($CdCl_2$, $Cd(NO_3)_2$, or $^{109}CdCl_2$) in MCS. Media containing L-cysteine, L-^{35}S-cysteine or a combination of Cd^{2+} and cysteine were prepared similarly. However, in no case was $^{109}Cd^{2+}$ used together with L-^{35}S-cysteine (i.e. there was no double labelling). Unsuccessful attempts to double label were made using ^{3}H-cysteine and $^{109}Cd^{2+}$, where ^{3}H can be counted on a different channel from that used for ^{35}S or $^{109}Cd^{2+}$ in the scintillation counter but the results were difficult to analyse, since the very high $^{109}Cd^{2+}$ counts masked the much lower ^{3}H counts of the cysteine.

On the whole the use of ^{35}S-cysteine to find the association of the -SH groups of cysteine with Cd^{2+} or Cd -binding proteins, proved more satisfactory in this study. The pH for all the media used was maintained at 6.0 ± 0.1 and all treatments were carried out at 20 ± 1 °C. All treatments whether of mass cultures or single cell were carried out in glass vessels.

2.3. Single cell treatment.

Chapters 3 and 5 deal with studies using individually selected and handled cells. In these studies a known number of amoebae were transferred to the treatment vessel with a minimum volume of MCS. The cells were allowed to attach to the glass surface and then the MCS was removed by gently sucking it out using a fine tipped Pasteur pipette. The MCS removed was immediately replaced by a known volume of the treatment medium. Similarly, at the end of each treatment the treatment medium was sucked off and replaced by fresh MCS or fixative if the cells were required for E.M. studies. By adding the different solutions to the cells instead of transfering the cells to the solution disturbance through handling was reduced to a minimum. Where it was necessary to follow the individual cell progress after treatments, the cells were grown singly in microtest trays (Flow Labs, disposable, non-toxic tissue culture trays with 24 cups/tray and 3ml vol./cup) with MCS and tetrahymena. The cells were transferred using a fine tipped Pasteur pipette to trays 3 times a week, while MCS and food were changed daily.

2.4. Mass cell treatment

Chapter 4 deals with the biochemical studies where the cells were treated in mass. For each treatment a known number of cells was placed in the glass treatment vessel with adequate MCS. The cells were allowed to attach to the glass surface, the MCS was poured off and the treatment medium added. To diminish loss of cells during pouring the tipped medium was centrifuged 1-2 min at 300 g in an MSE bench centrifuge and any cells recovered were returned to the treatment vessel.

In these studies the cell concentration was always maintained at 500 cells/ml of medium. Cell samples were taken using a 1 ml Oxford pipette. To ensure that the sample was taken from an evenly distributed suspension of cells all cells were detached from the vessel surface before sampling by gently squirting the culturing solution over them. Those samples taken in order to follow the individual cell progress (i.e. attachment, movement, division and viability) were placed in clean solid watch glasses and allowed to attach, the solution was sucked off using a fine tipped Pasteur pipette and the cells washed 3 or 4 times in MCS using the same method. Subsequently the cells were grown singly in microtest trays as described earlier (section 2.3). Samples or whole cultures required for biochemical analysis were centrifuged in an MSE bench centrifuge at 300 g for 1-2 min. This minimum time and force of centrifugation was used to minimize cell damage. The cell pellet was then washed 4 times in MCS by centrifugation. The final pellet of cells was processed as required.

2.4.1. Preparation for scintillation counting.

In treatments with radioactive materials, the sampled cells were washed in MCS by centrifugation as described above and re-suspended in 1ml MCS. To produce a cell homogenate the cells were taken 5 times through a 23 gauge syringe needle. Half of this homogenate (0.5 ml) was mixed with 5 ml Insta gel (Packard Instrument Co., Ltd., U.K.) in a 6 ml polypropylene counting vial and the radioactivity of the material in each vial counted for 4 min in a scintillation counter (Intertechnique ABAC SL40). Two 0.5 ml aliquots from the medium supernatant and the last wash supernatant were prepared similarly for scintillation counting.

2.4.2. Cell fractionation.

In experiments involving cell fractionation, the cell number was kept at 80,000 cells/batch and at a cell concentration of 500 cells/ml. The cells were harvested and washed 4 times in MCS by centrifugation as described earlier, then the cell pellet was fractionated as follows:

All the following steps were carried out at 4 ^{o}C. The cell pellet was resuspended in 1 ml of cold raffinose buffer (0.2M raffinose in phosphate buffer at pH 6.1$\pm$0.1). Cells were then disrupted by passing the suspension 5 times through a 23 gauge syringe needle. Another 2.5 ml of cold raffinose buffer was added to the cell homogenate and the total homogenate was centrifuged for 10 min at 300 g in an MSE bench centrifuge. This fraction when examined under the light

microscope after staining with Borax/Toluidine blue (1% Borax, 1% Toluidine blue) appeared to be a mixture of nuclei and cell membranes*. After removing the first pellet for further study the supernatant was centrifuged for 1½ h at 200,000 g in MSE Superspeed 75 centrifuge. The pellet obtained under these conditions should contain the microsomal fraction (including mitochondria) as judged by comparison with the separation procedure of Ord and Mawson (see below). The supernatant contained the cytosol fraction.

The further step of separating the nuclei from the cell membranes (of the first pellet was carried out) according to the polyethyleneglycol and dextran partition method modified by Hourani et al (1973): the nuclei / cell membrane pellet was resuspended in 10 ml of the upper phase solution (dextran saturated polyethyleneglycol) and mixed vigorously for 10 min. An equal volume of the lower phase solution (polyethyleneglycol saturated dextran) was added to the suspension and mixed by shaking. The two mixed phases were then reseparated by centrifugation at 4000 g for 10 min using the MSE Superspeed 75 centrifuge. The membranes appeared at the interphase while the nuclei and other debris appeared in the pellet.

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* Electron microscopic examination of material from the first pellet obtained by centrifuging an homogenate of amoebae under similar conditions were found by Ord and Mawson to be composed of nuclei and outer cell membranes, with little contamination by other cell organelles except for occasional food vacuoles when fed amoebae were used (personal communication, M. J. Ord).

The supernatant was poured into another centrifuge tube, the two phases were remixed and separated twice more to sediment any non-membrane material trapped at the interphase. The material at the interphase was then aspirated using a Pasteur pipette. The aspirated material was mixed with 5 fold of 0.05 M Tris-HCL buffer (pH 7.2) and centrifuged at 48000 g for 15 min using the MSE Superspeed 75 centrifuge. This step was repeated once more to remove any of the upper and lower phase solutions adherent to the membranes. The pellet collected represented the membrane fraction. Each of the membrane and the nuclear fractions were resuspended in 0.5 ml raffinose buffer and prepared as in section 2.4.1. for scintillation counting. This separation procedure resulted in a loss of a large percentage of $^{109}Cd^{2+}$ counts to the various solutions used for the separation (Table 4.2.b). Attempts to separate the nuclear fraction from the membrane fraction were therefore discontinued and the nuclear/cell membrane fraction was treated as one fraction. Counts from the initial separation had shown the percentage of the $^{109}Cd^{2+}$ counts in the cell membrane fraction after such separation was very low in comparison with those of the other cellular fractions (Table 4.2.b)

To determine the $^{109}Cd^{2+}$ content of the different cellular fractions the pellets of the nuclear / cell membrane fraction and the microsomal fraction were each suspended in 1 ml raffinose buffer. Two 0.5 ml aliquots from each fraction were individually mixed with 5 ml Insta-gel in 6 ml polypropylene disposable counting vials. Each vial was then counted for 4 min in the scintillation counter. As in section 2.4.1. aliquots of 0.5 ml from the medium and the last wash supernatants were prepared for scintillation counting.

2.5. Gel separation.

2.5.1. Gel preparation and column packing.

In experiments where further separation of the cytosol fraction was required, a 90 cm x 1.5 cm Whatman glass column packed with Sephadex G 75 (Pharmacia) was used. Before packing, a suspension of 15-20 g of the Sephadex beads in 200-400 ml of 10 mM Tris buffer at pH 8.0±0.2 was autoclaved, at 15 lb for 15 min, in order to facilitate the swelling of the beads and the expulsion of air bubbles. This suspension was cooled to room temperature overnight before adding sodium azide to a final concentration of 0.02%. During the packing of the column great care was taken to avoid trapping air bubbles in the Sephadex bed. All samples of cytosol fraction and calibration solutions were eluted on the Sephadex G 75 column using 10 mM Tris buffer at pH 8.0+0.2 containing 0.02% sodium azide. The protein fractions obtained from the separation of samples, or calibration solutions, were collected in 2 ml aliquots in a fraction collector (LKB 8000) at an approximate speed of 1 ml (16 drops) /min. 90 to 100 separated protein fractions were collected for each run (separation).

When the cytosol fraction of amoebae treated with radioactive material was separated on the column, 0.5 ml aliquots were taken from each of the separated protein fractions collected, these were prepared for scintillation counting as described previously (section 2.4.1.).

2.5.2. Calibration of the column.

Prior to any separation of samples the newly packed column was calibrated with different proteins of known MW. The compounds used for calibration were:

1) Blue dextran 2000, used to mark the void volume (Sigma Ltd.).
2) Ovalbumin, grade V, salt free, MW 45,000 (Sigma Ltd.).
3) ∝-Chymotrypsinogen-A, type II, from bovine pancreas, salt free, MW 25,000 (Sigma Ltd.).
4) Myoglobin, type I, from horse skeletal muscle, MW 17,800 (Sigma Ltd.).
5) Cytochrome-c, type II-A, practical grade, from horse heart, MW 12,500 (Sigma Ltd.).

Ten mg of each of the above compounds was dissolved in 2.5 ml of glass distilled water and eluted with 10 mM Tris buffer on the Sephadex G 75 column. To check the constancy of the column between separations, a calibration run using blue dextran, ovalbumin, and cytochrome-c only was carried out more frequently. Fig 2.1. shows the LKB Uvicord trace of the UV absorbance peaks obtained when the above mixture of compounds was separated on the Sephadex G 75 column. Wavelengths of 280 and 254 nm produced practically identical absorption traces. The column was also calibrated using the cytosol fraction obtained from the Cd^{2+} treated rat liver, (given kindly by Dr. M. Webb, of the MRC, Carshalton, Surrey).

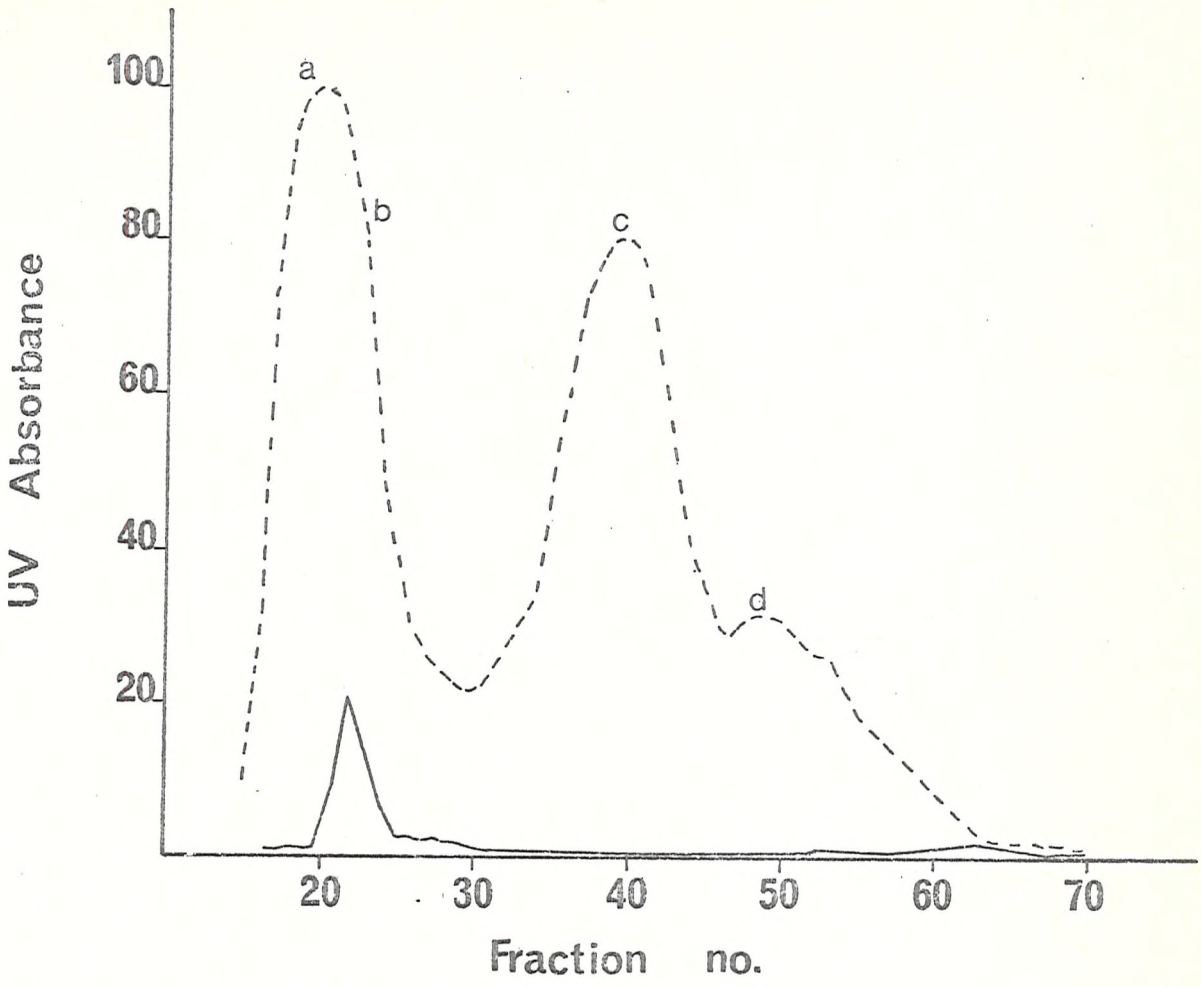

Fig. 2.1. Calibration of the Sephadex G 75 column, showing the UV absorbance of each of the eluted proteins used in calibrating the column (- - - - -).
(a) blue dextran, marking the void volume; (b) ovalbumin, 45,000 MW; (c) myoglobin, 17,800 MW; and (d) cytochrome c, 12,500 MW.

Also shown is the UV absorbance for the eluted protein fractions from the applied 2.5 ml amoeba cytosol fraction (———).

Similar curves were obtained with both 254 nm and 280 nm UV wavelengths.

2.5.2.1. Preparation of the cytosol fraction from the liver of Cd^{2+} treated rat.

The rat had received 4 oral doses of 2.5. mg Cd^{2+}/kgm weight. When the rat was sacrificed, the liver was removed and frozen immediately and kept at -20°C until used for fractionation. The total weight of the frozen liver was 8.75 g, and the weight of the liver tissue prepared was 6.6 g. The tissue fractionation was carried out at 4°C. The liver was homogenized in 3 volumes of ice cold 10 mM Tris buffer using an MSE homogenizer, until no intact cells could be observed under the light microscope. The homogenate obtained was then centrifuged for 1 h at 200,000 g in an MSE Superspeed 75 centrifuge. A total of 18 ml supernatant (cytosol fraction) was obtained.

2.5.2.2. Gel separation of the rat liver Cd -thionein.

Three ml of the rat liver cytosol fraction prepared above, was eluted on the Sephadex G 75 column using 10 mM Tris buffer. The separated protein fractions were collected in 2 ml aliquots as indicated previously, (section 2.5.1.). This separation was repeated 2 times using 254 nm wavelength. Between the runs the supernatant (liver cytosol fraction) was kept at 4°C. The Cd^{2+} level was measured in each of the protein fractions collected using, a Unicam SP 90 A, Atomic absorption spectrophotometer. The atomic absorption spectrophotometer was calibrated with a series of different concentrations of $CdCl_2$ standard solutions. Fig. 2.2. shows the UV absorbance trace obtained with the Cd^{2+} treated rat liver

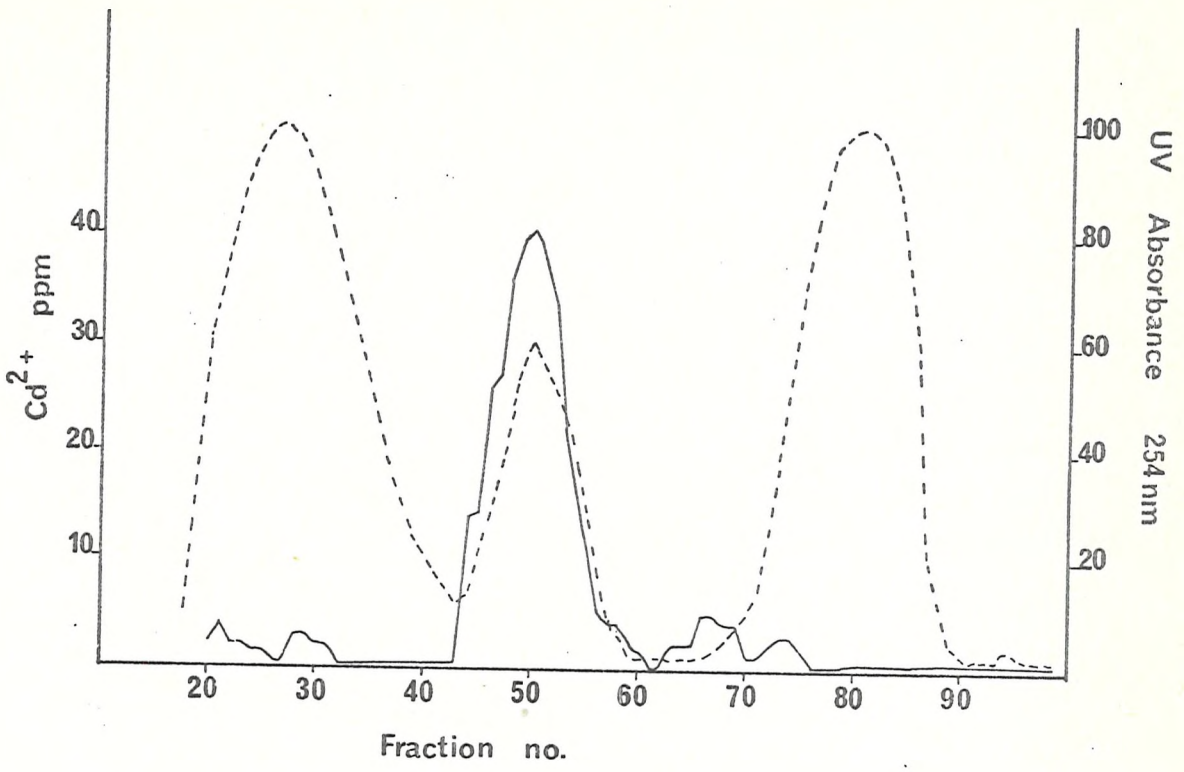

Fig. 2.2. Calibration of the Sephadex G 75 column using liver Cd-thionein from a Cd^{2+} treated rat, showing the UV absorbance for the eluted protein fractions collected from the applied 2.5 ml liver cytosol fraction (– – – –). Also shown is the Cd^{2+} concentration (μg/l) measured in each collected protein fraction using the atomic absorption spectrophotometer (————). The Cd-thionein peak is between fractions 45 - 55 as indicated by the high UV absorbance and the high Cd^{2+} concentration.

cytosol fraction was separated on the Sephadex G 75 column. It also shows the Cd^{2+} levels measured in the collected protein fractions obtained. Fractions 45 to 55 contain the rat liver Cd^{2+}-thionein.

2.5.3. Gel separation of amoebae cytosol fraction.

For experiments where the results of cytosol gel-separation for different batches of treated amoebae were to be compared, the following procedures were always carried out in order to ensure comparability:

a) The total cells/batch was 80,000.

b) The concentration of cells was 500 cells/ml.

c) The cytosol fraction obtained was in each case made up to 3.5 ml with raffinose buffer.

d) 2.5 ml of the cytosol fraction was then separated on the Sephadex G 75 column.

Thus, the protein levels in the separated fractions of each experiment were comparable with those of the other experiments. (see Fig. 2.1.).

2.6. Uptake and incorporation of L-^{35}S-cysteine by A.proteus.

To determine the total uptake of L-^{35}S-cysteine by amoebae the method described for finding the total $^{109}Cd^{2+}$ uptake was used, (section 2.4.). To measure the incorporated L-^{35}S-cysteine, however, the filter paper disk method (Mans & Novelli 1961) was applied as follows:

Whatman disks No. 3MM chromatography paper, 2.1 cm diameter were used. Each disk was numbered with the sample number and mounted on a straight pin. The pins facilitated handling of the disks as well as preventing their adherence during washing. The diameter of these disks fitted that of the scintillation counting vials used. Samples of 0.1 ml of cell suspension were removed at intervals using an Oxford pippette, and placed directly on the disks. The disks were then dried in a warm air oven at 50°C for 1 h. Thereafter, the disks were placed in 150-200 ml of ice cold 10% trichloroacetic acid (TCA) containing 1 mM unlabelled cysteine solution and incubated overnight at 4°C. The acid stops further enzymic reaction and quantitatively precipitates the protein into the matrix of the disks. The addition of the unlabelled cysteine was to dilute the specific activity of the radioactive cysteine washed from the disks into the solution and therefore to lower the values for the control disks. On the second day the washing solution was discarded and the disks were resuspended in an equal volume of 5% TCA plus 1 mM cysteine solution and incubated for 30 min at 90°C to hydrolyse and extract RNA. The disk suspension was cooled to room temperature and the recovered disks were resuspended in an equal volume of fresh 5% TCA solution and incubated for 30 min at room temperature. The solution was discarded and replaced by an equal volume of a 1:1 (v/v) ethanol/ether mixture and incubated for 30 min in order to extract the TCA and the lipid material. This wash was repeated once more. The recovered disks were dried in a warm air oven for 2 h or until completely dry. Each disk was then placed in a standard 20 ml low potassium glass counting vial with 5 ml of Schram's scintillation fluid (Schram 1963). Each vial was then counted for 4 min in the scintillation counter.

2.7. Electron microscopy.

After treatments cells wanted for E.M. studies were transferred to solid watch glasses with a minimum volume of solution. The cells were washed 3 times by replacing the MCS using a fine tipped Pasteur pipette prior to fixation.

2.7.1. Fixation.

The following double fixation procedure was carried out at room temperature.

1) The MCS was replaced with Karnovsky's fixative (paraformaldehyde-gluteraldehyde made up in 0.1 M sodium cacodylate buffer at pH 7.1, following the basic preparations of Karnovsky (1965). This fixation was given for 1 hr.

2) The cells were washed in two changes of 0.1 M sodium cacodylate buffer, 5 min each.

3) Cells were postfixed in 1% OsO_4 made up in 0.1 M sodium cacodylate buffer at pH 7.1, for 1 h.

4) The cells were washed in two changes of 0.1 M sodium cacodylate buffer, 5 min each.

5) The cells were blocked in 2% agar to facilitate further handling.

2.7.2. Agar blocking.

Six to eight amoebae were grouped on a thin agar layer and the excess solution was withdrawn using a fine tipped pipette.

The grouped cells were covered with a drop of melted 2% agar using a glass rod. After the agar had set a block of agar containing the cells was carefully cut out using a sharp scalpel blade.

2.7.3. Dehydration and staining.

The agar blocks containing the grouped cells were dehydrated through the following series of ethanols:

20% for 10 min.
35% for 10 min.
50% (containing 2% uranyl acetate) for 60 min.
70% for 10 min.
90% for 10 min.
100% for 10 min.
100% for 10 min.

If necessary the blocked cells could be left overnight in 70% ethanol. To overcome any destaining which occurs overnight the blocked cells were first returned to 50% ethanol containing 2% uranyl acetate for 20-30 min prior to completion of the dehydration schedule. When dehydrated, the blocks are ready for embedding.

2.7.4. Embedding.

The blocked cells were embedded in Spurr resin (Spurr 1969) according to the following procedure. The dehydrated agar blocked cells were transferred to 1:1 Spurr and absolute ethanol mixture in a

solid watch glass for 30 min. They were then transferred to 100% Spurr resin and left overnight. Finally each block was placed in the tip of a plastic TAAB capsule, with the cells as near as possible to the bottom surface of the capsule. Fresh Spurr resin was used to fill each capsule and the resin was polymerised in a 70°C oven over 2 days.

2.7.5. Sectioning.

The polymerised resin blocks containing the cells were trimmed and ultrathin sections of 600-900 Å (of a silver-gold colour) were obtained using freshly made glass knives, on an LKB III Ultratome. The sections formed a ribbon floating on the surface of the knife's water trough. The sections, which are compressed on cutting, were stretched by xylene vapour. The ribbons were picked up onto clean 200-mesh copper grids. Sections were examined on a Philips EM 300, using 80 or 60 KV. Thick sections (~1μm) were also obtained for light microscopy studies. These were mounted on glass slides and stained with 1% Borax/Toluidine blue stain. They were examined and photographed on a Zeiss Photomicroscope using Kodak Tri-X (400 ASA) film.

2.8. Chemicals and reagents.

$CdCl_2 . 2\frac{1}{2} H_2O$ MW 228.34, analar, BDH Chemical Ltd.

$Cd(NO_3)_2 . 4 H_2O$ MW 308.47, analar, BDH Chemical Ltd.

Raffinose $C_{31}H_{36}N_2O_8$ MW 582.61 BDH Chemical Ltd.

L-cysteine $CH_2(SH).CH(NH_2).COOH$ MW 121.16 Sigma Ltd.

L-cysteine-S35 hydrochloride $CH_2(SH),CH(NH_3^+).COOH\ Cl^-$

MW 157.7 in aqueous solution and sterilized. Half life 82.4 days. Amersham, Radiochemical Centre.

Cd-109, cadmium chloride in 0.1 M HCl carrier free, half life 453 ± 12 days. Amersham Radiochemical Centre

The specific activities, at the time of the experiments, of the radiochemical used were calculated using the formula:

$$N_t = N_0 \div \text{anti log } \left(\frac{0.3010\ t}{t_{\frac{1}{2}}}\right)$$

where: N_t is the specific activity at the time of the experiment.

N_0 is the reference specific activity provided by the radiochemical manufacturer.

t is the time between the reference day and the experiment day in days.

$t_{\frac{1}{2}}$ is the half life of the isotope in days.

To enable comparison between experiments carried out at different dates, the actual counts obtained were corrected to those expected for specific activities 120μCi/μg for $^{109}Cd^{2+}$ and 17μCi/μmol for ^{35}S using the formula: $x' = \frac{N_0\ x}{N_t}$

where: x is the actual count

x' is the corrected count

N_0 is either 120μCi/μg $^{109}Cd^{2+}$, or 17μCi/μmol ^{35}S.

N_t is the specific activity at the time of the experiment.

Raffinose buffer. 0.2 M raffinose in 10^{-3}M phosphate buffer, pH 6.15.

a) Stock solution 1; of K_2HPO_4 5.811 g in 500 ml distilled water giving a concentration of 0.0667 M.

b) Stock solution 2; of KH_2PO_4 9.078 g in 1000 ml distilled water giving a concentration of 0.0667 M.

c) Stock solution 3; one part of solution 1 mixed with nine parts of solution 2. (0.0667 M phosphate buffer).

d) 1.5 ml of solution 3 diluted to 100 ml with distilled water to give 10^{-3}M phosphate buffer at pH 6.15.

e) 12.57 g raffinose made up to 100 ml with 10^{-3} M phosphate buffer.

Fixative No. 1 (Karnovsky's fixative) (Karnovsky 1965)

In order to prepare 25 ml fixative, 1g of paraformaldehyde was dissolved in 7 ml distilled water by heating up to 70°C, and then adding single drops of 1N NaOH until all particles dissolved. This solution was cooled before the addition of 5 ml of 25% ultrapure glutaraldehyde. The total volume was made up to 25 ml with 0.2 M sodium cacodylate buffer in a volumetric flask. Finally 10 mg of $CaCl_2$ was added to the solution. The final concentrations of the fixative constituents were 4% paraformaldehyde, 5% glutaraldehyde, and 0.1 M sodium cacodylate. The pH was adjusted to 7.1. This fixative was made up freshly before use. The preparation and subsequent use of Karnovsky's fixative were carried out in a fume cupboard. Care is required in handling this fixative due to the potentially hazardous effects of sodium cacodylate (Weakley 1977).

Fixative No. 2 (Osmium fixative).

1% of OsO_4 was prepared in 0.1 M cacodylate buffer at pH 7.1 Preparation and use of the fixative were carried out in the fume cupboard. The fixative can be stored in a dark bottle in a cool place but it must be discarded at the first sign of precipitation.

Uranyl acetate stain.

2% uranyl acetate was prepared in 50% ethanol. The solution was filtered using a 0.22 µm diameter millipore filter prior to use.

Spurr resin (1969).

The resin was prepared by thoroughly mixing the following components:

10 g ERL 4206 (vinyl cyclohexane dioxide)

6 g DER 736 (diglycidyl ether of polypropylene glycol)

26 g NSA (nonenyl succinic anhydride)

0.4 g SI (dimethylaminoethanol)

The preparation and subsequent use of this resin were carried out in a fume cupboard and in covered vessels.

Schram's fluid (1963).

4 g of 2,5-diphenyloxazole (ppo) and 0.2 g of 1,4-bis-2(5-phenyloxazolyl) benzene (popop) were dissolved in 1 litre of

toluene (scintillation grade).

Two phase polyethyleneglycol dextran solutions (Hourani et al 1973)

25.75 g of 30% (w/w) polyethyleneglycol 600 (BDH) in distilled water. (15 g polyethyleneglycol 600/35 g H_2O)
50 g of 20% (w/w) dextran T 500 (Pharmacia) in distilled water; (10 g dextran T 500/40 g H_2O).

These two solutions were mixed together with 83 ml of 0.2 M sodium phosphate buffer (pH 6.4), 43 ml distilled water and 1.8 ml of 0.5 M $MgCl_2$ solution in a separatory funnel. The mixed solutions were allowed to separate at 4°C (minimum time of 5 h). The two phases were collected separately and kept at 4°C until required.

Sodium cacodylate buffer ($(CH_3)_2$ AsO_2 Na. $3H_2O$).

A stock solution of 0.2 M concentration was prepared by dissolving 2.1403 g of sodium cacodylate (BDH) in 100 ml of distilled water and adjusting the pH to 7.1 using 1N HCl.

Chapter Three

Single Cell Studies

3.1. Introduction.

Most of the studies carried out on the toxic effect of cadmium in protozoa have been concerned primarily with the accumulation of Cd^{2+} within the cell, the reduction in growth rate, and lethality, e.g. Tetrahymen pyriformis (Carter & Cameron 1973), Colpidium campylum, Vorticella mircostoma and Operculuria species (Sudo and Aiba 1974: Dive & Leclerc 1975).

In this chapter cadmium toxicity to Amoeba proteus is investigated. The cells were exposed to a wide range of Cd concentrations over a range of different exposure times. For each treatment it was found that the percent of survivals depended on both the concentration of Cd given and the time of exposure to that concentration.

In the mammalian system it is a well established phenomenon, that pretreatment with a low dose of Cd induces protection against a subsequent high dose of Cd . It was found that the Cd level of the pretreatment dose must be above a certain minimum to induce such a phenomenon, (Webb 1975). Such protection was initially attributed to the production of a low MW metal binding protein (Metallothionein) in response to the pretreatment (Terhaar et al 1965; and Leber & Miya 1976). Later, however, the presence of metallothionein in itself was found not to be sufficient to explain this protection, (Webb & Etienne 1977; and Webb & Verschoyle 1976). This will be discussed further in chapter four.

To investigate whether this protection phenomenon occurs in amoebae, two different doses of Cd were required. First, a low concentration of Cd which gives high percent survival (80% to 90%, i.e. LD_{10-20}) was used. Such a dose will be termed, the pretreatment dose. Then, a high Cd concentration which gives a low percent survival (20% to 30% survival, i.e. LD_{70-80}) was used. This second concentration leaves an up to 80% margin for improvement. This dose will be termed, the main treatment dose.

During the experiments outlined above a light microscopy study on the effects of Cd on cell morphology was carried out. These observations together will cell lethality (percent of survivals) due to Cd treatments and the possible induction of protection by pretreatment with low dosesof Cd comprise the investigations made in this chapter.

3.2. Cd is toxic to Amoeba proteus.

Cd is known to be toxic to most living systems. In order to determine whether Cd is toxic to Amoeba proteus the cells were exposed to a wide range of Cd doses. In this work the term dose represents an exposure to a certain concentration of Cd for a certain length of time.

Variations in the culture conditions prior to and during each experiment can have a considerable effect on the percent survivals (Lorch 1973). To minimise such variations cultured cells were subjected to the following rigid protocol. They were fed daily on Tetrahymena

up to 24 h before an experiment. They were well washed and starved for 24 h prior to treatment. Temperatures were maintained at 20°C through all cultures and treatments and the pH was maintained at 6.0 ± 0.1. During treatments the cells were shaken continuously at 20°C in a water bath shaker to produce an even distribution of metal ions and cells. In order to avoid possible damage to the cells by excessive shaking the minimum speed which could prevent the cells adhering to the treatment vessel was determined and used. This was found to be 40 strokes/min. The cell concentration in the Cd treatment media was always fixed at 50 cells/ml. During treatment 2 ml aliquots of cell suspension were shaken in 25 ml beakers in a Grant temperature controlled, water bath shaker. At the end of each treatment the cells were removed from the Cd treatment medium washed 4 times in MCS and singled into microtest trays (Flow laboratories disposable non-toxic tissue culture plastic trays with 24 cups/tray and 3 ml/cup) and left until death or completion of three cell cycles. Thus for a given Cd treatment the result obtained was the proportion of the original cell population which survived that dose. These were then expressed as the percent survivals.

The results are presented in table 3.I and fig 3.1. These give the range of the doses used, the total number of cells treated and the average percent survivals for each dose. They show that Cd toxicity in Amoeba proteus depends on two parameters, the concentration of Cd and the length of the exposure time. When either of these parameters is increased the percent survival is decreased; conversely, when either of the parameters is decreased the percent survival is increased. However, there is clearly no direct proportional relationship

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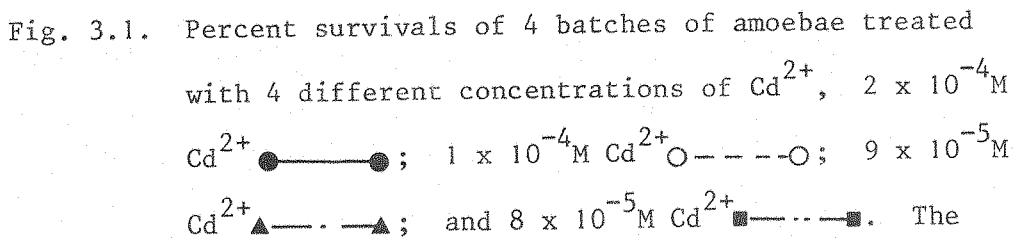
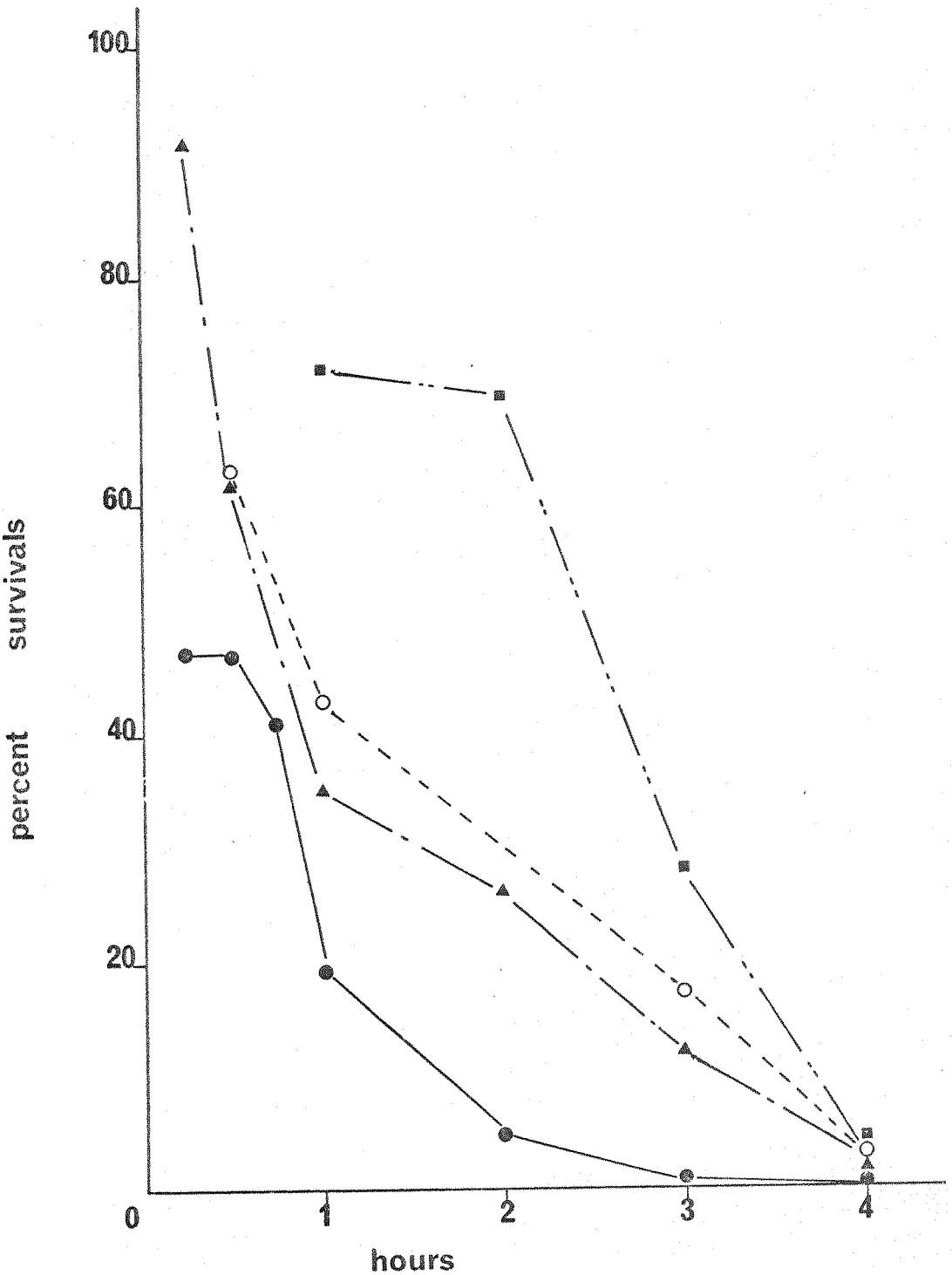


Fig. 3.1. Percent survivals of 4 batches of amoebae treated with 4 different concentrations of Cd^{2+}, 2×10^{-4}M Cd^{2+} ●——●; 1×10^{-4}M Cd^{2+} O- - - -O; 9×10^{-5}M Cd^{2+} ▲—·—▲; and 8×10^{-5}M Cd^{2+} ■—··—■. The different Cd^{2+} concentrations were given over a range of exposure times between 15 min and 4 h. These results correspond with those shown in table 3.1.

This figure indicates that Cd^{2+} toxicity in amoeba is dependent on both the Cd^{2+} concentration given and the time of exposure to that concentration (Cd^{2+} dose).


100
80
60
40
20
0
1
2
3
4
percent survivals
hours

between the ion concentration and the exposure time. For example, cells which received 8 x 10^{-5}M Cd^{2+} for 1 h did not show much difference in percent survival from cells which received the same concentration for 2 h; but when the exposure time for the same concentration was increased for 2 h to 3 h or from 2 h to 4 h, there was a dramatic decrease in percent survival. On the other hand, exposure of cells to 1 x 10^{-4}M Cd^{2+} for 1 h resulted in approximately twice the percent survivals obtained when the dose given was 2 x $10^{-4}Cd^{2+}$ for 1 h, but exposure to 1 x 10^{-4} Cd^{2+} for 3 h resulted in 18x the percent survivals obtained when the dose given was 2 x 10^{-4} Cd^{2+} for 3 h.

The doses given in table 3.I can be arbitrarily divided into two groups on consideration of the percent survivals produced:

a) Those doses which fall below an apparent 'threshold' and which produce a range of intermediate percent survivals. Such doses are: 8 x 10^{-5} Cd^{2+} for up to 3 h, 9 x 10^{-5} Cd^{2+} for up to 3 h, 1 x 10^{-4} Cd^{2+} for up to 3 h, and 2 x 10^{-4} Cd^{2+} for up to 1 h.

b) Those doses which fall above a 'threshold' and produce complete or near complete lethality. Such doses are 8 x 10^{-5}M, 9 x 10^{-5}M, 1 x 10^{-4}M Cd^{2+} each given for 4 h; 2 x 10^{-4}M Cd^{2+} given for 2 h; 3 x 10^{-4}M Cd^{2+} given for 1 h; and 1 x 10^{-3}M given for 15 min.

Table 3.I. Percent survivals of Cd^{2+} treated amoebae.

Cd^{2+} conc.	exposure time	total No. of cells used	% survivals
0.0	0.0	600	~ 100%
8×10^{-5}M	1 h	150	72%
	2 h	150	70%
	3 h	150	28%
	4 h	150	2%
9×10^{-5}M	15 min	150	92%
	30 min	150	62%
	1 h	150	35%
	2 h	150	26%
	3 h	150	12%
	4 h	150	2%
1×10^{-4}M	30 min	150	63%
	1 h	150	43%
	3 h	300	18%
	4 h	100	0.0%
2×10^{-4}M	15 min	200	47%
	30 min	200	47%
	45 min	200	41%
	1 h	1450	19.5%
	2 h	600	5%
	3 h	300	1%
	4 h	300	0.0%
3×10^{-4}M	1 h	300	0.0%
1×10^{-3}M	15 min	300	0.0%

3.2.1. Selection of a 'Main treatment dose'.

From the results shown in table 3.1. and fig. 3.1, the dose 2×10^{-4}M Cd^{2+} for 1 h exposure time was found to be approximately 80% lethal (LD_{80}). Repeated experiments using this dose gave percent survivals of $19.5^{+}_{-}9.8$ (95% C.I.) for batches of 100 cells each. This dose allows therefore, a range of up to 80% for improvement. In addition to killing 80% of the cells this dose was useful as a main treatment dose because the time of exposure was short enough to enable an experiment (pretreatment and main treatment) to be carried out during one cell cycle.

3.2.2. Selection of 'pretreatment doses'.

Amoeba proteus must attach to a substratum in order to catch and feed on their live food (Mast and Hahnert 1935). During the main treatments of 3.2.1. where the times of exposures to Cd^{2+} were short (i.e. 1 to 4 hours), the cells were not required to feed and were not given any food. It was considered more important to have an even distribution of cells and metal ions throughout the treatment vessel, and to create such conditions minimum shaking which kept cells from attaching was used. However, for pretreatment doses where exposures to low concentrations of Cd^{2+} over much longer periods (several days) were used, it was necessary to feed the cells. In these therefore, the cells were allowed to attach to the vessel surface and given a minimum quantity of food. At intervals, during each exposure the cells were detached from the vessel surface and samples of 2 to 4 ml of suspended amoebae were removed. The detachment of the cells was affected by gently squirting the medium over the amoebae using

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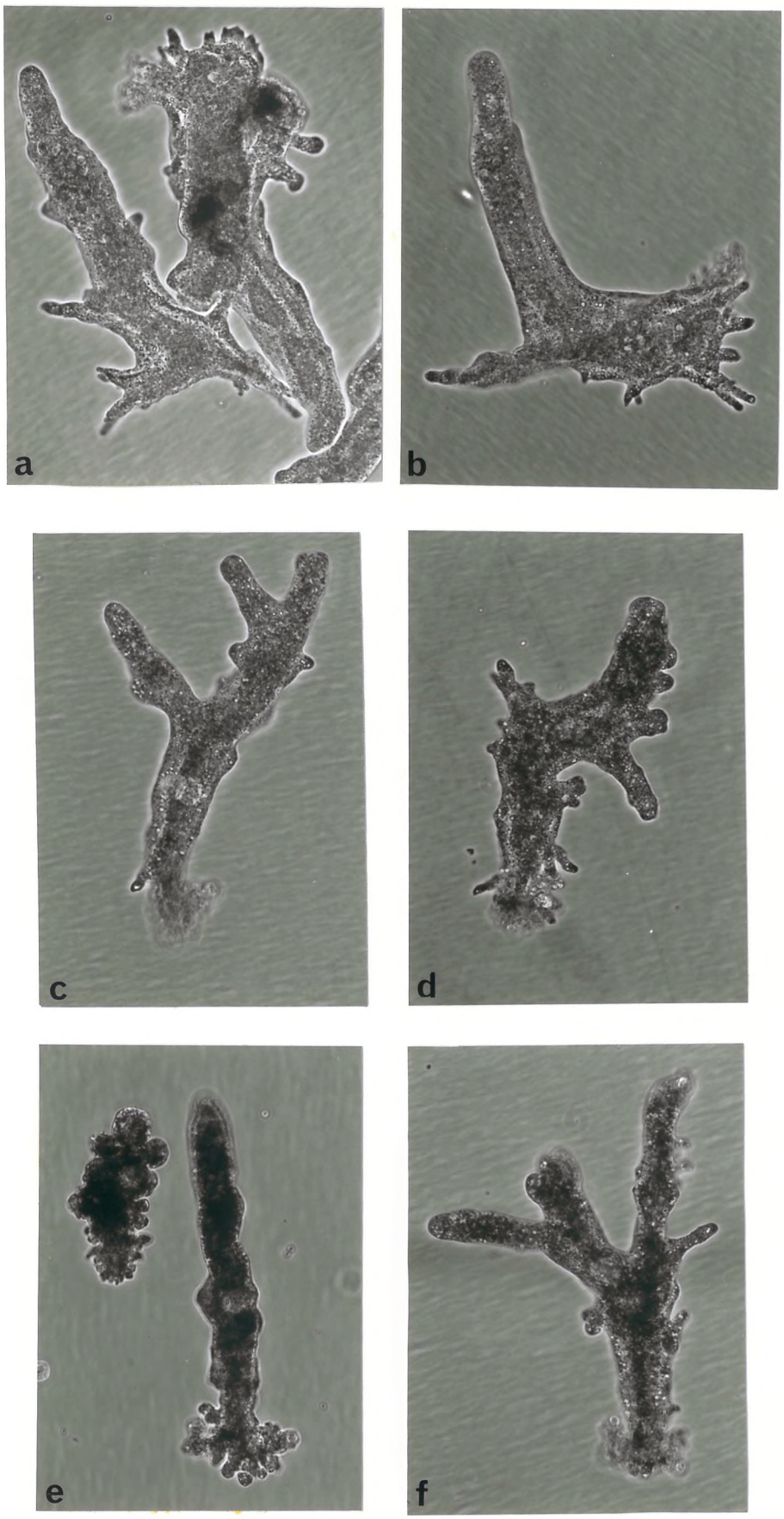
Fig. 3.2. Adaptation of amoebae to low concentrations of Cd^{2+}. Magnification all 127 x.

a and b) control amoebae cultured in MCS.

c and d) Cd-adapted amoebae cultured continuously in 10^{-7}M Cd^{2+} for 9 months.

e and f) Cd-adapted amebae cultured continuously in 10^{-8}M Cd^{2+} for 12 months.


a
b
c
d
e
f

a Pasteur pipette. The Cd treatment medium was sucked out from each sample using a fine tip pipette and fresh MCS was added in order to wash the cells. The cells were washed 4 times before they were set out singly in microtest trays in order to follow their individual progress and determine the overall percent survivals. Table 3.II gives the range of the low doses used, the total number of cells sampled, and the average percent survival for each dose.

The results given in table 3.II show that cells exposed to 10^{-5}M and 10^{-6}M Cd^{2+} demonstrated a decrease in percent survivals with increased times of exposure. Very shortly after 4 days in 10^{-5}M Cd^{2+} and 9 days in 10^{-6}M Cd^{2+}, many cells assumed a spherical shape and detached from the vessel surface. Most of these detached cells underwent cytolysis during washing procedures. Eventually an exposure time was reached with each concentration which gave complete lethality.

Cells treated with lower concentrations of Cd^{2+} e.g. 10^{-7}M and 10^{-8}M showed a slight decrease in percent survivals after a considerably longer time of exposure, 30 days and 77 days respectively. These cells were kept in cultures in the presence of Cd^{2+} for even longer periods than those shown in table II for the purpose of E.M. studies. Even after 12 months in 10^{-8}M Cd^{2+} and 9 months in 10^{-7}M Cd^{2+}, the cultures remained apparently healthy. The cells appeared to adapt to the low concentrations of Cd^{2+} and cell division and other cellular activities were similar to those for control amoebae (fig. 3.2).

Table 3.II. Percent survivals of Cd treated amoebae.

Cd^{2+} conc.	exposure time	total number of cells used	% survivals
10^{-10}M	24 h	200	97%
	6 months	200	94%
10^{-8}M	24 h	200	95%
	7 days	200	96%
	24 days	200	83%
	40 days	200	92%
	50 days	200	84%
	57 days	200	90%
	77 days	200	79%
10^{-7}M	24 h	200	80%
	7 days	200	48%
	27 days	200	50%
	30 days	200	45%
	34 days	200	74%
10^{-6}M	3 days	200	83%
	4 days	150	73%
	9 days	200	50%
10^{-5}	3 days	200	38%
	4 days	150	11%

The three concentrations, 10^{-10}M, 10^{-8}M and 10^{-7}M Cd^{2+} with an exposure period of 24 h were chosen to be used separately as pretreatment doses in those experiments requiring pretreatment followed by main treatment doses.

3.3. The combination of pretreatment and main treatment.

In this set of experiments the attached cells were given the pretreatment as described in section 3.2.2., and after each pretreatment the cells were either given the main treatment directly or allowed a recovery time. A recovery time is the interval between a pretreatment and a main treatment exposure. Thus, after a pretreatment the cells were removed from their pretreatment medium, washed 4 times in MCS and cultured as normal until the main treatment time. This was given after 0.0, 1, 2, 3, 4, 5, 6 and 7 days. Before all main treatments, with the exception of 0.0 day recovery time, the cells were starved for 24 h, see diagram I. During main treatments the cells were shaken in the water bath shaker (at 40 strokes/min and 20°C), and the cell concentration was again fixed at 50 cells/ml. Fig.3.3. shows a comparative study of percent survivals of cells given a pretreatment and a main treatment, and cells given the main treatment only.

This experiment showed that pretreatment of amoebae with a low dose of Cd produced a short lived protection against the subsequent main treatment, LD_{80} dose. This protection was maximum when 1 day recovery time was allowed. When the main treatment was given after 2 days or more the percent survival dropped back to the level of the non-pretreated cells, Fig. 2.2..

Diagram I. Shows the scheme followed in the pretreatment/main treatment experiment.

Amoebae from stock (wheat) culture transferred to

↓

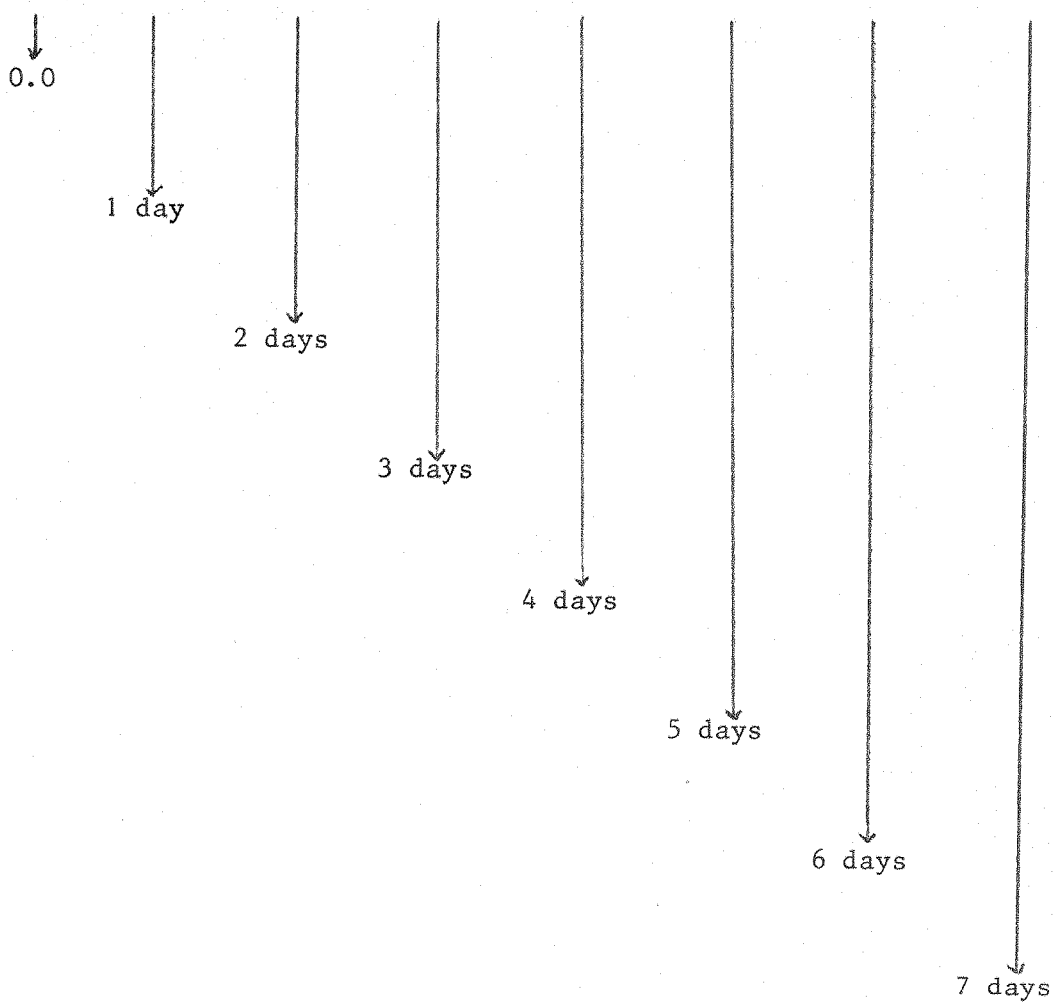
MCS + food (tetrahymena), for a minimum period of 2 weeks prior to any treatment.

↓

Pretreatment given to amoebae, set at an approximate cell concentration of 50 cells/ml with food, for 24 h.

↓

Amoebae returned to MCS with food for the following recovery time intervals:



Amoebae given the main treatment dose (LD_{80}).

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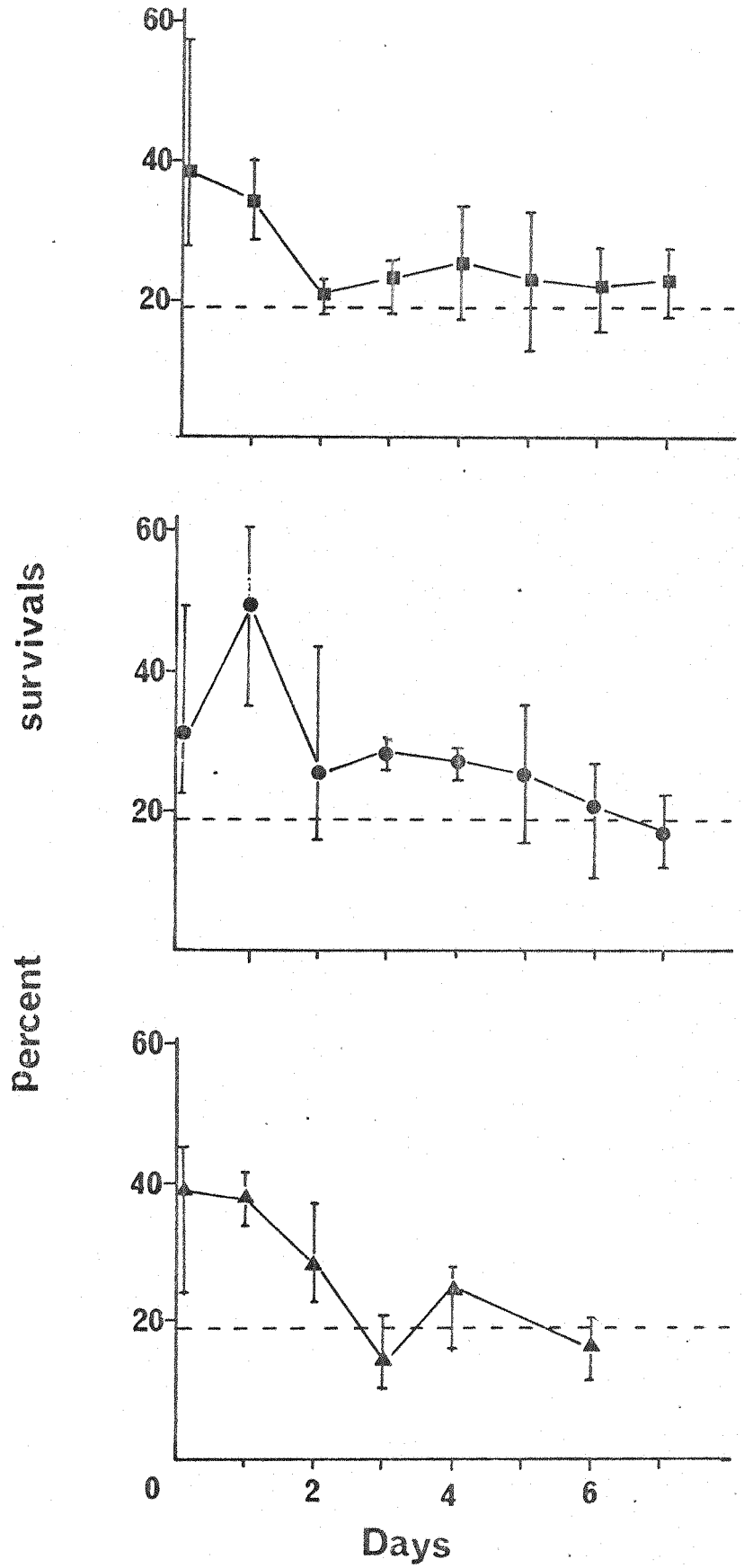
Fig. 3.3. These curves demonstrate the improvement in percent survivals found when amoebae were given a pretreatment with a low dose of Cd^{2+} prior to a main treatment with a high dose of Cd^{2+} (2×10^{-4}M Cd^{2+} for 1 h) compared with amoebae which received the main treatment alone.

Percent survivals are ploted against the time in days between the pretreatment and the main treatment (recovery time).

(- - - -) indicates the percent survivals found for the main treatment alone (2×10^{-4}M Cd^{2+}; (a) amoebae pretreated with 10^{-7}M Cd^{2+} for 24 h prior to the main treatment ■—— ; (b) amoebae pretreated with 10^{-8}M Cd^{2+} for 24 h prior to the main treatment ●—— ; and (c) amoebae pretreated with 10^{-10}M Cd^{2+} for 24 h prior to the main treatment ▲—— .

The main treatment dose gave percent survivals of $19.5 \pm 9.8\%$ (95% C.I.) for repeated batches of 100 cells each. The pretreatment doses 10^{-7}M Cd^{2+} for 24 h, 10^{-8}M Cd^{2+} for 24 h and 10^{-10}M Cd^{2+} for 24 h, gave percent survivals of $86 \pm 6.6\%$, $90 \pm 8.5\%$, and $92 \pm 6.2\%$ (95% C.I.) respectively for repeated batches of 20 cells each. Bars represent the range of percentages determined


60
40
20
60
40
20
Percent survivals
60
40
20
0
2
4
6
Days

The cell cycle in amoebae is of the order of two days, thus from two days recovery time onward many of the cells which were exposed to the main treatment were not the original pretreated cells, but daughter cells of the original pretreated cells. This could have some bearing on the drop of the percent survivals back to the non-pretreated cell level. Each of these experiments were carried out in duplicate. When Cd^{2+} was given as $CdCl_2$ instead of $Cd(NO_3)_2$ there was no difference in the results obtained.

3.4. The use of Zn^{2+} in pretreatment of amoebae.

In mammals pretreatment with Zn^{2+} has been shown to protect the whole animal or specific organs e.g. testis, from the toxicity of a subsequent high dose of Cd^{2+} or other toxic heavy metal ions (Gunn et al 1961, 1968 a, b, c; Webb 1972 b; Kar et al 1960). Therefore Zn^{2+} (as $ZnNO_3$) was used in the pretreatment of amoebae prior to their subsequent exposure to the high Cd^{2+} dose, LD_{80}, in an attempt to further test the possibility of inducing a protection against Cd^{2+}. Unfortunately, Zn^{2+} produced different side effects on amoebae which prevented the completion of the experiment. Most of the cells exposed to Zn^{2+}, at 1×10^{-5}M and 3×10^{-5}M made up in MCS, were found to become detached from the vessel surfaces and assumed a stellate shape of narrow and pointed pseudopods. Some cells grouped together in floating clusters which were surrounded with flocculated cloudy material. The majority of the cells remained detached until death and lysis or until they were removed, washed and recultured in MCS when they returned to their normal condition. Even when lower concentration of Zn^{2+} was used or slight modification in the MSC constituents was made, the cells adherence to the vessel surfaces remained unsatisfactory, and since it is important for amoebae to attach to surfaces in order to carry on their normal living activities (Mast & Hahnert 1935) carrying out such experiments were not possible.

Excess Zn^{2+} is known to modify cell membranes by interfering with the enzymes or the fuctional groups located in the membrane. It is also known to be capable of reducing the phagocytic activity and motility of macrophages. Peritoneal macrophages obtained from guinea pigs fed with 40 ppm Zn^{2+} were shown to have extrusions and long-reaching pseudopodia. High Zn^{2+} diet was shown to inactivate the mobility of plasma membranes of the peritoneal macrophages (Chvapil et al (in Prasad 1976). Similar findings were observed in mice by Rabinovitch & Destefano (1973).

3.5. Light microscopy observations.

In the previous sections, the cells were exposed to a wide range of Cd^{2+} concentrations over different time intervals. Some cells died and cytolysed during treatments. However, those cells which survived until the end of the treatment were washed and singled in MCS as described earlier. On the basis of observations made during the time between singling the treated cells in MCS and their death or completion of three cell cycles, the cells were divided into four categories.

Category 1. Cells which died within 1 to 2 h in MCS. These cells became detached from the vessel surface and took a spherical shape with short and finely tapered pseudopods, an indication that they were not carrying out their normal activities such as feeding. Ultimately, this was followed by cell lysis. All the cells which received the extremely high doses e.g. 2×10^{-4}M Cd^{2+} for more than 1 h or

1 x 10^{-4}M Cd^{2+} for more than 3 h (doses in group b. of section 3.2.) fell into this category.

Category 2. Cells which remained single, but increased in size forming giant cells. These did not divide. They had a rosette shape, with short broad pseudopods. The cytoplasm was dense and granular. Only one nucleus per cell was observed.

Category 3. Cells which divided and formed 2 or 3 daughter cells. Between 2 to 4 days after division, all the daughter cells had cytolysed. The daughter cells were smaller in size than control amoebae and of rosette shape with dense granular cytoplasm and a single nucleus.

In determining the percent survivals after each Cd^{2+} treatment, cells of the above three categories were not considered as viable cells.

Category 4. Cells which divided and formed clones of 16 or more cells. In these clones most daughter cells were smaller in size than control amoebae and had dense granular cytoplasm with a single nucleus per cell. They were rosette shaped and tended to remain together as a single cluster of cells adhering to the vessel surface. When the cells were cultured for longer periods in MCS they returned to the normal (control) appearance. Most of the cells which received the low concentrations of Cd^{2+} e.g. 10^{-10}M, 10^{-8}M and 10^{-7}M Cd^{2+} over long periods fell into this category. Cell division was not suppressed during the culturing of cells in these low concentrations of Cd^{2+}

The proportions of the first, second, and third categories of cells were found to be higher in those experiments where the cells were given the higher range of Cd doses. On the other hand, the proportion of the third and fourth categories of cells were found to be higher in experiments where the cells were given the lower range of Cd^{2+} doses.

3.6. Summary.

In this chapter, the experiments carried out showed that Cd was toxic to Amoeba proteus. Toxicity was expressed as cell lethality. In amoebae the degree of Cd toxicity depended on two parameters, the concentration of the Cd given and the time of exposure to that concentration. Cd toxicity to amoebae was also revealed by certain morphological changes, e.g. the formation of rosette shaped cells with dense and granular cytoplasm; interference with the cell cycle and cell division with cells failing to divide yet increasing in size to form giant cells; delayed cell death, i.e. death of daughter cells after one or two cell cycles. Pretreatment experiments showed that low doses of Cd can induce a short lived protection against a subsequent higher dose (LD_{80}) of Cd . Such a phenomenon has been observed in the mammalian system (Terhaar et al 1965 and Leber & Miya 1976; Piscator 1964).

In the next chapter, the investigations of Cd toxicity in amoebae will be continued through a biochemical approach in attempts to answer questions such as: how does Cd affect amoebae, i.e. does it effect the organism intracellularly or extracellularly,

for example can it reduce or prevent the uptake of Cd through changes in the cell membrane? What is the nature of the transitory protection against a subsequent high dose of Cd which was produced by Cd pretreatment?

Chapter Four

Biochemical Studies

4.1. Introduction.

The results obtained in the previous chapter showed that Cd^{2+} was toxic to Amoeba proteus. This chapter investigates the uptake of Cd^{2+} by amoebae, its accumulation and release characteristics and its distribution within the cell, i.e. with which cellular components does Cd^{2+} become associated? It was hoped that answers to these questions might also throw some light on the short lived protection induced by the low dose pretreatment with Cd^{2+} (section 3.3) since the transitional nature of this protection suggested that some biochemical changes may take place in amoebae on exposure to such doses, a suggestion supported by morphological changes observed on electron microscopic examination (Chapter 5).

The available literature provides very little, if any information on biochemical and physiological changes in protozoa, other simple systems have provided more data in these areas (Middlekauff et al 1956; Nakamura & Ashida 1959; Novick & Roth 1968; Chopra 1971, 1975; Smith & Novick 1972; Lindegren & Lindegren 1973; Kondo et al 1974; Doyle et al 1975; Mitra et al 1975). However, most of the information available on the biochemical changes in response to Cd^{2+} toxicity were obtained from studies carried out on mammals, vertebrates, and mammalian tissue cultures (see chapter 1).

In the previous chapter it was shown that treatments with high doses of Cd^{2+} resulted in high levels of lethality with cytolysis occuring frequently during or immediately after very short exposure periods. This type of "rapid death" resulting from the sudden gross toxicity of the cells produced a situation unsuitable for following

the course of Cd^{2+} uptake and accumulation. Therefore, it has been necessary in subsequent work to confine studies to the lower concentrations where cell death did not occur even during extended exposure periods. Thus, in the experiments carried out in this chapter the concentration of Cd^{2+} used was 10^{-8}M; a concentration sufficiently high to give the temporary protection detailed in chapter 3 (section 3.3), without causing cell death.

4.2.1. The uptake of $^{109}Cd^{2+}$ by Amoeba proteus

The available literature reports have shown that Cd^{2+} exerts its toxic effect on the living system intracellularly, e.g. in mammals (Kapoor et al 1961), bacteria (Doyle et al 1975), yeast (Lindegren & Lindegren 1973), and protozoa (Carter & Cameron 1973; Sudo & Aiba 1974, Dive & Leclerc 1975).

To identify and measure the Cd^{2+} uptake by amoebae, the isotope $^{109}Cd^{2+}$ was used in its chloride salt form, $CdCl_2$, at 10^{-8}M concentration. Cells used in the $^{109}Cd^{2+}$ uptake experiments were provided with food until the treatment time. This differs from the conditions in all previous experiments where the cells were starved during the 24 h period to the treatment, though, the food given was cut to a minimum quantity. During treatment the cells were allowed to attach to the vessel surface, the medium was not changed and no food was given. Samples of 2 ml were removed at different intervals using a 1 ml Oxford pipette. Each sample was centrifuged in a 15 ml centrifuge tube using the MSE bench centrifuge at 300 g for 1 to 2 min. (This low speed and short time of

centrifugation was used to avoid damaging the cells). The pellet of cells was resuspended in 15 ml MCS and recentrifuged as above. This washing procedure was repeated a further 3 times before the cells were resuspended in 1 ml MCS for homogenization. The cells were ruptured by repeated (5 times) passage through a 23 gauge bore syringe needle. Duplicates of 0.5 ml aliquots of the treatment medium supernatant, 0.5 ml of the last wash supernatant, and 0.5 ml of the ruptured cells homogenate, were mixed separately with 5 ml Insta-gel in 6 ml disposable polypropylene counting vials. These were counted in a scintillation counter for 4 min/vial. for counting. Simultaneously with the removal of samples for counting, 1 ml samples of the treated culture were examined under the light microscope to check the number of cells/ml, and their general condition.

The results of a typical uptake experiment are given in fig 4.1., general characteristics are as follows: A rapid increase in $^{109}Cd^{2+}$ uptake by the cells during the first 1½ h of exposure; no increases in the $^{109}Cd^{2+}$ uptake between 1½ h and 24 h, i.e. the curve shows a plateau; a slow but steady increase in the $^{109}Cd^{2+}$ uptake between day 1 and day 3. After the 3rd day of exposure until the end of the experiment the curve shows another plateau. This experiment was repeated twice with all the three curves giving a similar pattern for $^{109}Cd^{2+}$ uptake.

4.2.2. Further examination of the pattern of $^{109}Cd^{2+}$ uptake by amobae.

(A) Since the change in $^{109}Cd^{2+}$ uptake could be due to the

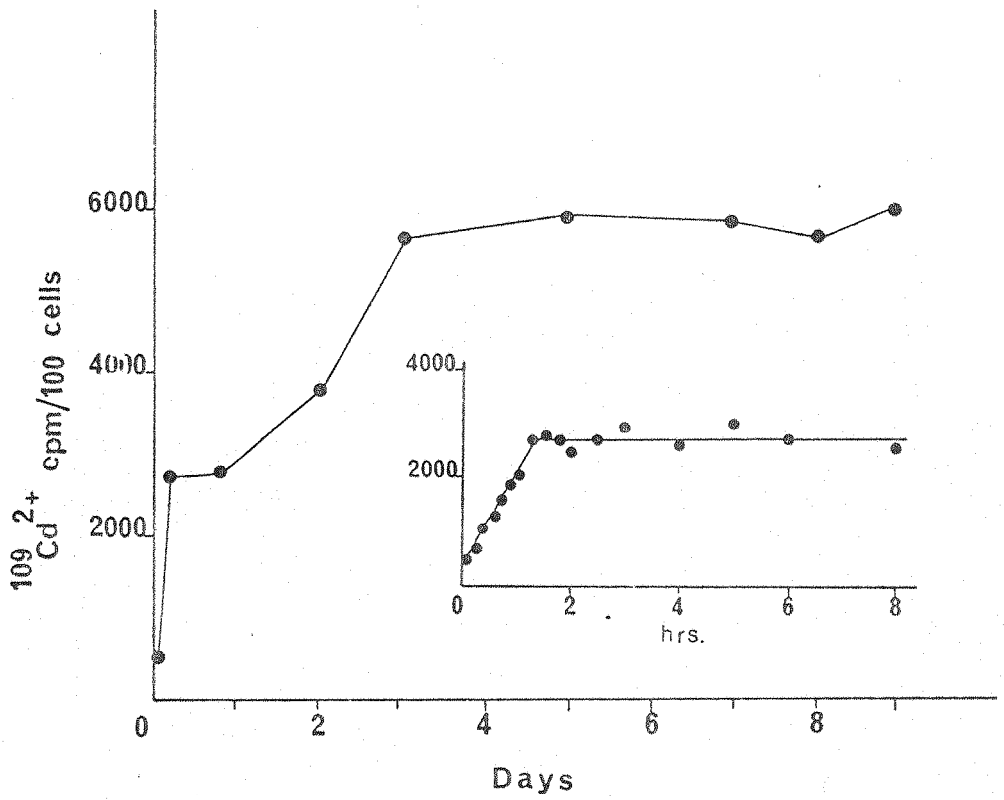


Fig. 4.1. Uptake of $^{109}Cd^{2+}$ by control amoebae. Counts of $^{109}Cd^{2+}$/100 cells were plotted against time (days). The cell concentration was 500 cells/ml 10^{-8}M $^{109}Cd^{2+}$ treatment medium.

Insert: showing the course of $^{109}Cd^{2+}$ uptake during the first 8 h of exposure (a rapid increase followed by a plateau).

removal of appreciable quantities of $^{109}Cd^{2+}$ from the treatment medium, it was important to find whether the replacement of the 10^{-8}_{M} $^{109}Cd^{2+}$ treatment medium by an equal volume of 10^{-8}_{M} $^{109}Cd^{2+}$ fresh treatment medium during the course of these studies altered the characteristics of the $^{109}Cd^{2+}$ uptake? i.e. "Is the observed pattern of $^{109}Cd^{2+}$ uptake a function of changes in the treatment medium rather than a true pattern of uptake by the cells?".

The following 24 h experiments were carried out to test the first two characteristics of the curve, i.e. the initial rapid uptake and the plateau period. A treatment vessel was set up using the same experimental conditions as in the previous experiment, except that at 2½ h and at 4½ h (from the beginning of the treatment) the 10^{-8}_{M} $^{109}Cd^{2+}$ treatment medium was replaced with fresh 10^{-8}_{M} $^{109}Cd^{2+}$ treatment medium. To do this the old medium was poured off leaving the majority of cells attached to the glass walls of the treatment vessel. Any loose cells poured off with the medium were recovered by centrifugation and were returned to the treatment vessel. Since at this point some samples had already been taken reducing the total volume of the treatment medium, care was taken to ensure that the same cell concentration was maintained by replacing the old medium with an equal volume of fresh medium. Sampling and preparation for scintillation counting were carried out as for the previous experiment. Table 4.1.b. gives the results of such an experiment. Thus, the pattern of $^{109}Cd^{2+}$ uptake over the 24 h period was very similar to that shown in table 4.1.a. and fig. 4.1. where the treatment medium was not changed.

(B) A third set of uptake experiments were made to check the authenticity of the "uptake plateau". In these, the $^{109}Cd^{2+}$ uptake experiment of section 4.2.2. was repeated using old treatment medium which had been collected from a previous experiment (10^{-8}M $^{109}Cd^{2+}$, 500 cells/ml, 48 h exposure). As shown in table 4.1.c., there was no change in the pattern of $^{109}Cd^{2+}$ uptake.

The volume of one amoeba has been estimated as approximately 2×10^6 μm^3 (Cohen 1957). Since the cell concentration in the uptake experiments was 500 cells/ml of 10^{-8}M $^{109}Cd^{2+}$ treatment medium, it is clear that the ratio volume to volume of medium to protoplasm in the treatment cultures is of the order of 1000:1. Hence for an increase of 1000 $^{109}Cd^{2+}$ count in* an equivalent volume of treatment medium. This consideration provides a ready explanation of the lack of effect on the $^{109}Cd^{2+}$ uptake curve by either replacing the treatment medium with an equal volume of fresh treatment medium or using a pre-used ^{109}Cd treatment medium in which the $^{109}Cd^{2+}$ level has dropped, i.e. despite a marked $^{109}Cd^{2+}$ uptake by the amoebae from the treatment medium the actual change in $^{109}Cd^{2+}$ concentration in that treatment medium is relatively very small. These experiments have demonstrated that the pattern of $^{109}Cd^{2+}$ uptake by amoebae (i.e. rapid uptake and plateau characteristics) were not caused by small variations in the concentration of $^{109}Cd^{2+}$ in the treatment medium.

* amoebae, one could expect a decrease of only one ^{109}Cd count in

Table 4.1. The ratios of $^{109}Cd^{2+}$ counts in 500 cells (1 mm^3 estimated packed volume) to $^{109}Cd^{2+}$ counts in 1 mm^3 of treatment medium (initially at $10^{-8}M$ $^{109}Cd^{2+}$ concentration).

Time	$^{109}Cd^{2+}$ counts ratio in experiment (a)	$^{109}Cd^{2+}$ counts ratio in experiment (b)	$^{109}Cd^{2+}$ counts ratio in experiment (c)
0.0	10:1	7:1	11:1
15 min	15:1	14:1	17:1
30 min	24:1	19:1	21:1
1 h	34:1	21:1	27:1
2 h	44:1	34:1	37:1
6 h	49:1	43:1	48:1
1 day	47:1	39:1	41:1

Table 4.1. demonstrating the increase in the ratio of $^{109}Cd^{2+}$ counts in 1 mm^3 packed volume of cells/$^{109}Cd^{2+}$ counts in 1 mm^3 of treatment medium with the time of exposure in the three $^{109}Cd^{2+}$ uptake experiments carried out.

Where, a) Cells treated with $10^{-8}M$ $^{109}Cd^{2+}$ treatment medium, with no change of medium during the experiment (section 4.2.1.)

b) Cells treated with $10^{-8}M$ $^{109}Cd^{2+}$ treatment medium, with treatment medium replaced twice during the experiment (section 4.2.2.)

c) Cells treated with a preused treatment medium i.e. a medium initially at $10^{-8}M$ $^{109}Cd^{2+}$ but from which some $^{109}Cd^{2+}$ had already been removed in an earlier experiment (section 4.2.3.)

Amoebae are capable of accumulating Cd , such that the intracellular concentrations reached many times the levels of the surrounding treatment media. These uptake experiments suggest that the toxic effects of very low concentrations of Cd present in the environment, concentrations which may generally be regarded as relatively harmless, can be greatly magnified within living systems, which accumulate Cd such as the amoeba. This accumulative nature of Cd has been observed in a wide range of organisms, vertebrates, invertebrates, microorganisms and plants (Taylor 1977 also see chapter 1).

4.2.3. $^{109}Cd^{2+}$ Uptake by amoebae continuously cultured in unlabelled Cd^{2+} containing medium, i.e. Cd-adapted amoebae.

This set of experiments investigates the course of $^{109}Cd^{2+}$ uptake by amoebae which had been growing and dividing for prolonged periods in the presence of low levels of unlabelled Cd^{2+}. The amoebae used in these experiments had been kept in Cd containing medium at either 10^{-8}M for 12 months, or at 10^{-7}M for 9 and 11 months. During these long periods the culturing conditions, such as changing of medium and feeding on tetrahymena, were carried out as normal except for Cd being added to the media (MCS) to a concentration of 10^{-8}M or 10^{-7}M.

In the $^{109}Cd^{2+}$ uptake experiment carried out on the amoebae which had been growing in 10^{-8}M Cd^{2+} for 12 months, the treatment medium used was 10^{-8}M $^{109}Cd^{2+}$. The rest of the experimental conditions, sampling and preparations for scintillation counting, were as for previous experiments. Fig. 4.2.a & b, shows the uptake

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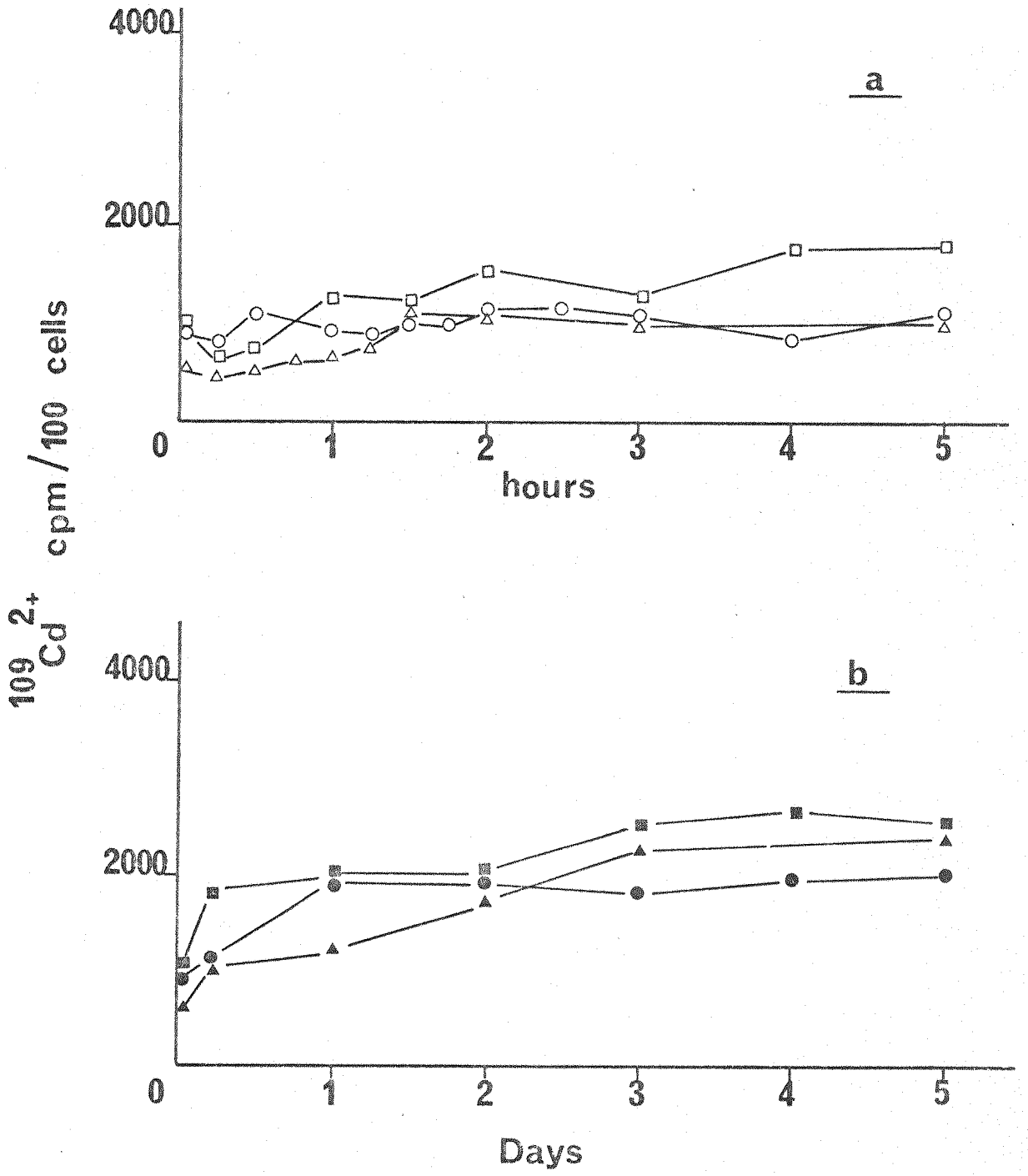
Fig. 4.2. Uptake of $^{109}Cd^{2+}$ by amoebae already cultured in 10^{-7}M Cd^{2+} for 9 months □■ and 11 months ○● and in 10^{-8}M Cd^{2+} for 12 months △▲ , from 10^{-7}M 10^{-7}M, and 10^{-8}M $^{109}Cd^{2+}$ treatment media respectively.

(a) Show a slight and consistent increase in $^{109}Cd^{2+}$ counts per 100 cells over the first 5 h of exposure.

(b) Shows the gradual increase in $^{109}Cd^{2+}$ counts per 100 cells over a period of 5 days. The slow steady pattern of increase was maintained throughout the experiment.


4000
2000
0
1
2
3
4
5
a
hours
$^{109}Cd^{2+}$ cpm / 100 cells
4000
2000
0
1
2
3
4
5
b
Days

of $^{109}Cd^{2+}$ by these cells. Unlike the pattern of $^{109}Cd^{2+}$ uptake shown in fig. 4.1., in this curve there is no initial rapid uptake of $^{109}Cd^{2+}$ but a slow and gradual increase in $^{109}Cd^{2+}$ counts within the cells as the time of exposure increased.

This experiment was repeated using amoebae cultured in 10^{-7}M Cd^{2+} for 9 months and 11 months. The cells were exposed to 10^{-7}M $^{109}Cd^{2+}$ treatment medium, made up with one part of labelled $^{109}Cd^{2+}$ plus nine parts of unlabelled Cd^{2+}. The rest of the experimental conditions were as indicated previously. Fig. 4.2. a & b again show a slow and gradual increase of $^{109}Cd^{2+}$ counts within the cells with the time of exposure. Each experiment was carried out in duplicate for confirmation.

These experiments showed that cells which had been cultured in the presence of Cd^{2+} for a long time did not show the initial rapid uptake of $^{109}Cd^{2+}$ observed for control cells, fig. 4.1. It suggests that these cells had already reached either the 2nd plateau or the 2nd slow uptake stage as shown in fig. 4.1. Thus the labelled $^{109}Cd^{2+}$ measured in the cells in these experiments may either represent a very slow $^{109}Cd^{2+}$ uptake by the cells or a gradual exchange of $^{109}Cd^{2+}$ for the unlabelled Cd^{2+} already present within the cell.

4.3. The retention of $^{109}Cd^{2+}$ by Amoeba proteus:

In the previous section it was demonstrated that $^{109}Cd^{2+}$ was taken up and accumulated by amoebae. In this section, the experiment carried out was to investigate whether the $^{109}Cd^{2+}$

taken up is retained by or lost from the cells on removal from the $^{109}Cd^{2+}$ treatment medium.

An experiment was set up in which the cell concentration was 500 cells/ml of 10^{-8}M $^{109}Cd^{2+}$ treatment medium, (the experimental conditions and the feeding/starving protocol were carried out as in the uptake experiments). The cells were exposed to the 10^{-8}M $^{109}Cd^{2+}$ treatment medium for 48 h, after which it was poured off and the cells harvested. To avoid any cell loss the treatment medium was centrifuged, and any cells recovered were returned to the treatment vessel. The harvested cells were washed 4 times and resuspended in MCS at the original cell concentration, i.e. the volume of the MCS added was equal to the volume of the discarded treatment medium. Another change of medium was carried out on the 10th day from the beginning of the experiment with the cells washed and resuspended in fresh MCS, as above. At different intervals 2 ml samples were removed and prepared for scintillation counting (section 2.4.1.)

The results obtained are shown in fig. 4.3. This curve can be divided into 3 parts:

1) The $^{109}Cd^{2+}$ treatment period (first 48 h of the experiment). The curve shows the uptake of $^{109}Cd^{2+}$ by the cells.

2) The first change (this period was between day 2 and day 10); the cells were removed from $^{109}Cd^{2+}$ treatment medium and returned to MCS. The curve shows a decrease of the $^{109}Cd^{2+}$ counts/cells. This decrease corresponded to a slight increase in the $^{109}Cd^{2+}$ counts/ml of MCS.

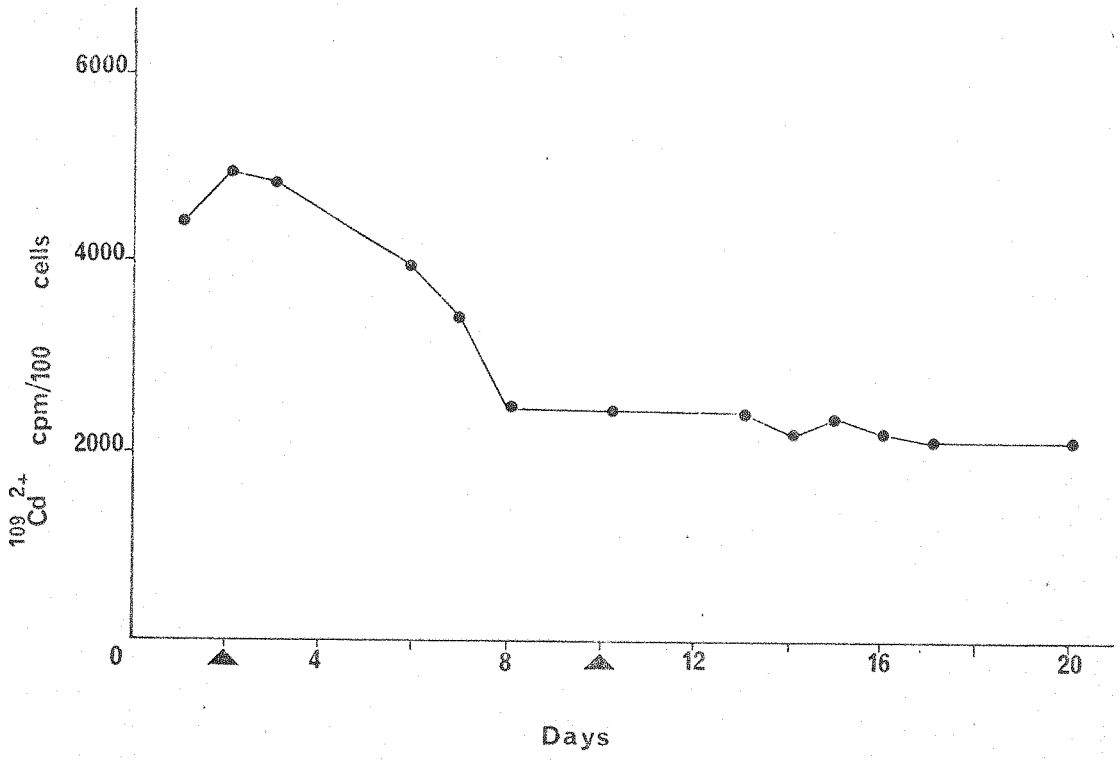


Fig. 4.3. Retention of $^{109}Cd^{2+}$ by amoebae. From 0 to day 2 the cells were in 10^{-8}M $^{109}Cd^{2+}$ treatment medium at a cell concentration of 500 cells/ml. On day 2 the cells were washed 4 times and transferred to MCS, maintaining the same cell concentration. On day 10 another change of medium was carried out.

The curve shows that approximately 50% of the $^{109}Cd^{2+}$ taken up by the cells was retained even after cell removal to MCS and over a period of 18 days.

3) The second change (this period was between day 10 and day 20), the cells were removed to fresh MCS. The curve shows a stable level of $^{109}Cd^{2+}$ counts/cells. The $^{109}Cd^{2+}$ counts/ml MCS was almost at background level.

This experiment demonstrates that after the removal of the cells from the $^{109}Cd^{2+}$ treatment medium to MCS, there was an initial loss of $^{109}Cd^{2+}$ from the cells, but that approximately 50% of the initial $^{109}Cd^{2+}$ taken up was retained within the cells. This may be Cd^{2+} which is firmly bound to the intracellular components. Similar results have been observed in Staphylococcus aureus where the cells were shown to retain 40% of the initial Cd^{2+} taken up, Chopra (1971).

4.4. The distribution of $^{109}Cd^{2+}$ within the cellular fractions.

The previous experiments showed that Cd^{2+} was taken up by amoebae, and that approximately 50% of this Cd^{2+} was retained by the cells for at least 18 days after their removal from the cadmium treatment medium to MCS (i.e. 50% remained in the amoebae for the duration of the experiment). In the following experiment, an investigation was carried out to find which cellular components are associated with the retained Cd^{2+}.

The cells for this investigation were treated with 10^{-8}M $^{109}Cd^{2+}$ treatment medium for 2 days. The experimental conditions and the feeding/starving protocol were as in the previous experiments. The cell concentration was again at 500 cells/ml. After treatment the cells were removed from $^{109}Cd^{2+}$ treatment medium, washed 4 times

and resuspended in MCS at the same cell concentration. The cells were kept in MCS for another 5 days. During the experiment there was no feeding. At each of the intervals shown in table 4.2. a & b a 10 ml sample was removed and the cells washed in MCS by centrifugation. The pellet of cells was then fractionated into the nuclear/cell membrane, microsomal, and cytosol fractions. Aliquots from each fraction were prepared for scintillation counting as described in chapter 2. (section 2.4.1.)

The results obtained from this experiment are given in table 4.2. a & b. These show that though there is an initial high association of $^{109}Cd^{2+}$ with the fraction containing cell membrane and nuclei, with time most of the $^{109}Cd^{2+}$ taken up gradually became associated with the cytosol fraction. The $^{109}Cd^{2+}$ level within the different fractions did not vary greatly on removals of cells from the $^{109}Cd^{2+}$ treatment medium to MCS; i.e. relatively high levels of $^{109}Cd^{2+}$ counts were maintained within the various fractions in the same distribution. The table 4.2.a. show the proportion, as percentages, of $^{109}Cd^{2+}$ counts associated with each of the cellular fractions at known exposure times. Thus, from 30 min to 5 h of exposure the lowest proportion of $^{109}Cd^{2+}$ counts was consistently found to be associated with the microsomal fraction. The proportion of $^{109}Cd^{2+}$ counts in the nuclear/cell membrane fraction decreased from 64% at 30 min to 17% by 24 h, after which it only changed slightly. The proportion of $^{109}Cd^{2+}$ counts in the microsomal fraction, gradually increased from 6% at 30 min to 23% by 24 h, after which this level was maintained although with minor fluctuation. The proportion of $^{109}Cd^{2+}$ counts in the cytosol fraction increased from 48% at 30 min

up to 60% on day one and thereafter remained at that level.

Initially, the proportion of $^{109}Cd^{2+}$ counts in the nuclear/cell membrane and the cytosol fractions were approximately the same, (46% and 48% respectively), at day one, i.e. 24 h, the proportion in the nuclear/cell membrane fraction had fallen to 17%, whereas that of the cytosol fraction had risen to 60%.

To find whether the 12% $^{109}Cd^{2+}$ counts of the nuclear/cell membrane fraction represented $^{109}Cd^{2+}$ associated with the cell membrane or $^{109}Cd^{2+}$ associated with the nuclear constituents a further separation of these two fractions was undertaken as described in section 2.4.2. i.e. The cell membrane fraction was separated from the nuclear fraction according to the method of Hourani et al (1973). The results of the separation are given in table 4.2.b. They show that after 2 days from the beginning of the experiment, the $^{109}Cd^{2+}$ counts in the cell membrane fraction were very low when compared with the other cellular fractions including the nuclear fraction. However, during the course of separation a high percentage of $^{109}Cd^{2+}$ counts were lost to the solutions used in the separation procedure. Since it was not possible to assess the relative losses of $^{109}Cd^{2+}$ from the separated nuclear and cell membrane fractions it was decided to discontinue this separation and to treat the nuclear/cell membrane fraction as one fraction. Though this leaves the role of the cell membrane and the Cd accumulation unclear, the 5-6% which consistantly separated with the nuclei does show that a minimum of 5-6% of the Cd is reaching and being retained by the nucleus.

Table 4.2.(a). The proportions, expressed as percentages, of $^{109}Cd^{2+}$ counts in the different cellular fractions obtained from $^{109}Cd^{2+}$ treated amoebae.

Time	Nuclear/cell membrane fraction	Microsomal fraction	Cytosol fraction
30 min	46%	6%	48%
1 h	47%	9%	44%
2 h	40%	11%	49%
5 h	34%	10%	57%
1 day	17%	23%	60%
2 days	12%	20%	68%
Cells removed from $^{109}Cd^{2+}$ treatment medium to MCS.			
3 days	15%	21%	64%
4 days	11%	26%	63%
7 days	14%	24%	62%

Table 4.2.(b)

As for (a) except that in these determinations the nuclear fraction was separated from the cell membrane fraction. Counts were then made on the $^{109}Cd^{2+}$ associated with the nuclear, the cell membrane and the separation solutions, and compared with the counts of the microsomal and the cytosol fractions of the initial centrifugation separation.

Time	Nuclear fraction	Cell membrane fraction	Separation solutions	Microsomal fraction	Cytosol fraction
2 days	6%	1.4%	11.6%	18%	63%
2 days	5.9%	1.3%	10.8%	20%	62%
2 days	5%	1.0%	10.0%	19%	65%

4.5. Gel separation of the cytosol fraction.

In the last experiments it has been shown that much of the $^{109}Cd^{2+}$ which was taken up and retained by the cells was associated with the cytosol fraction. The following set of experiments was carried out in order to investigate whether the $^{109}Cd^{2+}$ was firmly bound to certain protein molecules or was loosely associated with the cytosol fraction.

Batches of amoebae were set at 80,000 cells/batch and at 500 cells/ml of 10^{-8}M $^{109}Cd^{2+}$ treatment medium. The different batches were treated over the time intervals: 30 min, 1 h, 2 h, 5 h, 24 h and 48 h. Each batch was processed in the following manner. After each treatment the cells were harvested and washed 4 times in MCS by centrifugation. The pellet obtained was then fractionated into the nuclear/cell membrane, the microsomal, and the cytosol fractions. Aliquots from the different fractions were prepared for scintillation counting as given in the methods (section 2.4.1.). 2.5 ml of the cytosol fraction was separated on a Sephadex G 75 column. The eluted protein fractions were collected in 2 ml aliquots using a fraction collector. From each of the 2 ml aliquots collected, 0.5 ml was prepared for scintillation counting. Fig. 4.4. shows a time series study of the gel separation of the different cytosol fractions.

Two $^{109}Cd^{2+}$ binding protein peaks appeared: peak I appeared at the high MW level (over 45,000) and peak II appeared at the low MW level (10,000-12,000). The $^{109}Cd^{2+}$ counts in both peaks are seen

to increase almost in step with the time of exposure. The position of peak II on the curve was found to correspond with that of the rat liver Cd-thionein used in calibrating the column, fig. 2.2.

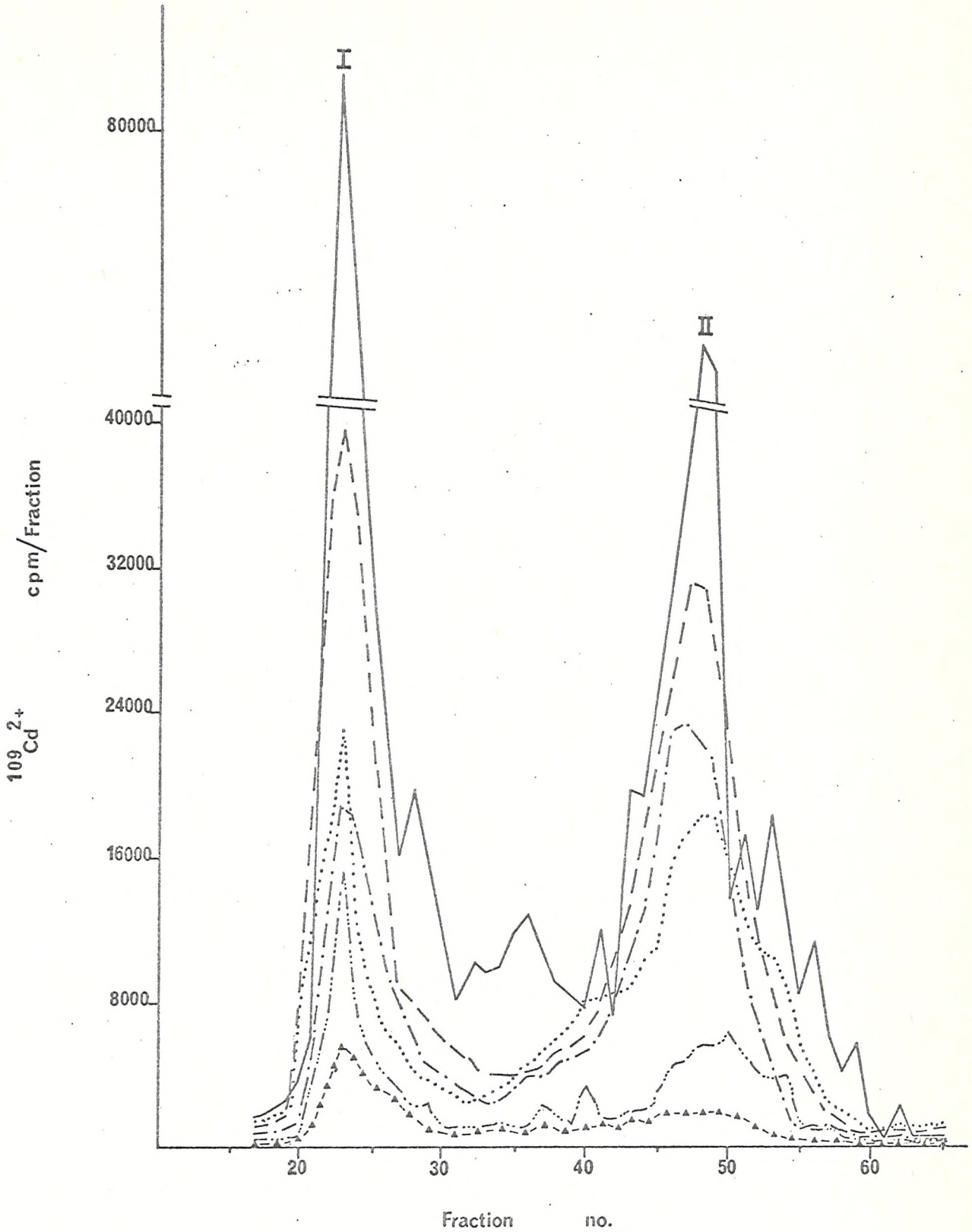
The UV absorbance trace measuring the protein level at peaks I and II (fig 2.1.) showed that there was a relatively high protein level at peak I but no reading was obtained for the protein level at peak II, indicating a very low level of protein at that peak. Protein estimation using the Lowry (1951) technique was attempted, but the protein levels were found to be below the sensitivity of the technique.

Comparison of the UV absorbance measurements for the protein level (fig. 2.1.) with the $^{109}Cd^{2+}$ counts measurements (fig. 4.4.) obtained for these peaks suggests that the protein of peak II has a greater binding capacity for Cd^{2+} than the protein of peak I. i.e. the proportion of Cd^{2+}/protein molecule for the protein at peak II is likely to be greater than that for the protein at peak I. Examination of the curves published by Nordberg et al (1971a) in fig. 1. (Sephadex G 75, separation of the liver protein from mice) on page 463 and fig. 2. A and B, (Sephadex G 100, separation of liver protein from mice) on page 465 and also the curves published by Shaikh & Smith (1976) in fig. 3. (gel filteration of liver supernatent from control and Cd-exposed rats) on page 332, reveals a similarity to the data obtained for the amoeba work. In their work the metalloproteins were also barely detectable by UV absorbance, indeed in the former paper (Nordberg et al 1971a) demonstration of the production of Cd-thionein was dependent on the atomic absorption of

Fig. 4.4. Gel separation of amoebae cytosol fractions obtained from 6 different batches of amoebae treated with 10^{-8}_{M} $^{109}Cd^{2+}$ over the following exposure periods: 30 min ▲——▲ ; 1 h —···— ; 2 h ····· ; 5 h —— · ——; 24 h — — — — ; and 48 h ———— . Data are plotted as total $^{109}Cd^{2+}$ counts/min/fraction against fraction numbers.

$^{109}Cd^{2+}$ was found to be incorporated into two peaks, peak I (protein(s) of MW over 45,000) and peak II (protein(s) of MW 10,000 to 12,000).

The increase in the total whole cell $^{109}Cd^{2+}$ counts/min/100 cells with time was: 147 at 30 min; 850 at 1 h; 1990 at 2 h; 3,190 at 5 h; 3,250 at 24 h and 3,840 at 48 h.


I
II
80000
40000
32000
24000
16000
8000
cpm/Fraction
$^{109}Cd^{2+}$
20
30
40
50
60
Fraction no.

Cd^{2+} bound to it.

There are three possible explanations for the increase in Cd-binding proteins of peaks I and II with time of exposure to $^{109}Cd^{2+}$.

a) The increase in $^{109}Cd^{2+}$ counts with time could represent an accumulative binding of $^{109}Cd^{2+}$ with time to pre-existing proteins.

b) The Cd-binding proteins of peak I and II could be induced proteins and thus synthesised in response to $^{109}Cd^{2+}$.

c) The Cd-binding proteins could have been pre-existing within the cells at lower levels which were then increased in response to $^{109}Cd^{2+}$.

To find out, which if any, of these apply the following experiments were carried out.

4.6. The use of L-^{35}S-cysteine as a marker in the study of Cd-binding proteins.

Many authors have shown that Cd-thioneins from mammals and other animals contain high levels of cysteine, 24.3 to 34.6%, (e.g. Webb 1979a; Margoshes & Vallee 1957; Kagi & Vallee 1960, 1961; Pullido et al 1966; Winge & Rajagopalan 1972, Weser et al 1973 a, b; Kojima et al 1975 and Noel-Lambot 1975). Further, several authors have demonstrated the incorporation of cysteine into newly produced Cd-thionein of animals *in vivo* and mammalian cell lines *in vitro* (e.g. Shaikh & Smith 1976, 1977; Gunn et al 1968 a, b; Bryan & Hidalgo 1976; Hidalgo et al 1978). Therefore, in the present study cysteine was used in combination with Cd^{2+} in order to elucidate the following points:

i) Is cysteine an important component of the amoebae Cd-binding proteins of peak I and/or peak II of fig. 4.4.?

ii) Do the Cd-binding proteins of peak I and/or peak II (fig. 4.4.) exist in control amoebae? i.e. are they present before the Cd^{2+} treatment?

iii) Would added cysteine affect the total Cd^{2+} uptake and its subsequent distribution within the cytosol fraction of amoebae?.

In amoebae two $^{109}Cd^{2+}$ peaks were found, section 4.5. (peak I of high MW and peak II of low MW). The Cd-binding protein of peak II, i.e. the low MW protein appeared analogous in position to the rat liver Cd-thionein used in calibrating the Sephadex G 75 column. In general the work on mammalian whole tissues does not show a high MW protein corresponding to the amoebae protein of peak I. This is because the mammalian high MW proteins were removed from the cytosol fraction before gel filtration by methods such as: heat-denaturation treatment (Webb 1972a), precipitation with rivanol (2-ethoxy-6,9-diaminoacridine lactate); (Piscator 1964, Nordberg et al 1972), or ultrafiltration through appropriate membrane filters (Riordan & Gower 1975). However, high MW proteins corresponding to that of amoebae of peak I were obtained from the gel filtration of cytosol fractions obtained from several mammalian cell lines in tissue culture (Rudd & Herschman 1978, Rugstad & Norseth 1978; and Hidalgo et al 1978), also from the gel filtration of cell homogenate obtained from the flagellate Crithidia fasiculata (Maclean et al 1972).

4.6.1. Uptake and incorporation of L-^{35}S-cysteine by Amoeba proteus.

In amoebae the uptake and incorporation of amino acids is greatly affected by their nutritional condition. Fed amoebae are likely to have within their amino acid pools high levels of various

amino acids including cysteine readily available for protein synthesis. Therefore, the L-^{35}S-cysteine taken up will be diluted by unlabelled cysteine already present within the cell. Starved amoebae on the other hand, require a complete diet containing all the essential amino acids in order to synthesize proteins. Therefore, even if there was some L-^{35}S-cysteine taken up by the starved cells, very little would be incorporated into proteins unless all other components also become available.

In this set of experiemnts 4 different batches of amoebae were used:

a) Starved control amoebae.

b) Fed control amoebae.

c) Starved Cd^{2+} treated amoebae, (the cells were kept for 48 h in 10^{-8}M Cd^{2+} containing medium, without food).

d) Fed Cd^{2+} treated amoebae, (the cells were kept for 48 h in 10^{-8}M Cd^{2+} containing medium with food.

All cells were treated with 10^{-9}M L-^{35}S-cysteine at a cell concentration of 500 cells/ml. For the total uptake experiment where counting was of homogenates of whole cell samples suspended in Insta-gel, both the ^{35}S-cysteine in the amino acid soluble pools and that incorporated into protein would be included in the counts. For the incorporation experiment where the proteins were precipitated with TCA (Trichloroacetic acid) on the filter paper discs and all the unincorporated amino acids were thoroughly washed away the counts would be of the ^{35}S-cysteine incorporated into protein only.

4.6.2. Total uptake of L-^{35}S-cysteine by A. proteus

During exposure to L-^{35}S-cysteine, 2 ml samples were removed from each of the 4 treated batches at a number of time intervals. These cells were washed 4 times in MCS and their final pellets prepared for scintillation counting as detailed in the Insta-gel preparations of section 2.4.1.

The total L-^{35}S-cysteine uptakes by the cell samples from the 4 different batches are shown in fig. 4.5. It is clear that the factor which affected the total uptake of L-^{35}S-cysteine was the nutritional status of the cell. The starved cells of both the Cd-treated and the control batches did not take up any appreciable amounts of ^{35}S-cysteine, whereas, the fed cells, again both the Cd-treated and the control batches, showed the same distinct pattern of uptake. There was an initial rapid uptake of ^{35}S-cysteine which lasted up to about 30 min when the levels of ^{35}S-cysteine in the cells suddenly fell to less than one third of their peak value. This resulting level was then maintained more or less constant until the end of the experiment. From the similarity of the curves for fed amoebae and of that for starved amoebae it is apparent that the treatment with Cd^{2+} had little or no effect on the total uptake of ^{35}S-cysteine.

4.6.3. Protein incorporation of L-^{35}S-cysteine by A. proteus

To find the level of incorporated L-^{35}S-cysteine, in each of the 4 treatment batches, 0.1 ml samples were removed at a number

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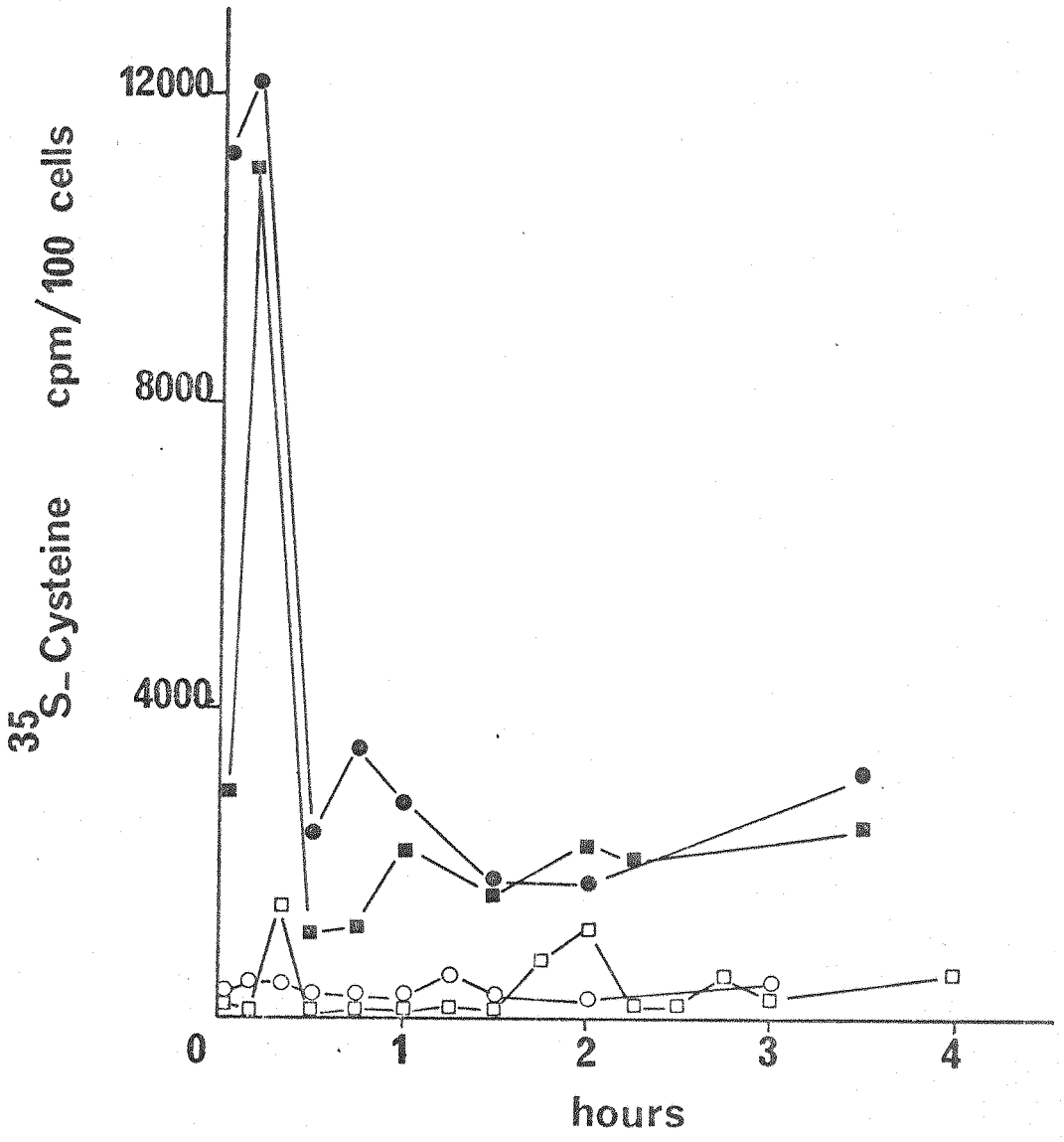
Fig. 4.5. Total uptake of L^{35}-S-cysteine by amoebae over a 4 h period.

Four batches of amoebae were exposed to 10^{-9}M L-^{35}S-cysteine, (a) control fed amoebae ●; (b) control starved amoebae ○; (c) Cd^{2+} treated fed amoebae ■; and (d) Cd^{2+} treated starved amoebae □.


12000
8000
4000
^{35}S-Cysteine cpm/100 cells
0
1
2
3
4
hours

of time intervals, placed on 3 mm Whatman filter discs and treated with TCA (trichloroacetic acid) according to the method described in chapter 2, (section 2.6.). The scintillation counting solution used in counting the incorporated L-^{35}S-cysteine precipitated on the discs was Schram's fluid (Schram 1963). It is noteworthy that the efficiency of this counting solution is approximately one fifth of that of Insta-gel which was used in the determination of the counts of total ^{35}S-cysteine taken up by the cells.

The incorporated ^{35}S-cysteine by the cell samples from the 4 different batches are shown in fig. 4.6. Similar to the results obtained for the total uptake of ^{35}S-cysteine it was found that the amount of ^{35}S-cysteine incorporated into proteins by the cell was dependent on the nutritional status of the cell. The fed cells (Cd-treated and control) showed a steady increase with time in the level of incorporated ^{35}S-cysteine. After 2 h the curves for the control and the Cd-treated fed amoebae seemed to diverge to some extent, and it may be that in the Cd-treated fed amoebae an effect of Cd^{2+} on protein synthesis could have prevented the same levels of incorporation of ^{35}S-cysteine obtained for the control fed amoebae. The significance of this observation, however, remains to be tested. The starved amoebae of both groups showed little or no increase in ^{35}S-cysteine incorporation from time zero until the end of the experiment, and thus, these curves demonstrate a lack of ^{35}S-incorporation by the starved cells. It is noteworthy that the Cd-treated starved amoebae had a higher 'background' level of ^{35}S-cysteine counts than did the control starved amoebae.

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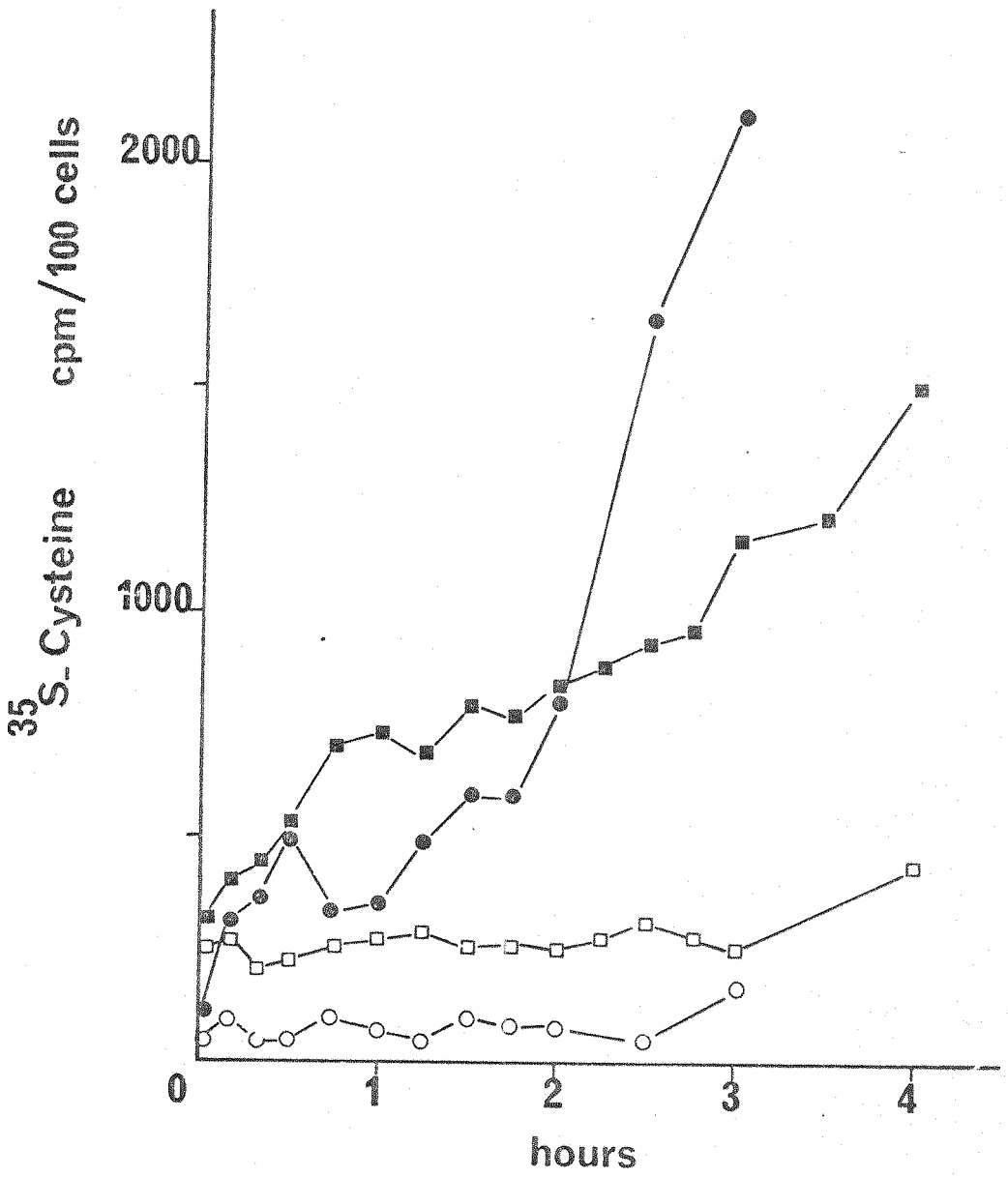
Fig. 4.6. The incorporation of L-^{35}S-cysteine by amoebae over a 4 h period.

The same batches of amoebae as in fig. 4.5. (a) control fed amoebae ●; (b) control starved amoebae ○; (c) Cd^{2+} treated fed amoebae ■; and (d) Cd^{2+} treated starved amoebae □; were used at the same time to measure the incorporated levels of L-^{35}S-cysteine.


35S- Cysteine cpm/100 cells
2000
1000
0
1
2
3
4
hours

It was concluded from these results that in order to obtain a reasonable level of ^{35}S-cysteine uptake and incorporation into the cells it was necessary to use fed cells. It was therefore, decided that over the last 24 h prior to treatments all cells should be given a minimum feeding. This is in line with the experimental procedure used previously in this chapter.

4.7. The incorporation of L-^{35}S-cysteine into the Cd-binding protein of peaks I and II in Amoeba proteus

In these experiments the combination of labelled L-^{35}S-cysteine and unlabelled Cd^{2+} was used. The experimental cells were given minimum feeding during the last 24 h prior to the experiment. During the experiment there was no food given. Batches of 80,000 cells/batch at a cell concentration of 500 cells/ml were treated with 10^{-8}M Cd^{2+} (unlabelled) and 10^{-9}M L-^{35}S-cysteine together for 48 h. As a control, a similar batch of cells was treated with 10^{-9}M L-^{35}S-cysteine only, over 48 h. After treatments, 2 ml samples were taken from each batch and prepared for scintillation counting to find the total ^{35}S counts in the whole cells. The rest of the cells were harvested, washed 4 times in MCS and fractionated as described in chapter 2. Aliquots of the nuclear/cell membrane, microsomal, and cytosol fractions from each batch were prepared for scintillation counting (section 2.4.1.). 2.5 ml of each of the cytosol fractions obtained were separated on the Sephadex G 75 column. The eluted protein fractions were collected and prepared for scintillation counting as in section 2.4.1.

The results obtained from this experiment are shown in fig. 4.7. Two ^{35}S-cysteine peaks appeared, the position of which corresponded with those of ^{109}Cd peaks I and II, shown in fig. 4.4. A third ^{35}S-cysteine peak appeared at a very low MW level representing the unincorporated L-^{35}S-cysteine (i.e. retained in the amino acid pools). In the curve for the control cells, i.e. cells exposed to ^{35}S-cysteine only, there were two major ^{35}S-cysteine peaks only. A ^{35}S-cysteine peak corresponding in position to ^{109}Cd peak I was obtained, but there was little to no ^{35}S-cysteine peak corresponding to ^{109}Cd peak II (of fig. 4.4.). The other major ^{35}S-cysteine peak appeared at a very low MW level representing unincorporated ^{35}S-cysteine. The other minor peaks shown are due to the incorporation of ^{35}S-cysteine into various proteins which are not Cd-binding proteins.

The difference in the size of ^{35}S-cysteine peak II (analogous to $^{109}Cd^{2+}$ peak II of fig. 4.4.) for the "Cd^{2+} plus ^{35}S-cysteine" treated cells as compared with the "^{35}S-cysteine" treated cells (fig. 4.7.) suggests that the Cd-binding protein of peak II has either greatly increased (if it was already present within the cells) or has been synthesized in response to Cd^{2+} treatment. Each of these treatments was repeated at least twice for confirmation.

4.8. The effect of L-cysteine on $^{109}Cd^{2+}$ uptake and distribution within the cytosol fraction of *Amoeba proteus*

In these experiments the combination of unlabelled L-cysteine and labelled ^{109}Cd was used.

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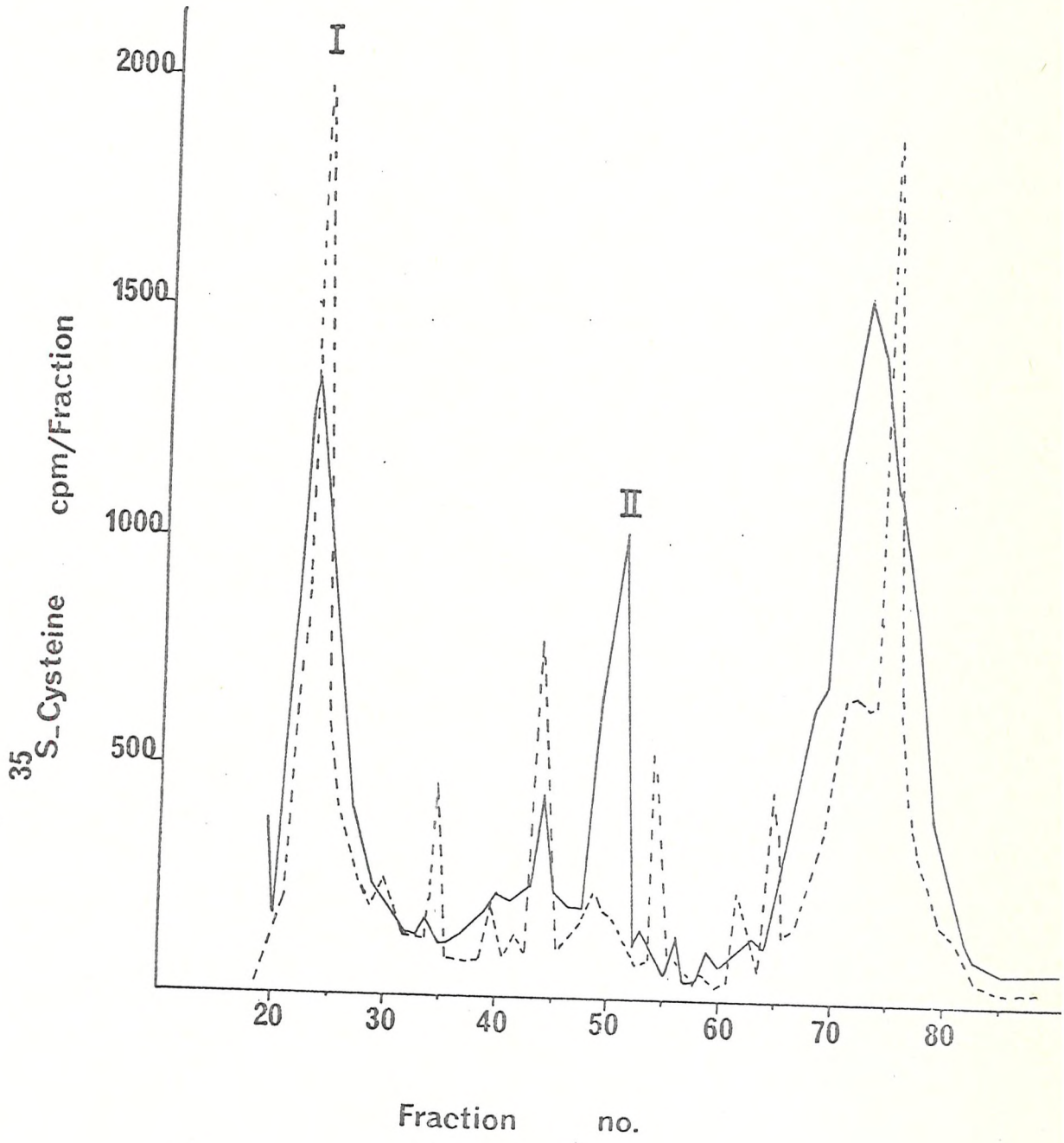
Fig. 4.7. L-^{35}S-cysteine distribution within the cytosol fraction of control and Cd^{2+} treated amoebae. Control amoebae given 10^{-9}M L-^{35}S-cysteine alone for 48 h (- - - -); amoebae given a dose of 10^{-8}M Cd^{2+} (cold) plus 10^{-9}M L-^{35}S-cysteine for 48 h (———). Data are plotted as total ^{35}S counts/min/fraction against fraction numbers.


I
II
2000
1500
1000
500
35S-Cysteine cpm/Fraction
20
30
40
50
60
70
80
Fraction no.

The aim of this experiment was to find out whether L-cysteine increased the uptake of Cd^{2+} in amoebae, (as is known to be the case in the mammalian system) and whether it affected the Cd^{2+} distribution between ^{109}Cd peaks I and II of figs. 4.4. and 4.7.

The experiment was set up as follows:

(i) 80,000 cells/batch at a cell concentration of 500 cells/ml was treated with $10^{-8}M$ $^{109}Cd^{2+}$ together with unlabelled $10^{-9}M$ L-cysteine for 48 h.

(ii) A control batch of amoebae was set up similar to the cysteine supplemented batch except that in this batch the cells were given $10^{-8}M$ $^{109}Cd^{2+}$, only, for the 48 h. After treatment both batches of cells were fractionated and aliquots (0.5ml) from the different fractions were prepared for scintillation counting as previously. 2.5. ml of the cytosol fraction from each batch was separated individually on the Sephadex G 75 column. 2 ml aliquots of the eluted protein fractions were collected using a fraction collector, and 0.5 ml from each was prepared for scintillation counting as before.

Results are shown in fig. 4.8. Both ^{109}Cd peaks I and II were found to have incorporated more $^{109}Cd^{2+}$ when the cells were treated simultaneously with L-cysteine and $^{109}Cd^{2+}$. However, the increase in $^{109}Cd^{2+}$ counts in peak II was much greater than that in peak I. These results indicate clearly that (i) the presence of L-cysteine increased the uptake of Cd^{2+} by amoebae. (ii) there was a disproportionate increase of $^{109}Cd^{2+}$ in the Cd-binding protein of peak II.

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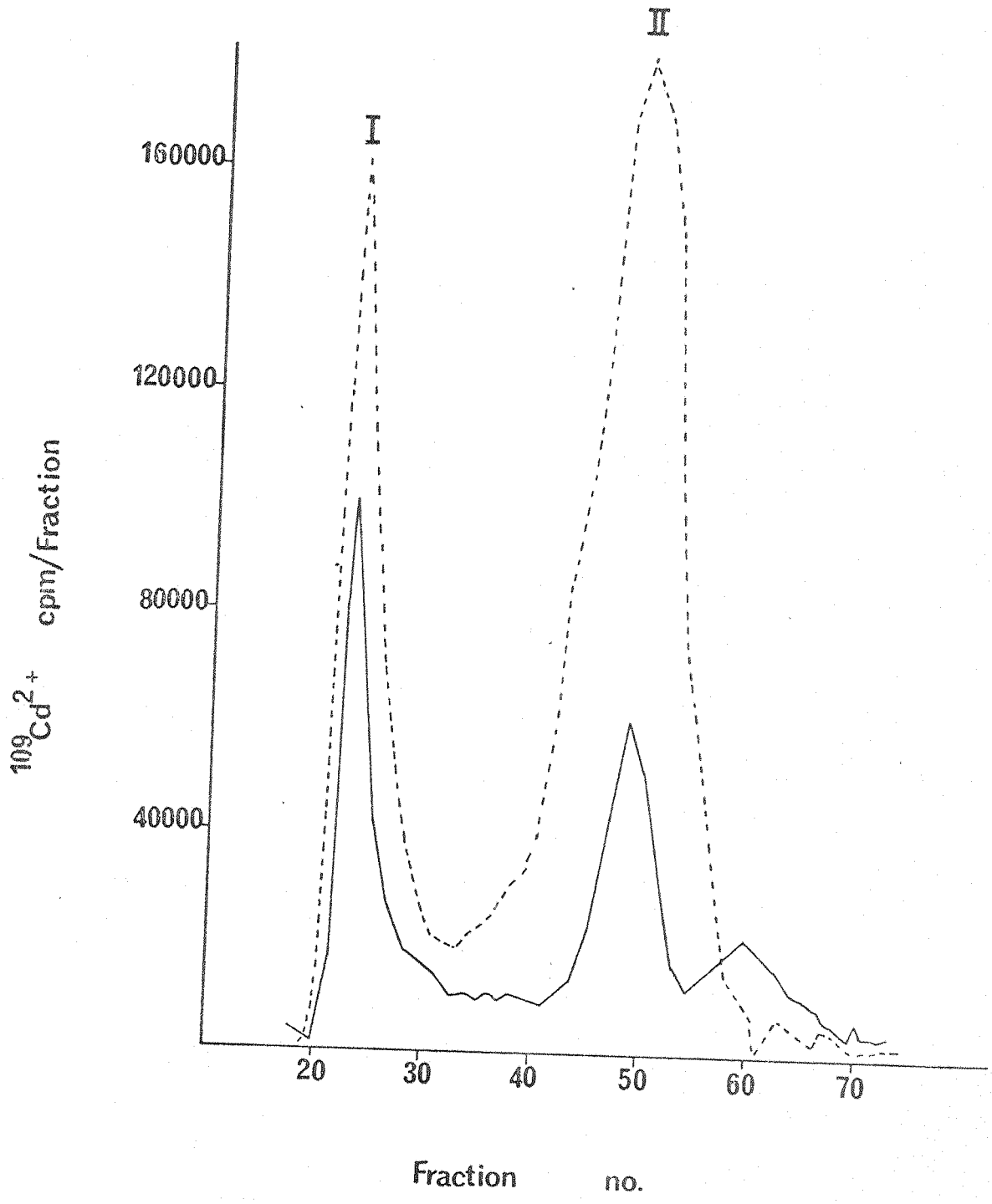
Fig. 4.8. The increase in $^{109}Cd^{2+}$ incorporation with added cysteine. Amoebae given a dose of 10^{-9}M L-cysteine (cold) plus 10^{-8}M $^{109}Cd^{2+}$ for 48 h (- - - -); amoebae given 10^{-8}M $^{109}Cd^{2+}$ alone for 48 h (———). Data are plotted as total $^{109}Cd^{2+}$ counts/min/fraction against fraction numbers.


II
I
160000
120000
80000
40000
$^{109}Cd^{2+}$ cpm/Fraction
20
30
40
50
60
70
Fraction no.

The UV trace obtained at both 280 nm and 254 nm wavelength for all the experiments carried out in sections 4.7. and 4.8. (i.e. figs 4.7. and 4.8.) remained similar to that shown in fig. 2.1. A comparison of the peaks from the UV trace for the protein levels with the peaks of activity from either ^{35}S-cysteine counts or $^{109}Cd^{2+}$ counts as shown in figs. 4.7. and 4.8. respectively, suggests that the proportion of cysteine/protein molecule and Cd^{2+}/protein molecule is higher in peak II than in peak I. i.e. the Cd-binding protein of peak II is richer in cysteine and Cd^{2+} than the protein of peak I.

4.9. The effect of freezing and thawing on the stability of Cd-binding proteins of peak I and II in amoebae.

When 2.5. ml of the ^{109}Cd treated amoebae cytosol fraction was frozen and thawed twice prior to gel separation, only ^{109}Cd peak I was obtained (though reduced in size) and no ^{109}Cd peak II. This suggests that the Cd-binding protein of peak II is susceptible to denaturation upon storage by freezing, therefore, all gel separations were carried out immediately after cell fractionation.

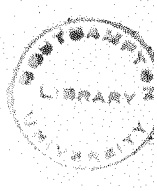
The mammalian metallothioneins have been reported to be thermostable (Webb 1972a). They resist heat-denaturation; heating at 80°C for 10 min which can denature 90% of the total protein of the tissue soluble fraction, however, they then become more susceptible to denaturation on storage (Webb 1979). Also a susceptibility of the hepatic Cd-thionein from a Cd treated rat to freezing and thawing treatments has been noted (M. Webb, personal communication). Therefore, when this protein (hepatic Cd-thionein from the rat) was

used in calibration for its position on the gel column, storage of the liver cytosol fraction was avoided i.e. the liver tissue was fractionated directly before the gel separation of its cytosol fraction.

4.10 Summary

The results obtained in this chapter have shown that Cd is taken up by amoebae in a biphasic pattern. Phase (1) rapid uptake followed by a plateau; phase (2) further uptake followed by a second plateau. Also amoebae were shown capable of accumulating Cd^{2+} to concentrations far greater than those of the surrounding medium. The ratio of the $^{109}Cd^{2+}$ counts in the protoplasm to the $^{109}Cd^{2+}$ counts in an equal volume of treatment medium can reach up to 49:1 (table 4.1.). Amoebae were also shown to retain 50% of their total $^{109}Cd^{2+}$ uptake even after 18 days from their removal from the $^{109}Cd^{2+}$ treatment medium.

The changes in the relative proportions of $^{109}Cd^{2+}$ counts in the different cellular fractions with time given in table 4.2. showed an initial phase, which can be recognised in the period up to 24 h of $^{109}Cd^{2+}$ treatment, where the relative proportions of $^{109}Cd^{2+}$ counts change considerably with time. These changes may reflect the course of $^{109}Cd^{2+}$ uptake by amoebae during phase (1), (rapid uptake followed by a plateau). After 24 h little further change in these proportions was observed. Considering the distribution of $^{109}Cd^{2+}$ whithin the cell after 48 h of treatment (i.e. after cells removal from the $^{109}Cd^{2+}$ treatment medium and when the relative proportions of $^{109}Cd^{2+}$



counts in the different fractions had stabilised), it is clear that most of the $^{109}Cd^{2+}$ taken up by the cells subsequently becomes associated with the cytosol fraction.

The results can also be expressed as $^{109}Cd^{2+}$ counts/volume of nucleus or cytoplasm. The cytoplasmic volume of A. proteus has been estimated as $1.0 - 1.6 \times 10^6$ μm^3 (Prescott 1955, James 1959) and the nuclear volume has been estimated as 1.5×10^4 μm^3 (Prescott 1955). Thus taking the median cytoplasmic volume, the nucleus occupies

$$\frac{1.5 \times 10^6}{(1.3 \times 10^6 + 1.5 \times 10^4)} \times \frac{100}{1} \% = 1.14\% \text{ of the cell volume.}$$

Table 4.2.a. shows that the proportion of $^{109}Cd^{2+}$ associated with the nuclear/cell membrane fraction stabilised in the region of 12%. Also in table 4.2.b., where the nuclear fraction was separated from the cell membrane fraction, the minimum proportion of $^{109}Cd^{2+}$ associated with the nuclear fraction was 5%. Thus we can set a range of 5 - 12% for the proportion of $^{109}Cd^{2+}$ associated with the nucleus. However, as calculated above, the nucleus represents only 1.14% of the cell volume. From this it can be calculated that the concentration of $^{109}Cd^{2+}$ in the nucleus must be 4.56 to 11.82 times that of the cytoplasm.

When the cytosol fraction of ^{109}Cd treated amoebae was separated on the Sephadex G 75, two Cd-binding protein peaks were found, one at a high MW, over 45,000 and the other at a low MW, approximately 12,000. The second peak (i.e. at 12,000 MW) is similar in position to that obtained for the rat liver Cd-thionein.

Further investigations using L-^{35}S-cysteine as a marker showed that the production of Cd-binding protein of peak II had been either induced or greatly increased (if it was already present in small quantities) in response to Cd^{2+}. The $^{109}Cd^{2+}$ incorporated into this protein increased several fold when cysteine was added. This could have been due to either an increase in the amount of $^{109}Cd^{2+}$ bound by the protein or to an increase in the total amount of protein.

Chapter Five

Ultrastructural study

5.1. Introduction.

Cadmium toxicity has been investigated at the ultrastructural level by many authors. Most of these investigations were carried out on various mammalian tissues; e.g. rabbits bone marrow, blood, liver, and kidney (Berlin et al 1961; Stowe et al 1972), rats liver, kidney and lung (Nishizumi 1972; Hoffmann et al 1975; Feader et al 1977; Takashima et al 1978; Fowler et al 1975; Hayes et al 1976) and mouse liver (Gamulin et al 1977). Cadmium has also been shown to cause ultrastructural changes in the gills of Jaera nordmani (crustacea) Bubel 1976), yeast (Lidegren & Lindegren 1973) and the slime mould Physarum polycephalum (Sina & Chin 1978, 1979).

The ultrastructural changes produced by Cd in A. proteus were found to be greatly dependent on the cadmium dose given (concentration and time of exposure). In this study the different doses of Cd^{2+} given can be classified into 3 groups:

a) High doses; Cd concentrations of 2×10^{-4}M given for 1 h and 1×10^{-4}M given for 1 h or 2 h.
b) Intermediate doses; Cd concentrations of 10^{-5}M and 10^{-6}M given for 3 and 6 days respectively.
c) Low doses: Cd concentrations of 10^{-8}M given for 1, 2, and 11 months and 10^{-7}M given for 1, 2 and 9 months.

A short exposure to a high concentration of Cd^{2+} resulted in a high level of cell death, but relatively less extreme ultrastructural changes could be observed. On the other hand, longer exposures to lower concentrations of Cd^{2+} demonstrated more ultrastructural changes yet the percent survivals were higher. In the latter case the cells

appeared to have a mixture of damaged and normal organelles. This might explain the cells ability to continue performing the necessary living activites despite the presence of some damaged organelles. Therefore, electron microscopic studies carried out on cells exposed to intermediate or low doses of Cd can be more revealing than those carried out on cells which receive high doses of Cd .

The major damages observed in Cd treated cells were mitochondrial damage, changes in the distribution of membrane bound ribosomes (RER), polyribosomes and free ribosomes (monosomes), and the accumulation of lipid droplets. Relatively minor changes were observed in the golgi bodies. Nuclear changes were not visualized by E.M. although the biochemical studies did indicate that an appreciable percentage of Cd was associated with the nuclear fraction at the early stages of exposure (section 4.4.)

5.2. General features of the mitochondria.

In A. proteus, as in other eukaryotes, the mitochondria consist of the following structures:

a) An outer limiting membrane: usually the outer membrane is freely permeable to charged and uncharged molecules of less than 10,000 MW. (Quinn 1976).
b) An inner membrane: this forms infoldings of variable length and of tubular shape into the stroma of the mitochondrion (cristae). Generally the inner membrane is only permeable to certain small uncharged molecules, such as H_2O, O_2, all less than 150 MW.
c) The inter-membrane space: this is the space between the inner and the outer membranes. In this space most of the anaerobic enzyme pathways are located (Quinn 1976).

d) An inner matrix surrounded and invaded by the inner membrane: the oxidation-phosphorylation reactions are carried out in the matrix or in the inner membrane of the mitochondrion (Quinn 1976). Changes in the matrix density was found to be dependent on the ADP level. At high ADP level, the matrix becomes condensed giving the mitochondrion a dark appearance, whereas, at low ADP level the matrix becomes expanded giving the mitochondrion a light appearance (Hackenbrock 1966). Another factor shown to affect the matrix density is Ca^{2+} uptake by the mitochondria in the presence of phosphates. This Ca^{2+} uptake causes various degrees of expansion within the inner mitochondrial components. These two factors affecting the matrix density are different from each other i.e. the addition of ADP did not affect the matrix density of a mitochondrion the matrix of which was expanded in response to the Ca^{2+} uptake in the presence of phosphate (Hackenbrock & Caplan 1969).

In A. proteus the matrix density is used to classify the mitochondria into three types (a) Type I, dark mitochondria, which are usually rod-shaped and have tubular cristae with a mean cristal width of 660 A. (b) Type II light mitochondria which have a more spherical shape and narrow more uniform cristae with a mean cristal width of 500 A (c) Intermediate mitochondria which have intermediate characteristics. Under normal conditions the proportion of the light mitochondria was equal to that of the dark mitochondria and there was usually 5-10% of the intermediate type. All mitochondria are claimed to be able to change from one type to another according to the cellular activity fig 5.1. (Flickinger 1968a). Besides the structural changes, various chemicals have shown to cause considerable changes in the ratio of the

different types of mitochondria (I & II) in A. proteus . For example, chemicals such as potasium cyanide, antimycin A and uncouplers (m-chlorocarbonyl cyanide phenylhydrazone, cccp, and pentachlorophenol, cpc) were found to turn large proportion of the mitochondria to type intermediate I or type intermediate II in density (Smith and Ord 1979), most Cd^{2+} treatments turned the majority of the mitochondria to type II, and intermediate type II.

Other forms of stress on amoeba, such as, starvation, anaerobiosis, enucleation, change in temperature, and change in pH, cause less obvious changes in the ratio of the different types of mitochondria, but in addition, they can produce structural changes.

5.3. Effects of Cd on the mitochondria.

In amoebae treated with the high doses of Cd some of the mitochondria became dilated and of irregular (lobular) shapes. There was a loss of internal structure, the cristae losing their identity resulting in a matrix of a coagulated appearance (fig. 5.3, 5.5 and 5.8). There were always some apparently normal mitochondria together with the damaged ones (figs. 5.4, 5.6 and 5.10). At these doses (i.e. 2×10^{-4}M for 1 h, 1×10^{-4}M for 1 or 2 h) the percents of survival were low, yet there were few visible ultrastructural changes. This suggests that the large number of visually normal mitochondria and other organelles may have had invisible damage caused by the sudden high toxicity of the Cd^{2+} dose. (Figs. 5.2.-10).

Similar changes were observed in amoebae treated with the intermediate doses of Cd (i.e. 10^{-5}M for 3 days and 10^{-6}M for 6 days). However, in these amoebae the mitochondrial damage was greater than that observed in the amoebae which had received the high doses. The damaged mitochondria were swollen and assumed irregular shapes, and in some, the outer and inner membranes were ruptured, the matrix was very light and appeared partially disintegrated (figs. 5.11 c and 5.12 a). In some mitochondria there was loss of cristae from the central region, resulting in a coagulated appearance (fig. 5.12 b). Together with the damaged mitochondria apparently normal ones were present (figs. 5.11 a & b).

More extensive damage was observed in the mitochondria of amoebae treated with the low doses of Cd (10^{-7}M and 10^{-8}M). After 1 and 2 months of exposure the overall appearance was one of disintegration, the mitochondria being obviously ruptured and "ghost like" (figs. 5.13 a, b; 5.16 c and 5.17 a). There were always some normal mitochondria of all types, dark, light and intermediate, but the greater number were of the light type (II), (figs. 5.13 c and 5.14 c). Together with the apparently normal and the extremely damaged (disintegrating) mitochondria, other stages of mitochondrial damage, including vacuolation, irregular outline, and rupture of outer and inner membranes, were observed in amoebae treated with the low doses of Cd (figs. 5.13 c, 5.14 a & b, 5.16 a & b, 5.17 a, b & c). After continuous exposure of amoebae to 10^{-8}M Cd^{2+} for 11 months and to 10^{-7}M Cd^{2+} for 9 months, it appeared that the amoebae were adapting to Cd^{2+} and recovering from the earlier damage. There appeared a level of apparently normal mitochondria similar to

that of the control, with the proportions of the 3 types of mitochondria returning to near control values. There were only a few mitochondria with a damaged appearance (figs. 5.15 a, b; and 5.18 a). No further studies were carried out on these "possibly Cd-adapted" amoebae except for the $^{109}Cd^{2+}$ uptake experiment described previousely (section 4.2.3.

Other authors have observed similar mitochondrial damage in the tissues of Cd treated rats, e.g. the mitochondria of the injured alveolar cells of the lung (Hayes et al 1976), of the liver parenchymal cells (Hoffmann et al 1975) and those of the kidney proximal tubular epithelium cells (Nishizumi 1972). Lindegren & Lindegren (1973) have reported a similar mitochondrial damage in Cd treated yeast, and also Bubel (1976) in the gill tissue of the crustacean Jaera nordmani.

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Ultrastructural changes in the mitochondria of Cd^{2+} treated amoebae.

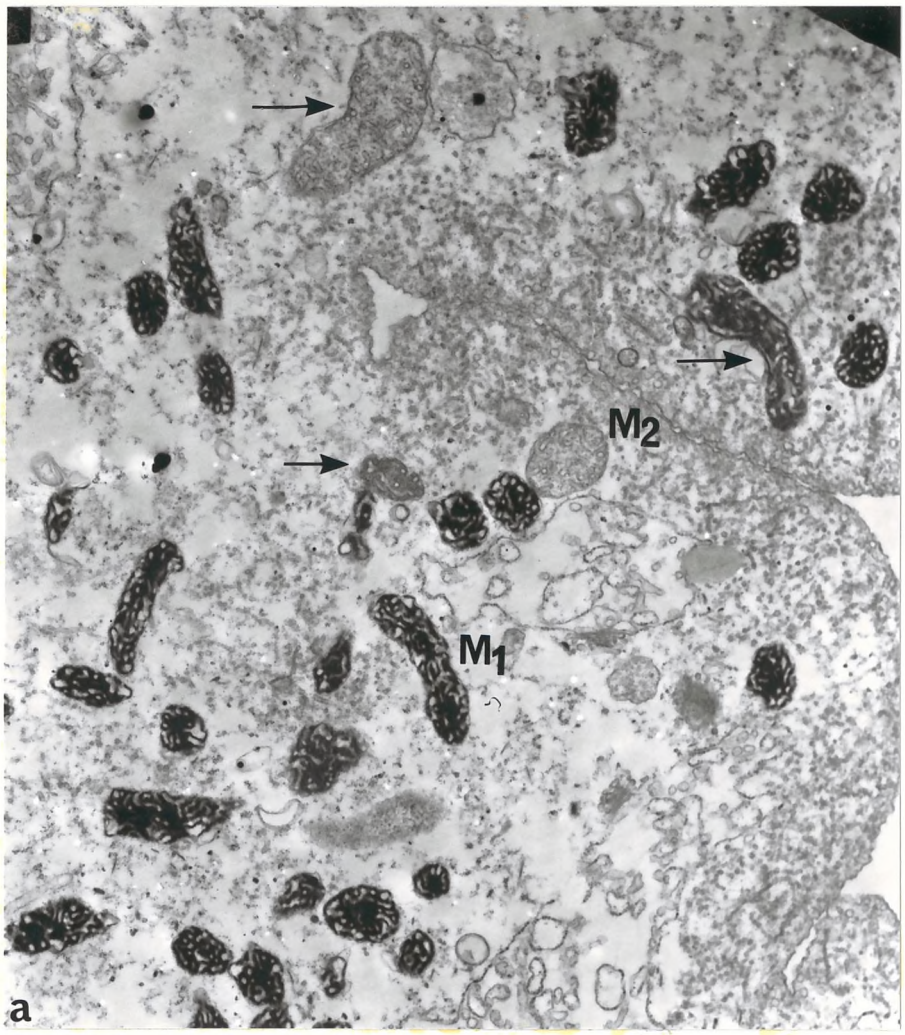
Fig. 5.1. Mitochondria of control amoeba

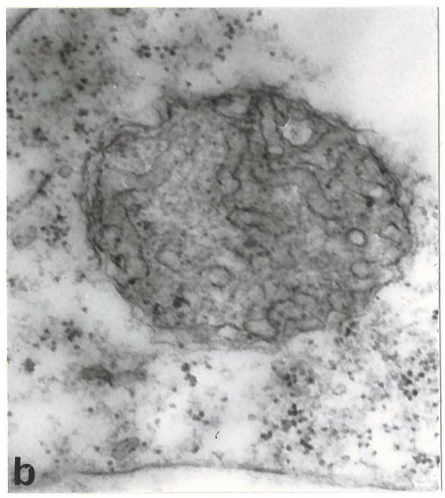
(a) Control mitochondria, type I, dark (M_1), type II, light (M_2), and mitochondria showing intermediate characteristics (arrow). Magnification 10000 x.

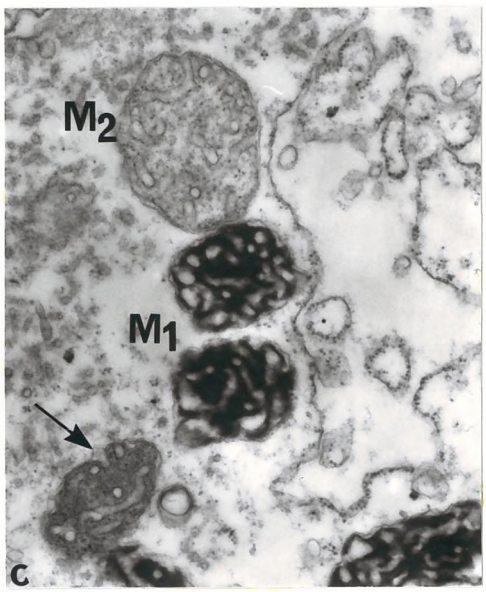
(b) Control mitochondria type II. Magnification 40000 x.

(c) Control mitochondria of the 3 types shown in (a) magnified to 20000 x.

5.1.







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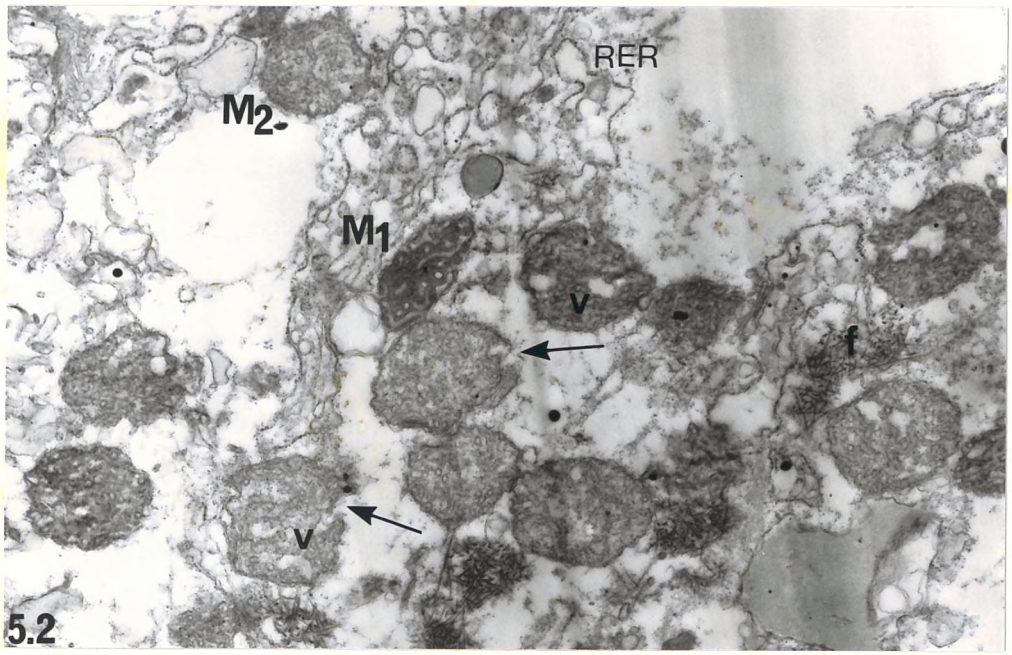
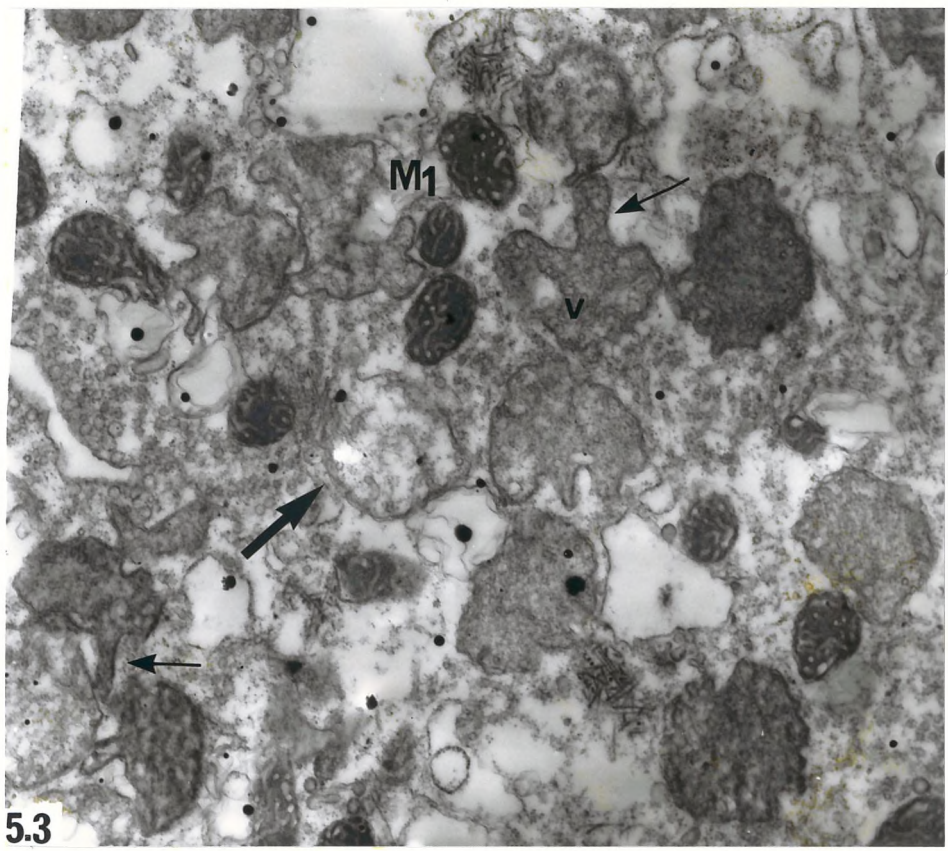
Amoebae treated with 2 x 10^{-4}M Cd^{2+} for 1 h.

Fig. 5.2. Group of mitochondria (types I, II, & intermediate). Some appear normal (M_1 and M_2). Whereas, others are damaged, typically by vacuolation of the matrix (v) and rupture of the outer and inner membranes (arrows). Also shown are clusters of microfibres (f) and RER. Magnification 12000 x.

Fig. 5.3. This micrograph illustrates further types of mitochondrial damage observed with the above Cd^{2+} treatment. Together with the apparently normal mitochondria (M_1) there are some disintegrating "ghost like" (large arrow), others showing considerable irregularity of outline (lobular) (small arrows) and others with vacuolated matrix (v). Magnification 15000 x.



5.2



5.3

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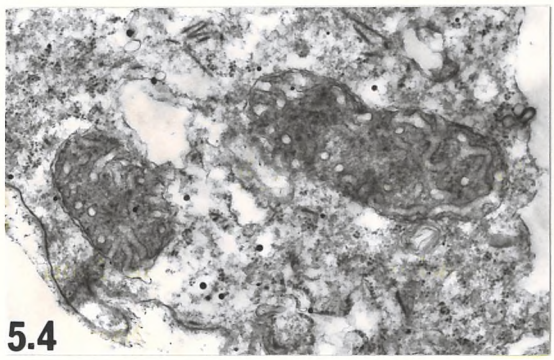
Fig. 5.4. Apparently normal mitochondria from amoebae treated with 2 x 10^{-4}M Cd^{2+} for 1 h. Magnification 20000 x.

Amoebae treated with 1 x 10^{-4}M Cd^{2+} for 1 h.

Fig. 5.5. A mitochondrion showing loss of cristae from the central region. Lipid droplets (L). Magnification 30000 x.

Fig. 5.6. Another sample of mitochondria from an amoeba which received the above Cd^{2+} treatment, some are damaged (large arrow) and others are apparently normal (small arrow). Notice the vacuolation of matrix in some mitochondria (v). Magnification 15000 x.

Fig. 5.7. Another field of mitochondria showing irregular (lobular) outline and vacuolated matrix, Notice the reduced golgi body (gb), a cluster of microfibres (f) and the free detached ribosomes. Magnification 20000 x.



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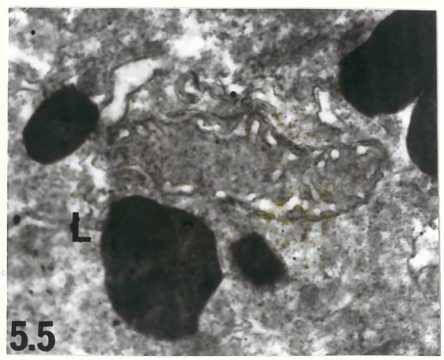

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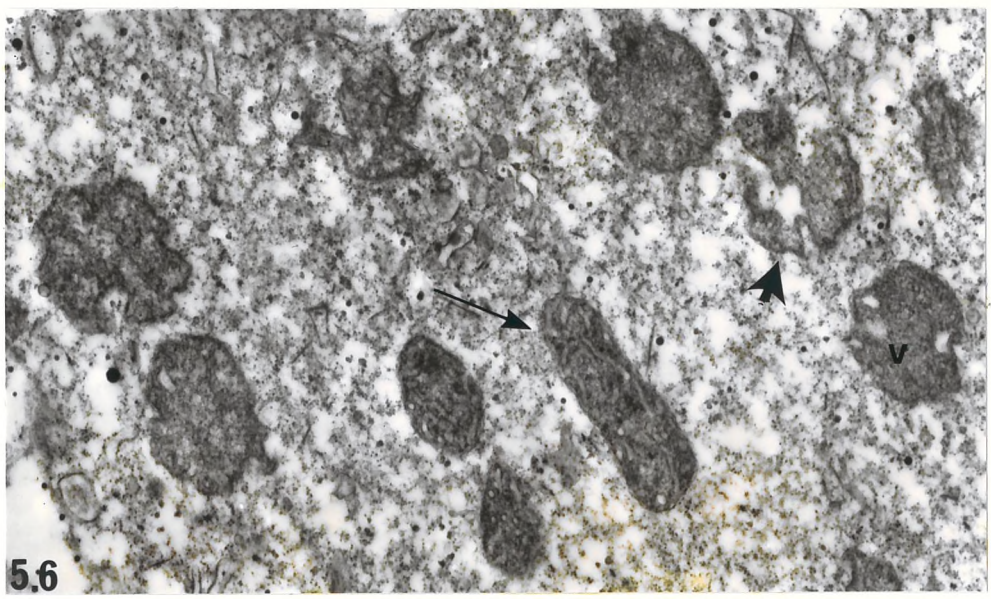

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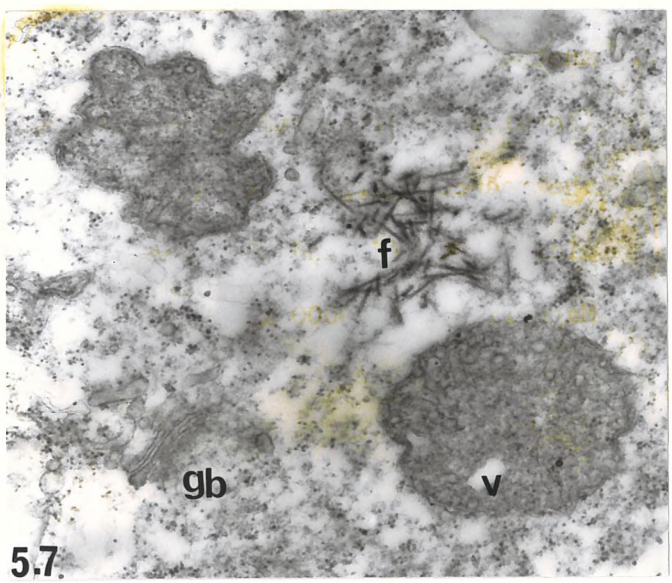

5.7

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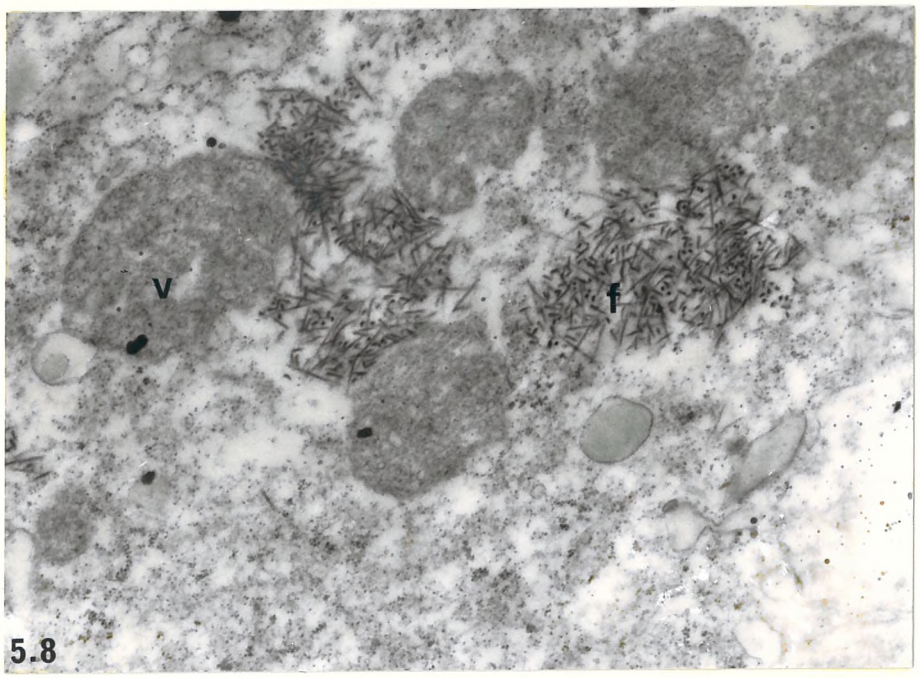
Fig. 5.8. Another field of mitochondria from an amoebae which received the above Cd^{2+} treatment showing loss of cristae/matrix texture, vacuolation of matrix (v) and irregularity of outline. Cluster of microfibres (f). Magnification 20000 x.

Amoebae treated with 1 x 10^{-4}M Cd^{2+}M for 2 h.

Fig. 5.9. "Ghost like" disintegrating mitochondria, some with membranous extensions (arrow). Lipid droplet (L). Magnification 30000 x.

Fig. 5.10. A near normal mitochondrion, type II (M_2) found together with the extremely damaged mitochondria of fig. 5.9. Magnification 30000 x.



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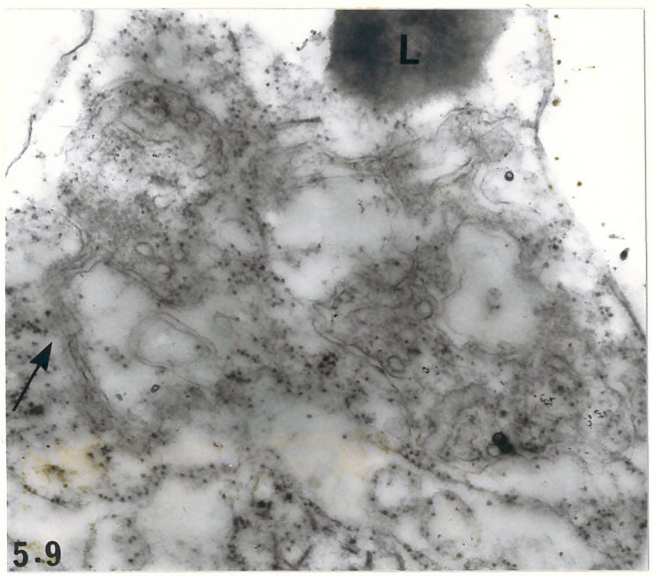

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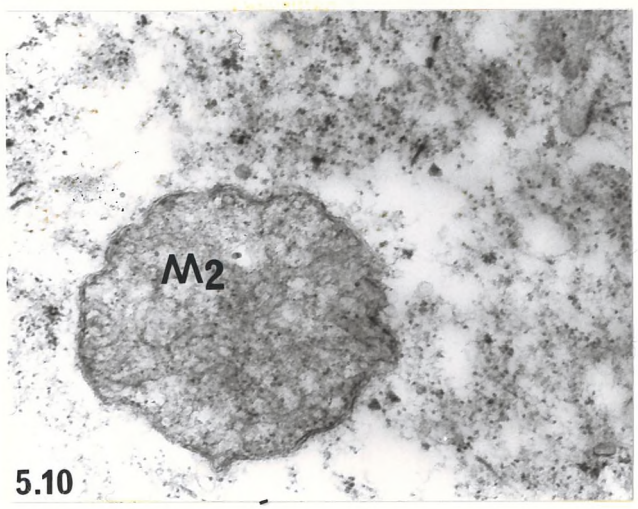

5.10

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Amoebae treated with 10^{-5}M Cd^{2+} for 3 days.

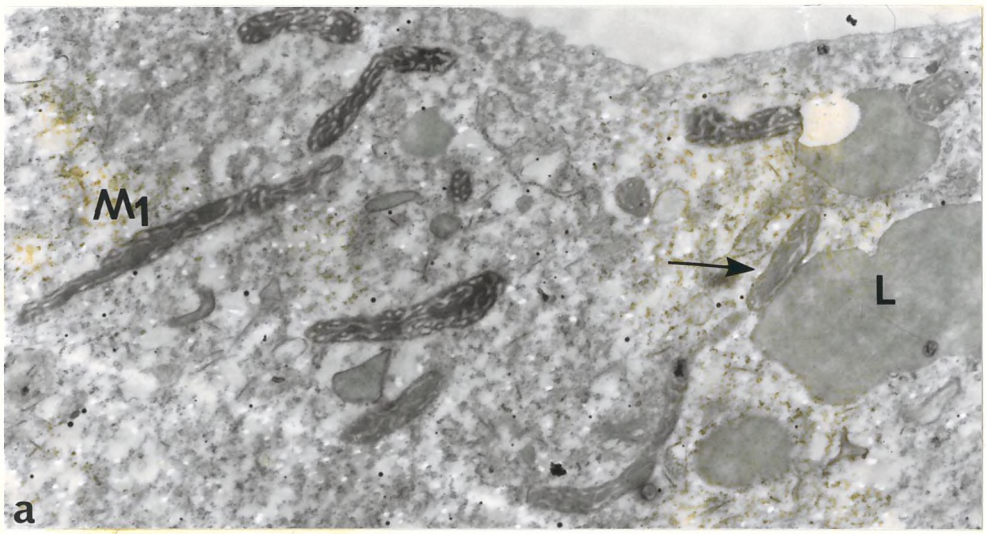
Fig. 5.11.

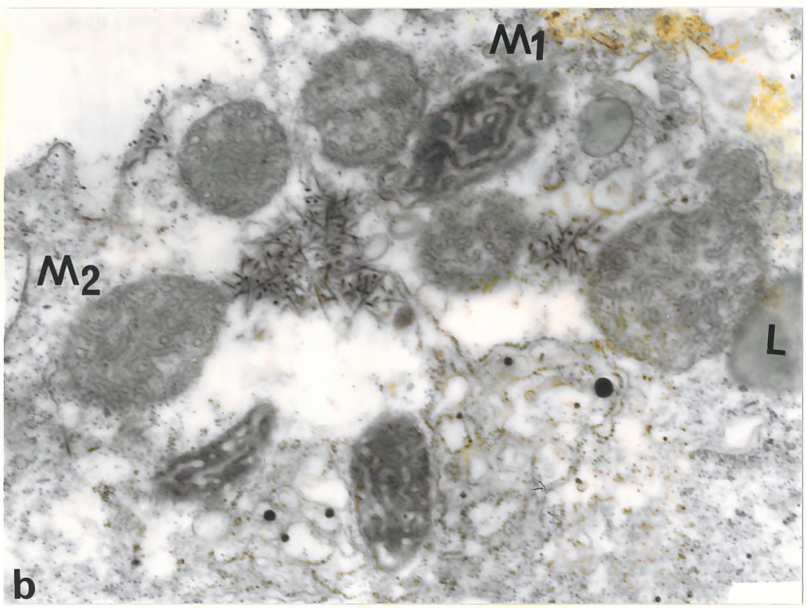
(a) A field of type I (M_1) and intermediate mitochondria (arrow). Although they are of a near normal appearance, some however seem elongated. Also shown are very large lipid droplets (L) which are characteristic of this treatment. Magnification 10000 x.

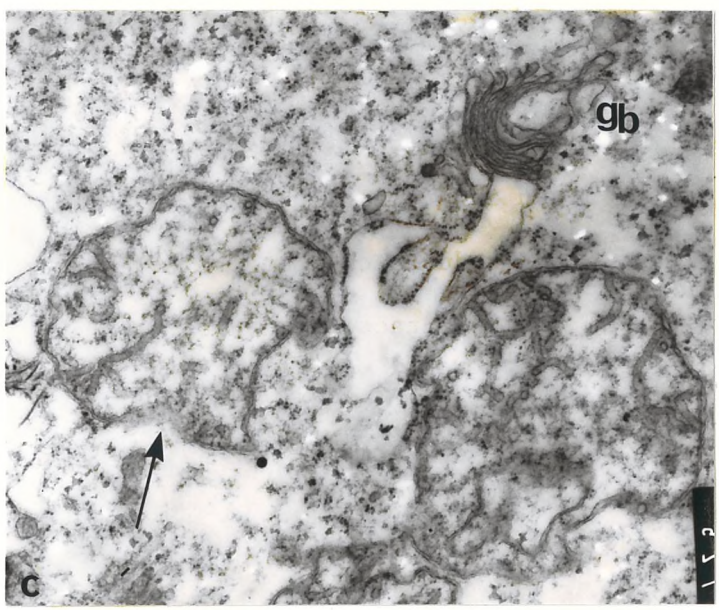
(b) Mitochondria (M_1 and M_2) of a normal appearance. Lipid droplet (L). Magnification 18000 x.

(c) "Ghost like" mitochondria showing ruptured outer and inner membranes (arrow), loss of matrix density and reduced cristae. Also shown is a golgi body (gb) having intact cisternae. Magnification 20000 x.

5.11.







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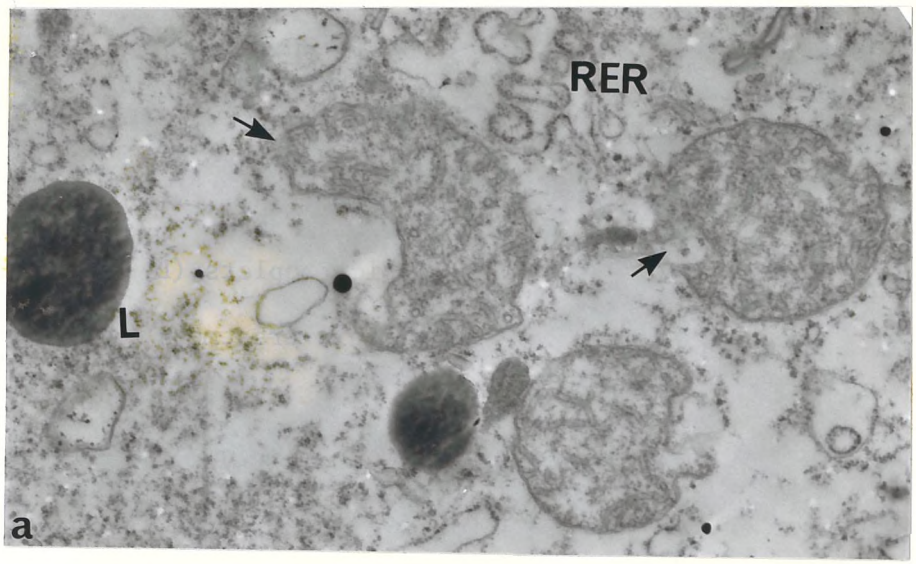
Amoebae treated with 10^{-6}M Cd^{2+} for 6 days.

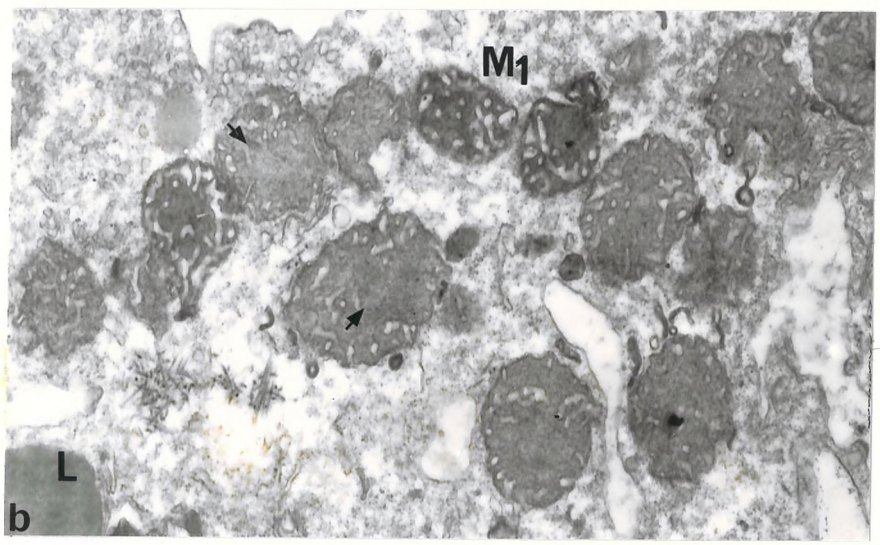
Fig. 5.12.

(a) Damaged mitochondria showing ruptured outer and inner membranes (arrow) and loss of matrix density. Also shown are lipid droplets (L), which are characteristic of this treatment, and RER. Magnification 15000 x.

(b) Mitochondria, type I (M_1) and intermediate, showing loss of cristae from the central region (arrows). Magnification 15000 x.

5.12.





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Amoebae treated with 10^{-7}M Cd^{2+} for one month.

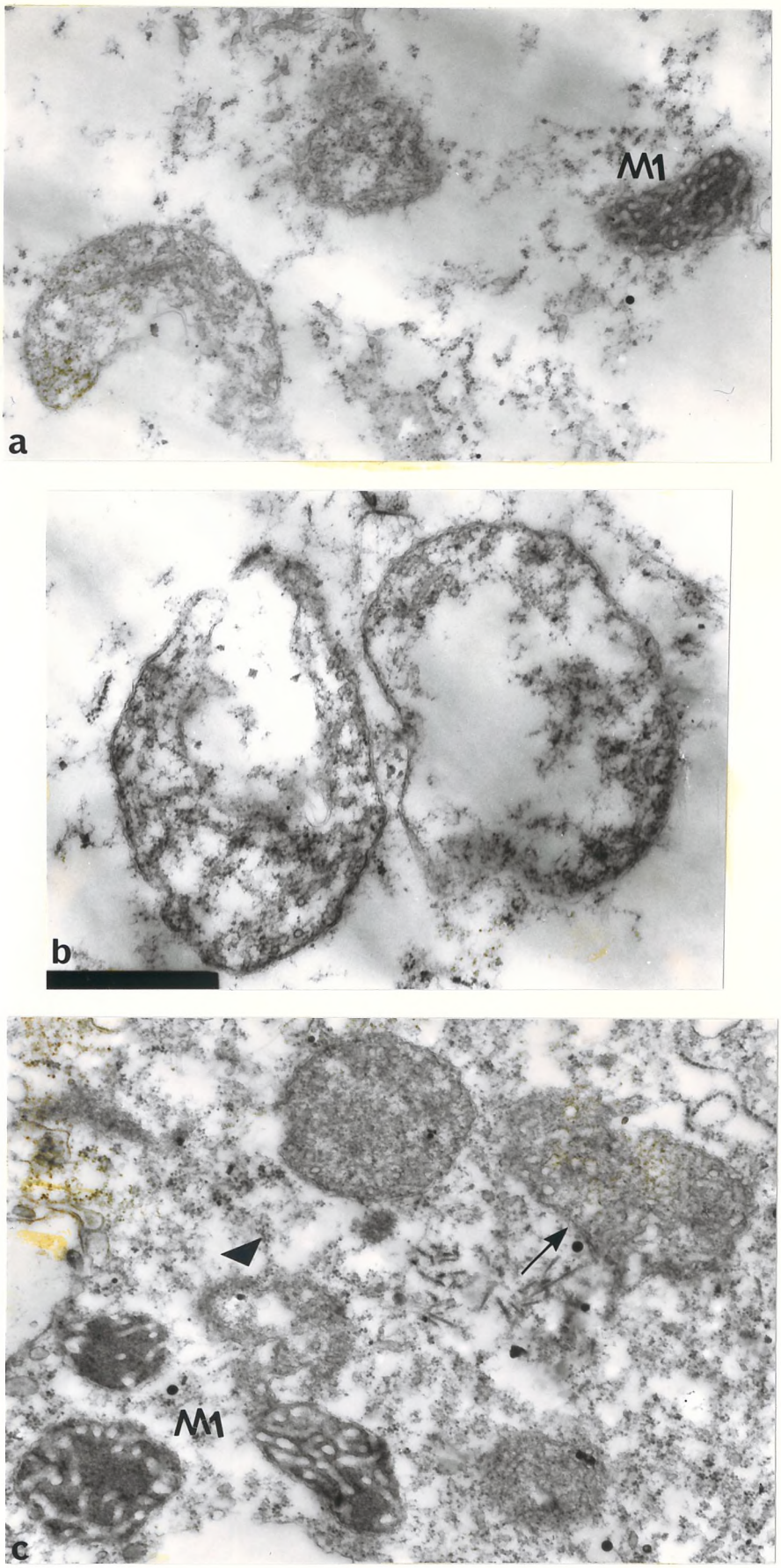
Fig. 5.13.

(a) Extensively damaged "ghost like" mitochondria showing disintegration of cristae and matrix structure and rupture of outer and inner membranes. Normal mitochondria are still present in the cytoplasm of amoebae treated with the above dose (M_1). Magnification 20000 x.

(b) Another sample of the extensively damaged mitochondria obtained in an amoeba treated with this dose. Magnification 25000 x.

(c) Apparently normal (M_1), damaged (arrow) and disintegrating (triangle) mitochondria are often found together in amoebae treated with the above dose. Magnification 20000 x.

5.13.



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Amoebae treated with 10^{-7}M Cd^{2+} for two months.

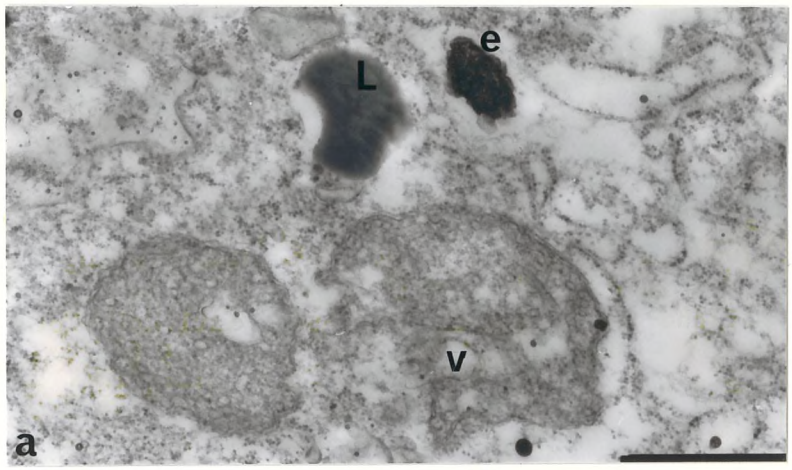
Fig. 5.14.

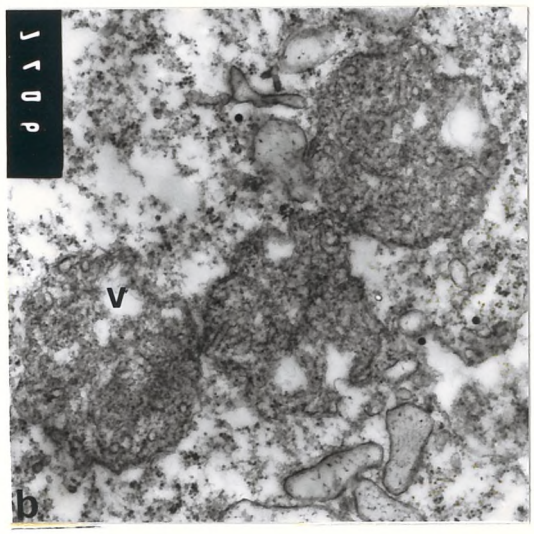
(a) and (b)

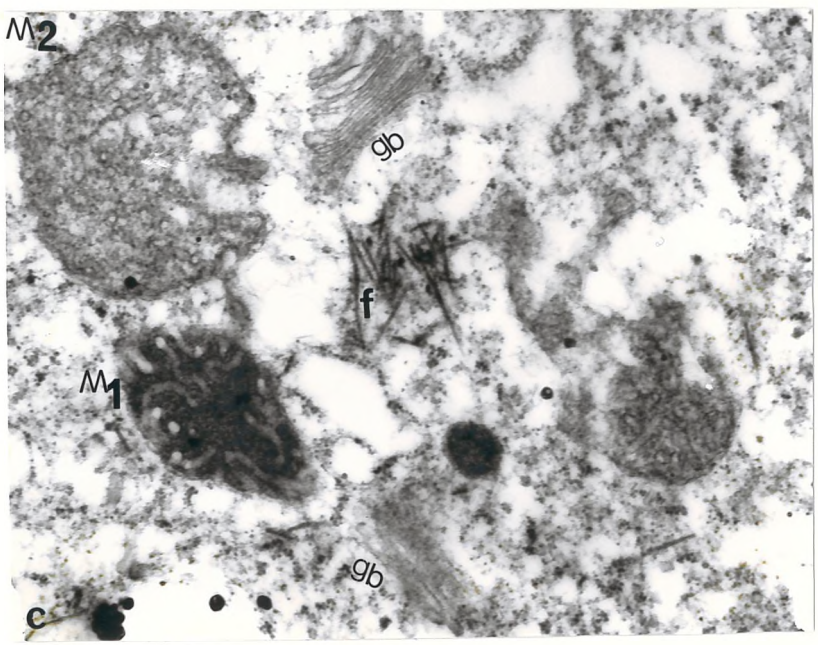
Two samples of type II mitochondria showing vacuolation of the matrix (v) and irregular outline. Notice the degree of damage in these mitochondria is less than that shown in figs. 5.13 a and b, where the amoebae received the same concentration of Cd^{2+} for one month only. Lipid droplet (L), endosymbiont (e). Magnification for both micrographs is 20000 x.

(c) A near normal (M_1) and a partially damaged (M_2) mitochondria. Also shown are golgi bodies (gb), one with intact cisternae and the other with a diffuse ill-defined appearance, a cluster of microfibres (f). Magnification 28000 x.

5.14.







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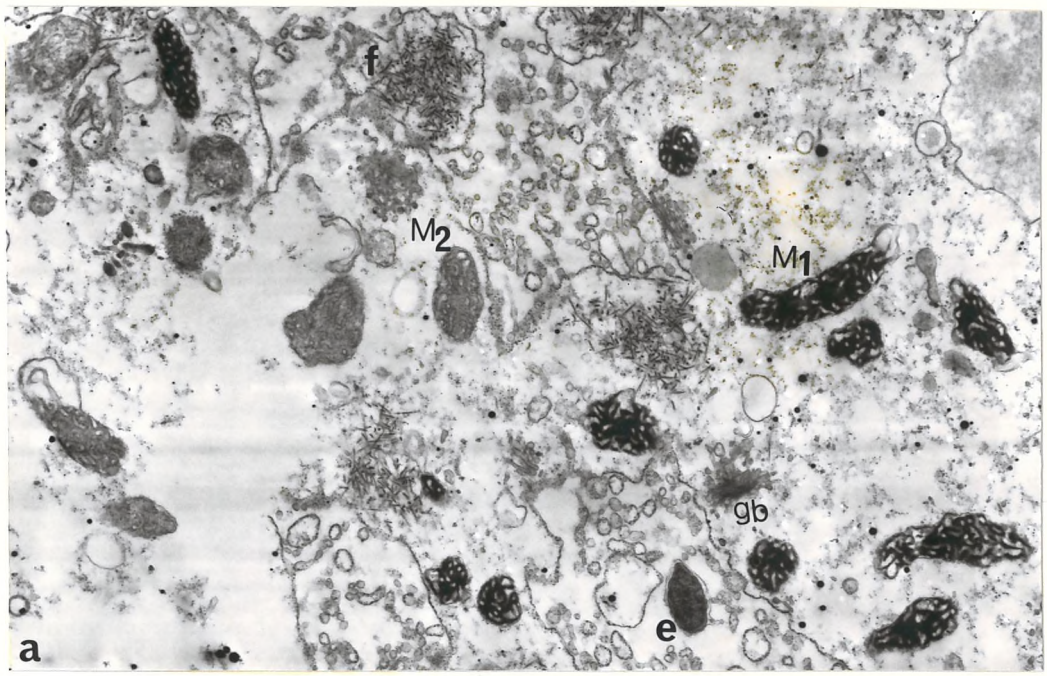
Amoebae treated with 10^{-7}M Cd^{2+} for nine months (Cd-adapted amoebae).

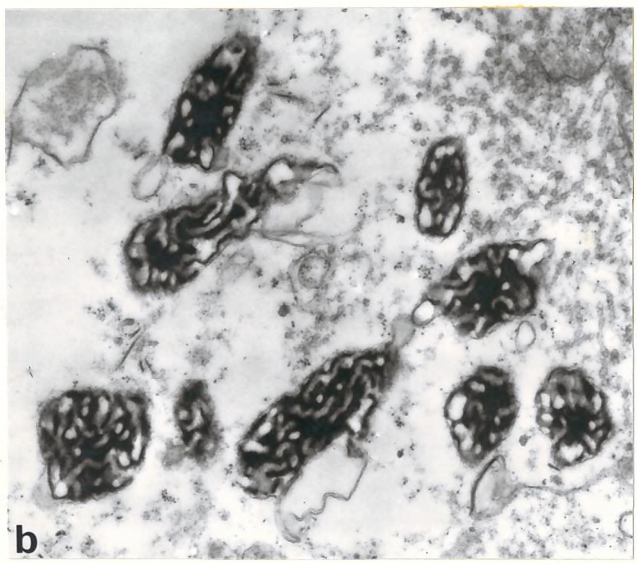
Fig. 5.15.

(a) A field of dark, type I, (M_1) and light, type II, (M_2) mitochondria of a near normal appearance. Also shown are endosymbiont (e), golgi body (gb) and microfibres (f). Magnification 12000 x.

(b) A group of dark, type I, mitochondria of a near normal appearance. Some of these mitochondria have membraneous extrusions. Mitochondria from both control and amoebae under stress were found to have such membraneous extrusions (personal communication M.J. Ord), however, the cause for their formation is not known. Magnification 15000 x.

5.15.





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Amoebae treated with 10^{-8}M Cd^{2+} for one month.

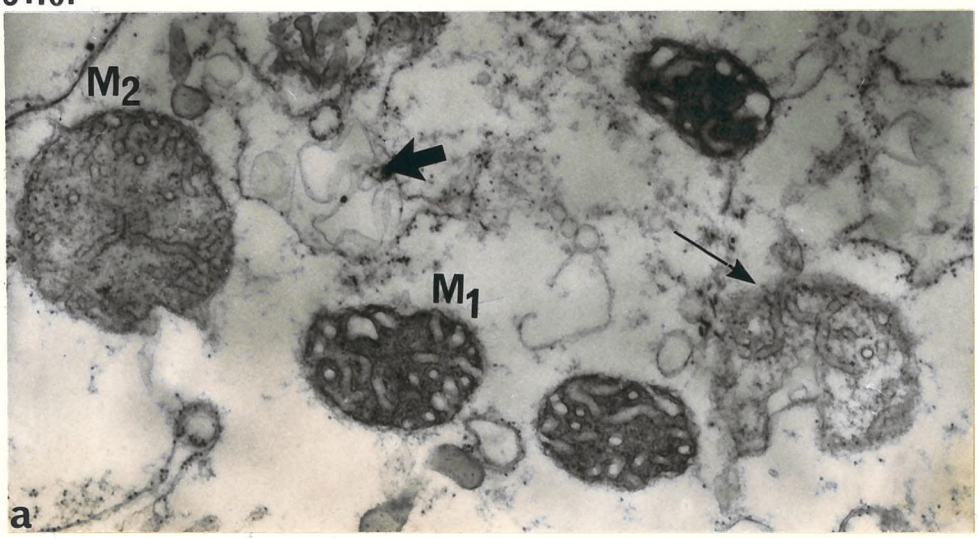
Fig. 5.16.

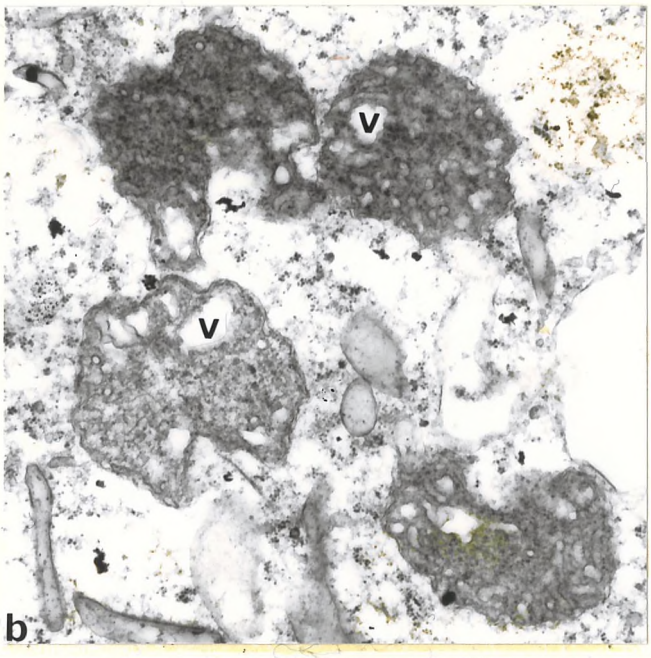
(a) Apparently normal mitochondria (M_1 and M_2) and a damaged mitochondrion (small arrow). Notice the folds of smooth membranes (large arrow). Magnification 20000 x.

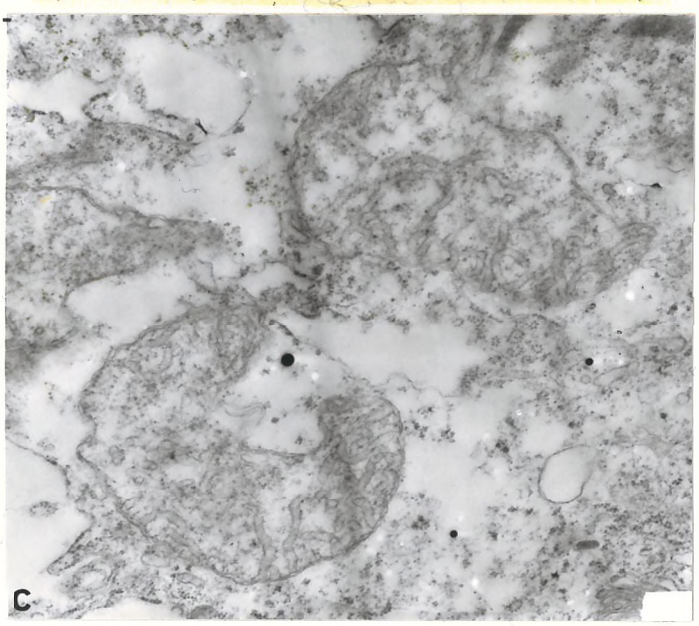
(b) Damaged mitochondria with vacuolated matrix (v) and irregular outline. Magnification 20000 x.

(c) Extensively damaged mitochondria showing rupture of outer and inner membranes, loss of matrix density and reduced cristae. Magnification 20000 x.

5.16.







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Amoebae treated with 10^{-8}M Cd^{2+} for two months.

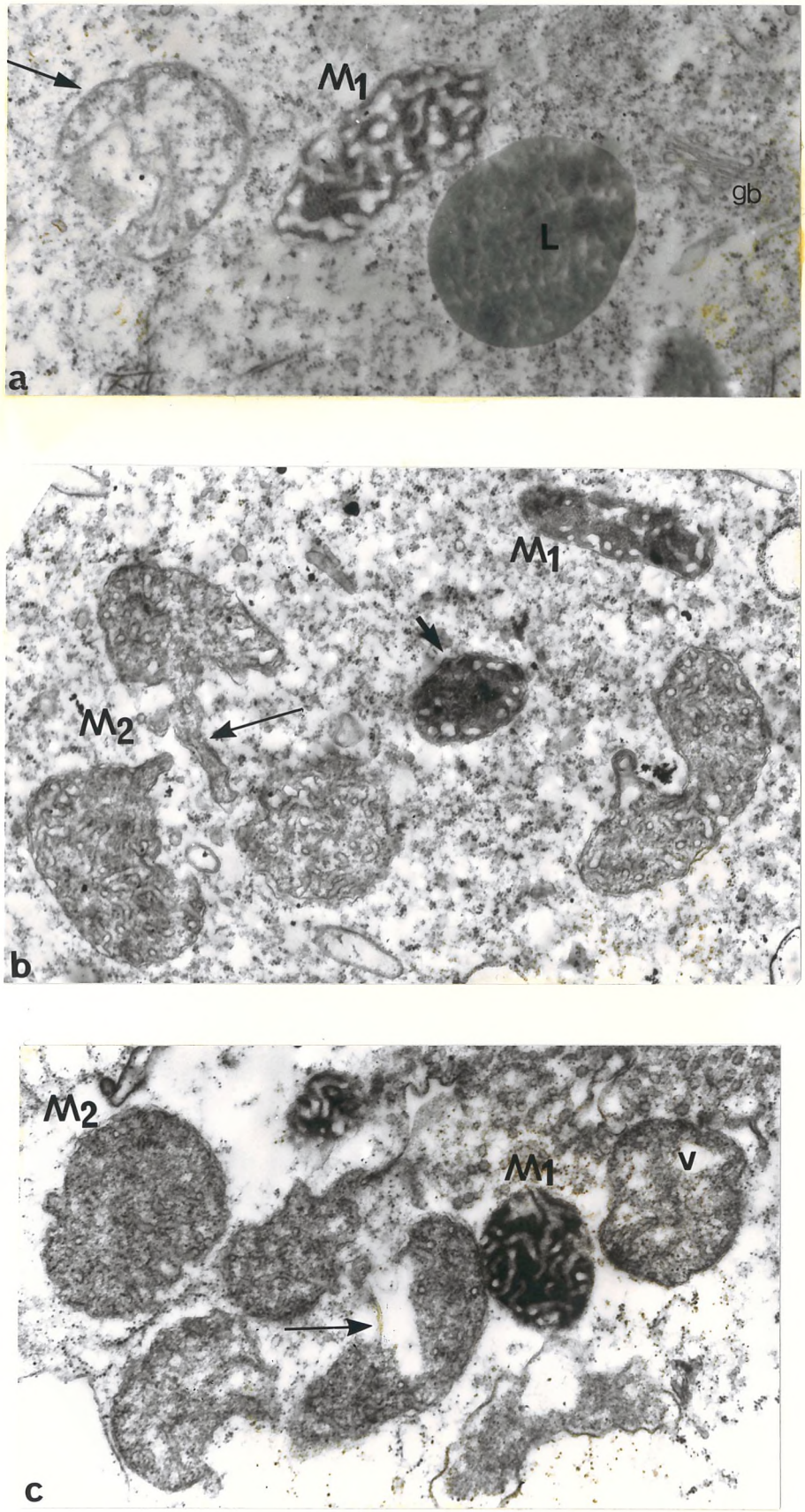
Fig. 5.17.

(a) A type I mitochondrion showing dilation of cristae (M_1), a disintegrating mitochondrion (arrow) a lipid droplet (L) and a reduced golgi body (gb). Magnification 20000 x.

(b) Mitochondria types I and II (M_1 and M_2). Type II mitochondria showing irregularity in shape, some with finger like extensions (long arrow). A type I mitochondrion showing loss of cristae/matrix texture from the central region (short arrow). Magnification 20000 x.

(c) A group of mitochondria type I and II (M_1 and M_2), The type I mitochondria are of a normal appearance, while some of type II mitochondria have a vacuolated matrix (v) and rupture of outer and inner membranes (arrow). Magnification 20000 x.

5.17.



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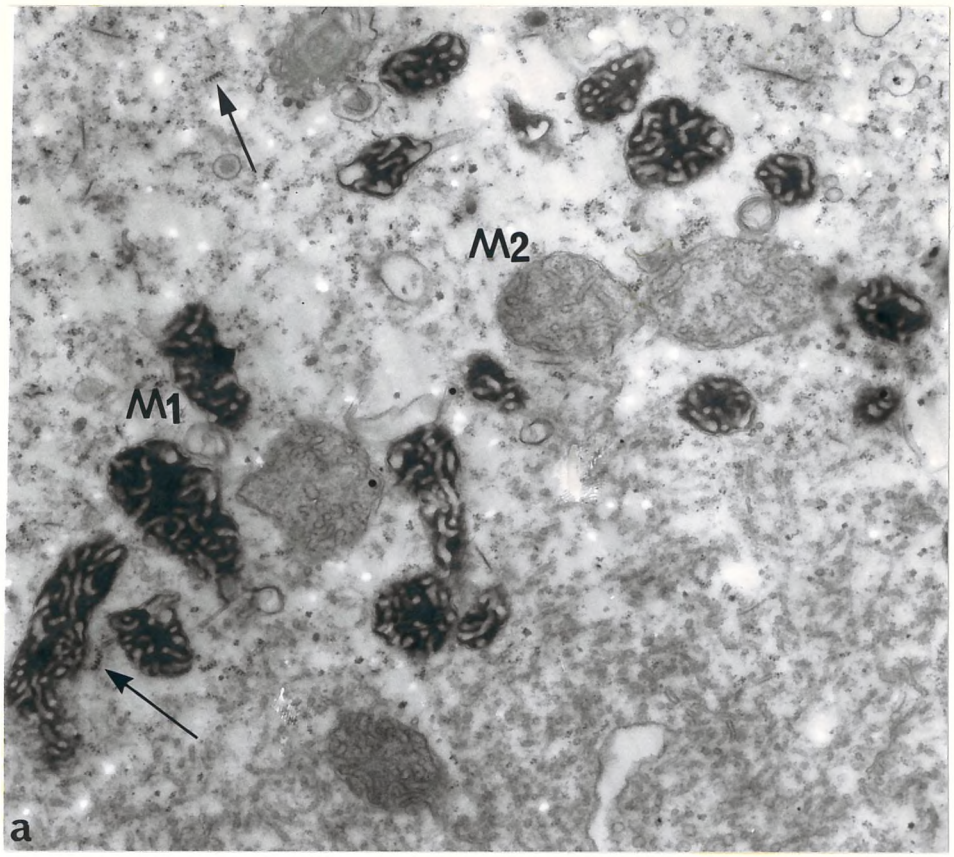
Amoebae treated with 10^{-8}M Cd^{2+} for eleven months (Cd-adapted amoebae.

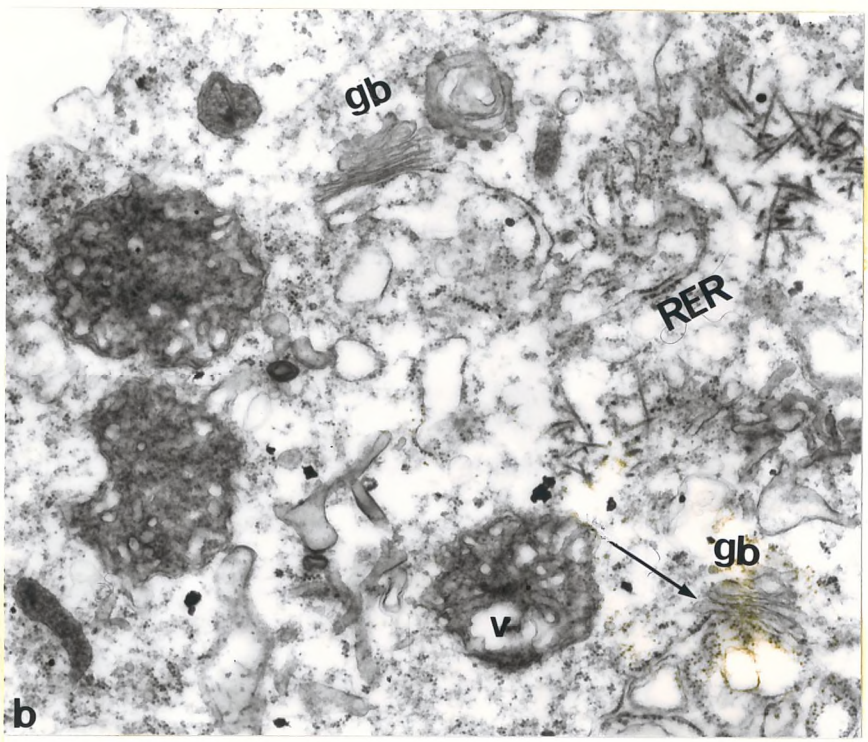
Fig. 5.18.

(a) A group of mitochondria types I and II (M_1 and M_2) of normal appearance. Notice the spiral cytoplasmic helices (nuclear helices which apparently have failed to uncoil during exit from the nucleus to the cytoplasm) (arrows). Magnification 15000 x.

(b) A few damaged mitochondria, some with vacuolated matrix (v) can still be seen together with the apparently normal mitochondria shown in (a). Also shown are golgi bodies (gb), one having reduced cisternae (arrow), and RER. Magnification 20000 x.

5.18.





5.4. Lipid droplets.

Spherical lipid droplets of 1.5 µm in diameter enclosed in a membrane are found in the cytoplasm of A. proteus (Daniels 1964). In starved amoebae the number of these lipid droplets has been shown to decrease. Prolonged starvation results in their loss from the cytoplasm. These lipid droplets are considered to function as reserves of food which can be utilised for energy production (Mast & Doyle 1935; Daniels & Bryer 1968).

Cells treated with low doses of Cd were found to have similar or slightly higher proportions of lipid droplets than control cells. Cells which receive any of the intermediate doses of Cd showed an appreciable increase in the number of lipid droplets of various sizes and shapes, figs. 5.19 - 21. Cells which received any of the high doses of Cd did not show great differences in their lipid droplets from those of the controls. This suggests that Cd at certain concentrations may affect the lipid metabolism in A. proteus.

It is interesting to note that Schroeder & Balassa (1965) have shown that in Cd fed rats there was an increase of lipid deposition in the aorta. Also in rats, acute Cd exposure resulted in a prominent increase of lipid droplets in the liver parenchymal cells (Hoffmann et al 1975) and doubled the lipid content in the alveolar cells of the lung. (Hayes et al 1976).

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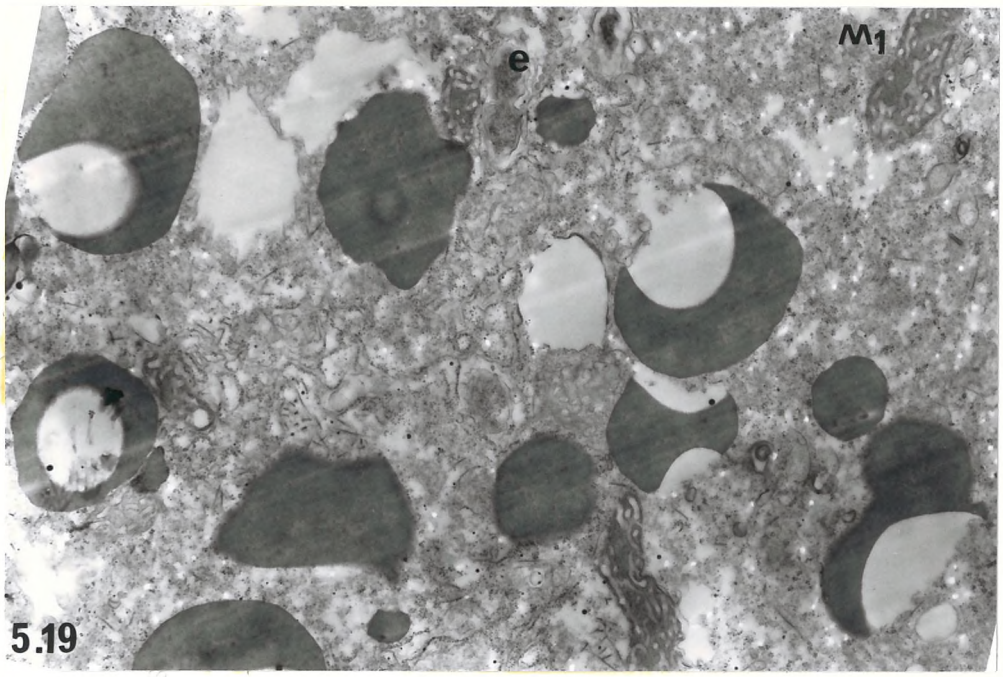
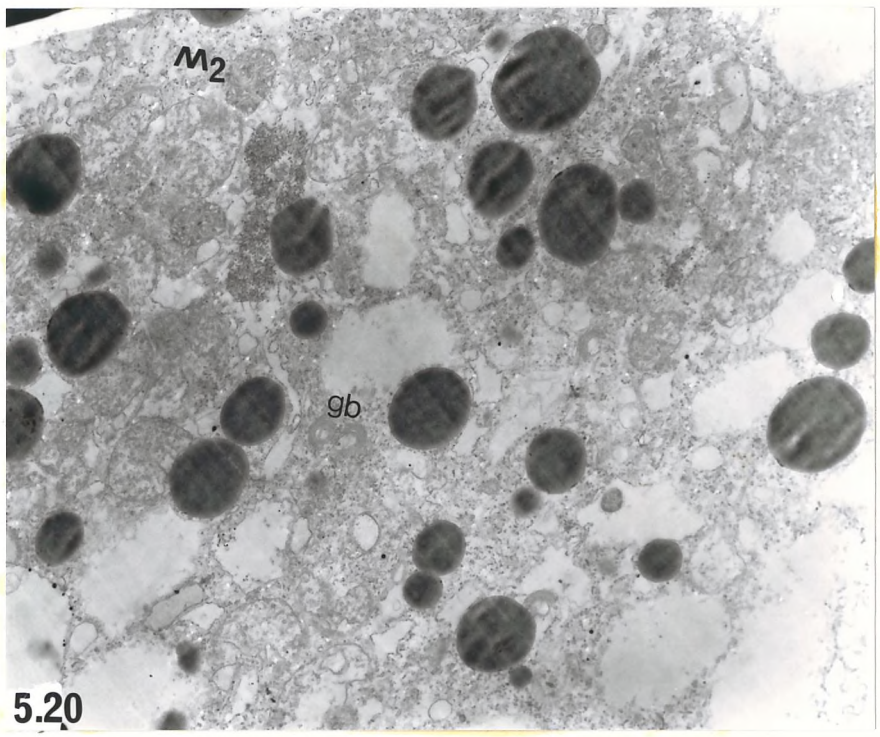
Accumulation of lipid by Cd treated amoebae.

Fig. 5.19. Amoeba treated with 10^{-4}M Cd^{2+} for 3 days, showing accumulation of large lipid droplets. Also shown are endosymbionts (e) and mitochondria (M_1). Magnification 12000 x.

Fig. 5.20. Amoebae treated with 10^{-6}M Cd^{2+} for 6 days showing accumulation of lipid droplets. Also shown are mitochondria (M_2) and golgi bodies (gb). Magnification 6000 x.



5.19



5.20

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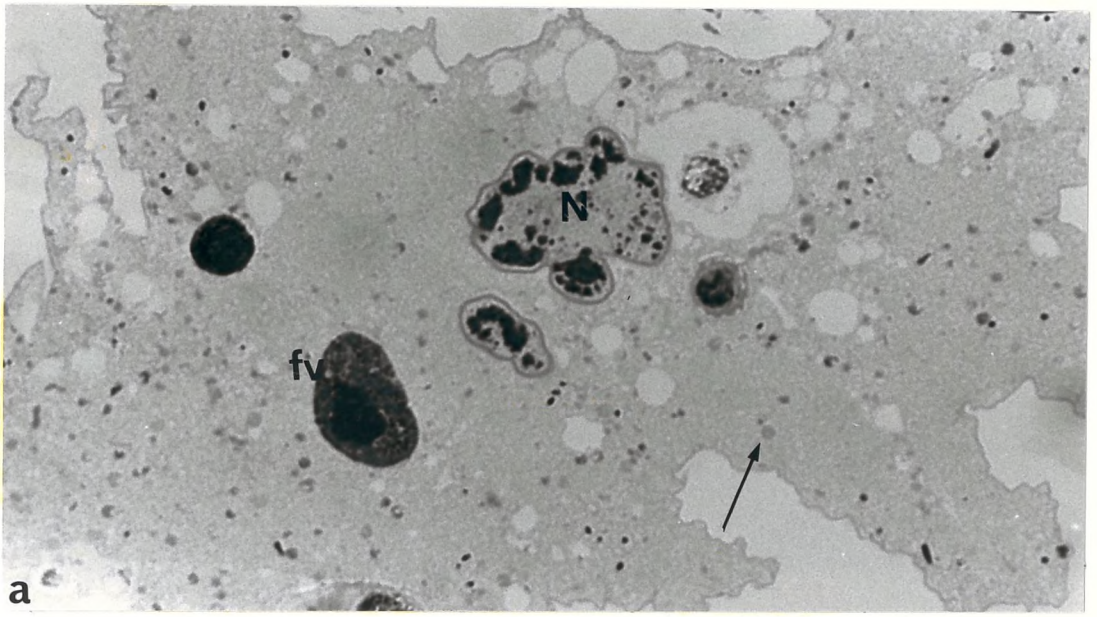
Fig. 5.21. The overall distribution of lipid droplets is also demonstrated in these light microscopy photographs of 1μm thick sections of Spurr embedded amoebae.

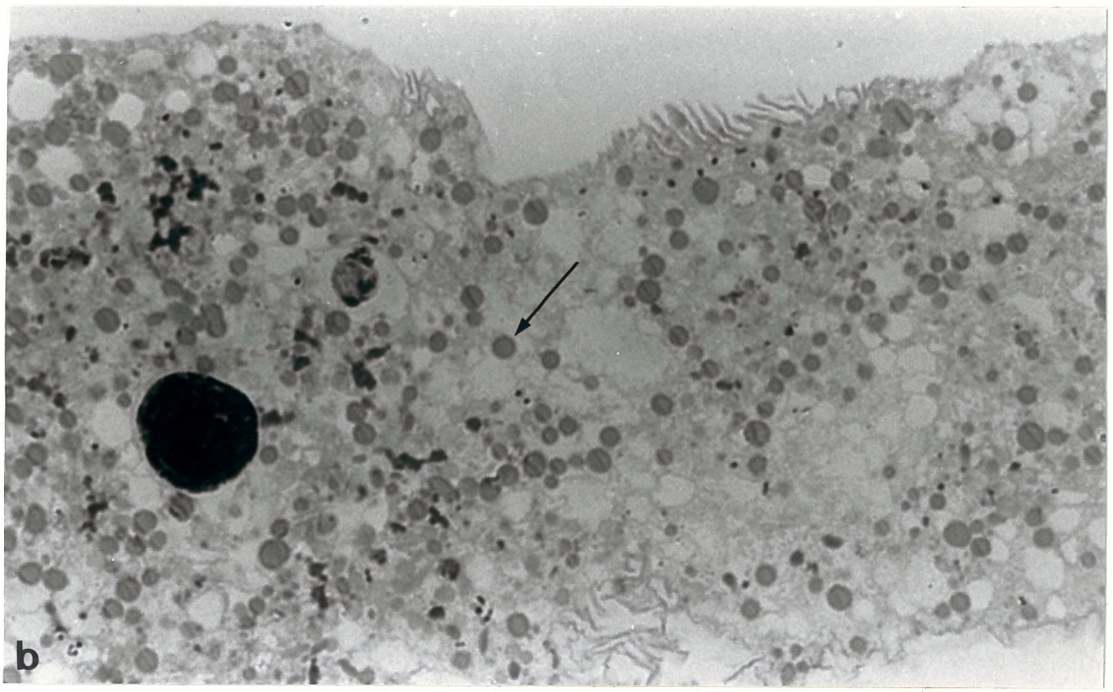
(a) Control amoebae having relatively few lipid droplets (arrow) scattered through the cytoplasm. Nucleus (N). Food vacuole (fv). Magnification 1275 x.

(b) Amoebae treated with 10^{-6}M Cd^{2+} for 6 days, showing a large accumulation of lipid droplets (arrow) throughout the cytoplasm. Magnification 1275 x.

This heavy lipid accumulation is mainly associated with the intermediate doses of Cd^{2+}.

5.21.





5.5. Golgi bodies.

The golgi body in A. proteus consists of folded layers of membrane in a goblet shape forming a number of cisternae, usually there are 6-8 cisternae in an amoeba golgi body (Flickinger 1973) (Fig. 5.22). Golgi bodies and golgi vesicles are often associated with smooth endoplasmic reticulum (SER) and lysosomes. The convex pole of the golgi body has been shown to possess acid phosphatase and thiamine pyrophosphatase activities. Cisternae towards the concave pole lack phosphatases but they contain filamentous material composed of acid mucopolysaccharides (Revel & Ito 1967; Stockem 1969). It has been shown that carbohydrates are attached to peptide precursors (to form glycoprotein) at the inner surface of golgi membranes in preparation for their ultimate transportation to the plasma membrane (Wise & Flickinger 1970 a & b).

Cd treatment of amoebae resulted in the following changes of the golgi body. The cisternae appeared diffuse and ill-defined, some golgi bodies showed some distortion(fig. 23 a & b) and others showed variation in the number of cisternae/golgi body. In general there was a reduction in the number of cisternae (fig. 5.24; 5.28; 5.30), but in some samples the number was found to increase (fig. 5.26). It is difficult to associate any of these changes with a specific dose of Cd , since all changes were demonstrated by almost all the Cd treated amoebae. However, golgi bodies with reduced number of cisternae were numerous in amoebae which had received prolonged Cd treatment (Figs.5.27-30). Similar changes were shown in amoebae subjected to various types of stress, chemical and mechanical (Ord 1979; Flickinger 1968 b).

Changes in the golgi body caused by Cd treatment in A. proteus

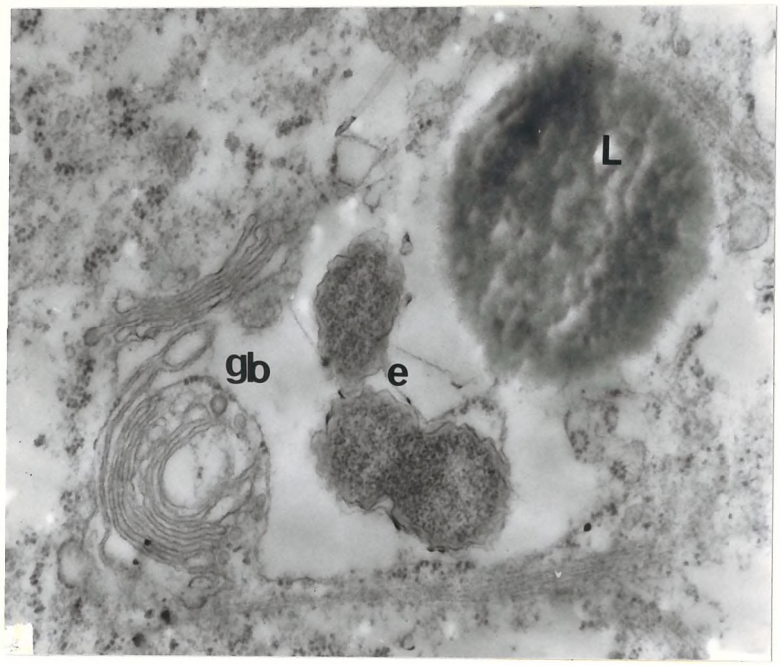
Fig. 5.22. Control amoebae showing golgi bodies (gb) one of a goblet shape and the other flat, endosymbionts (e), and a lipid droplet (L). Magnification 25000 x.

Fig. 5.23. Amoebae treated with 2×10^{-4} Cd^{2+} for 1 h.

(a) Showing golgi bodies (gb), some of which have a diffuse ill-defined appearance (arrows), lipid droplet (L). Magnification 15000 x.

(b) Another field showing a golgi body (gb) of a distorted appearance, it also associated with numerous vesicles. Magnification 250000 x.

5.22.



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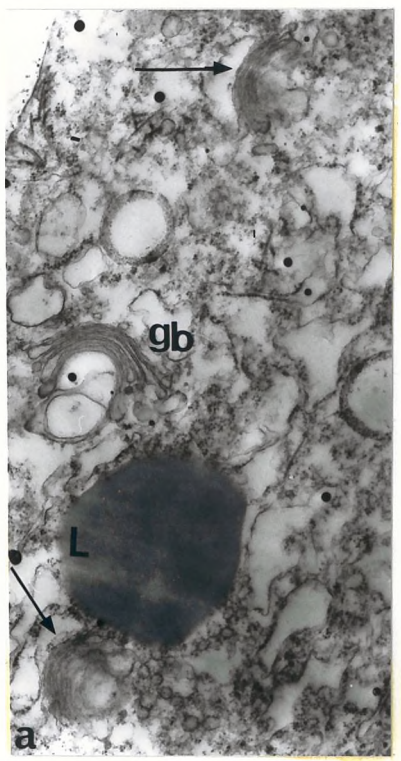


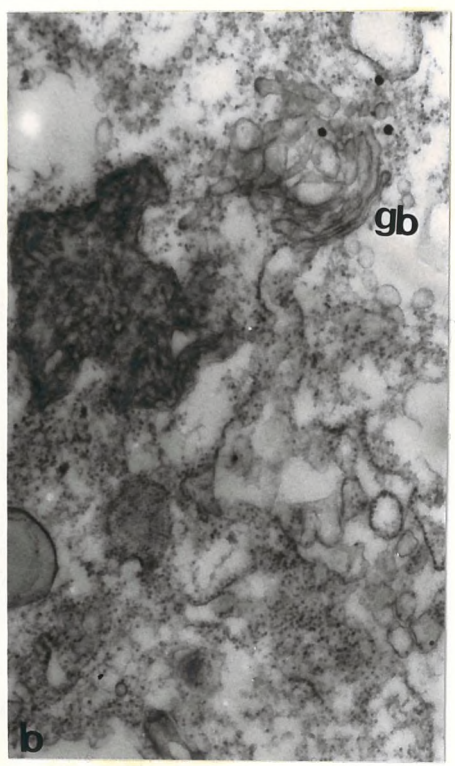
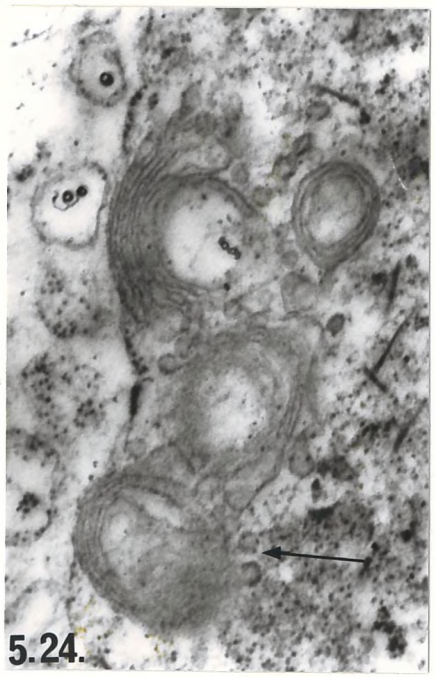


Fig. 5.24. A cluster of golgi bodies, some of which appear diffuse and ill defined (arrow), from amoeba treated with 1 x 10^{-4}M Cd^{2+} for 2 h. Magnification 20000 x.

Fig. 5.25. An intact golgi body (gb) and mitochondria, type I (M_1) from amoebae treated with 10^{-6}M Cd^{2+} for 6 days. Magnification 20000 x.

Fig. 5.26. A golgi body in an area of polysomes (P) from amoebae treated with 10^{-7}M Cd^{2+} for one month. Magnification 25000 x.

Fig. 5.27. Amoeba treated with 10^{-7}M Cd^{2+} for one month showing a flat golgi body (gb), a mitochondrion (M_2), and RER. Magnification 20000 x.



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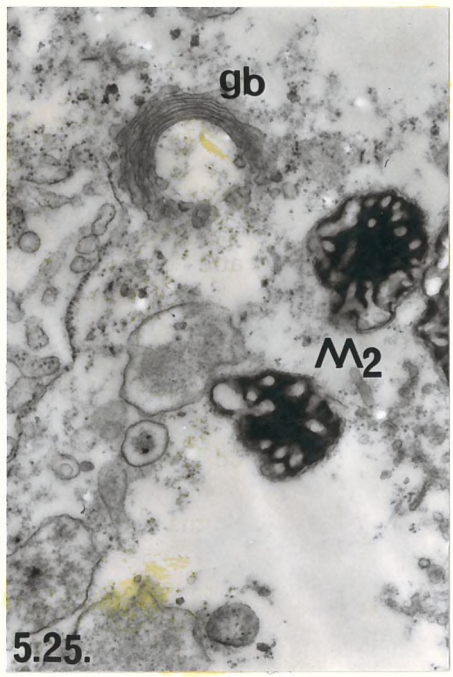

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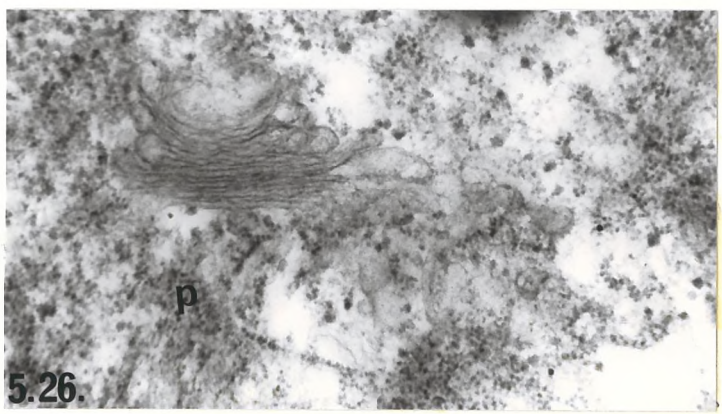

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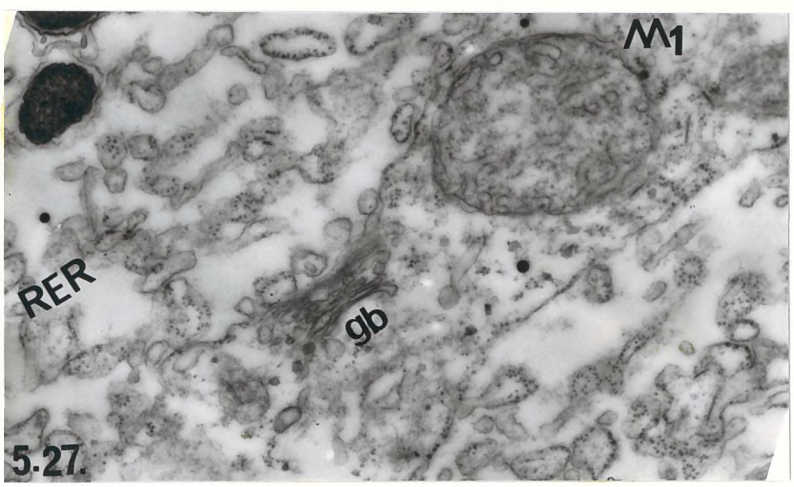
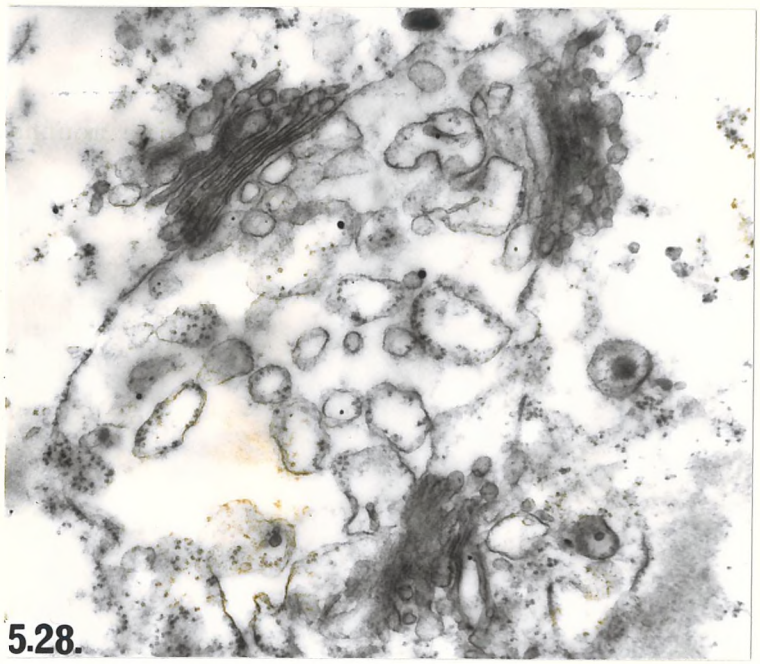


5.27.

Fig. 5.28. A group of golgi bodies and RER from an amoeba treated with 10^{-7}M Cd^{2+} for nine months (i.e. Cd-adapted amoebae). Magnification 25000 x.

Fig. 5.29. Golgi bodies from amoeba treated with 10^{-8}M Cd^{2+} for two months, notice the diffuse ill defined cisternae of some of them (arrow) and also the numerous free detached ribosomes.
Magnification 30000 x.



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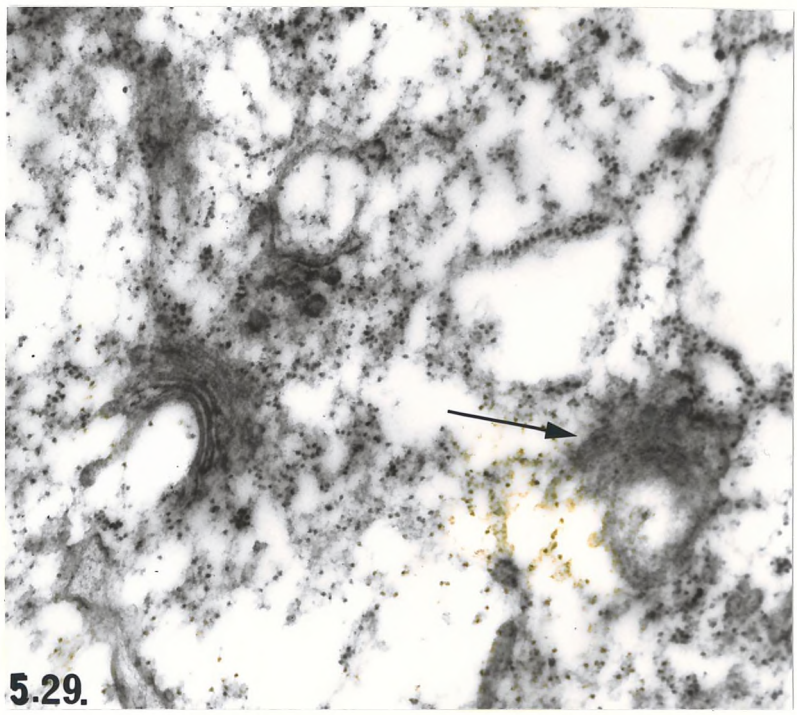

5.29.

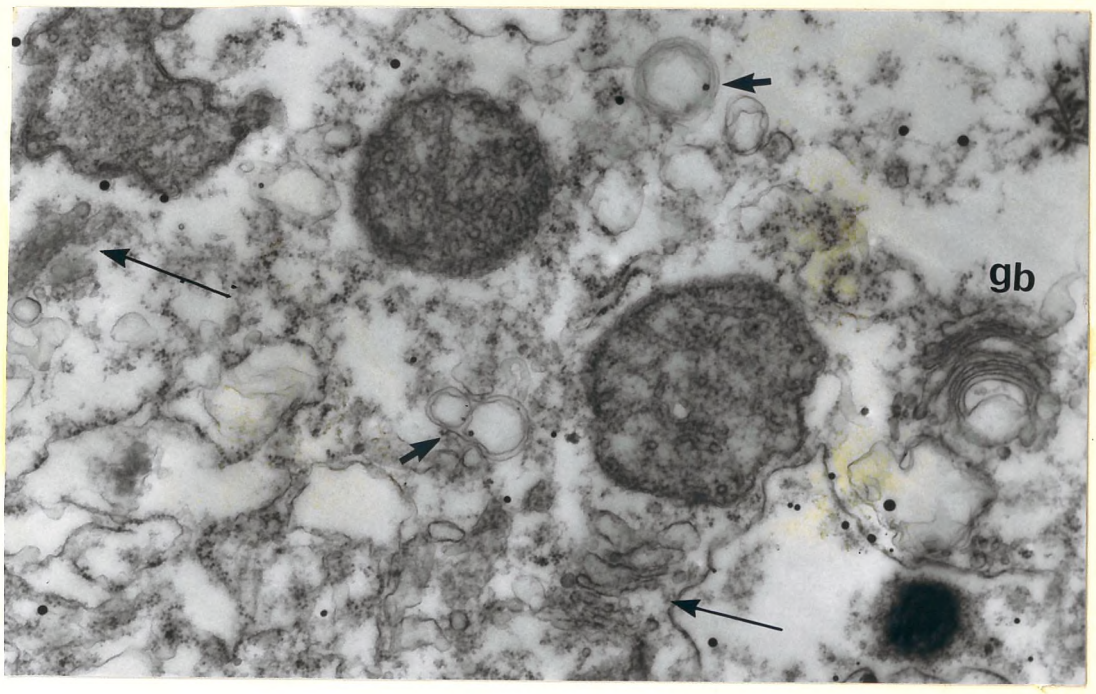

Fig. 5.30. Amoebae treated with 10^{-8}M Cd^{2+} for two months showing golgi bodies, some are intact (gb) and others are distorted, diffuse and ill defined (long arrows). Also shown are mitochondria, intact and damaged, and laminated folds of smooth membranes (short arrows). Magnification 25000 x.

5.6. Endoplasmic reticulum and the ribosomal distribution.

In eukaryotic cells a certain population of ribosomes is always found to be attached to the endoplasmic reticulum (ER), thus forming rough endoplasmic reticulum (RER). Another population of ribosomes is found attached to mRNA template forming polyribosomes, and a small population of ribosomes may exist as free unattached monosomes. The proportion of membrane attached ribosomes (RER) to free polyribosomes varies between different types of cell. It is usually higher in cells actively secreting proteins, such as liver or pancreas cells, where about 80% of the ribosomes are membrane attached. In nonsecretory cells such as skeletal muscle and cerebral cortex cells, only 10% and 20% respectively, of their ribosomes are membrane attached. Although proteins can be synthesized in both membrane attached and free polyribosomes, most proteins are known to be synthesized on membrane bound ribosomes. Examples of proteins synthesized by both membrane attached and free polyribosomes are; soluble proteins, such as ferritin and arginase of liver cells and serine dehydratase in rat liver, (Quinn 1976), detached free ribosomes (monosomes) are believed to be nonfunctioning.

The smooth endoplasmic reticulum (SER) is the other form of ER, consists of folds of smooth membranes frequently associated with golgi bodies and RER. The proportion of RER to SER depends primarily on the cellular activity; cells actively producing proteins such as liver and pancreas cells are known to have higher proportion of RER. Changes in the proportion of SER appear to be associated with drug detoxification processes; the amount of SER in mammalian liver cells can increase 10 fold following the administration of certain barbiturate drugs (Quinn 1976).

. Light and electron microscopic studies using radioisotopically labelled proteins revealed an overall route of transportation of newly synthesized proteins from the site of synthesis to their ultimate destination. The newly synthesized proteins accumulated in the cisternae of RER passes through to the region SER tubular folds to the golgi cisternae. Then the small golgi vesicles enclosing the synthesized materials leave the golgi body to other parts of the cell. Excreted materials may be externalized via golgi vesicles which fuse with the plasma membrane. Thus the golgi body could be a supplier of the plasma membrane (Jamieson & Palade 1967 a and b).

In A. proteus functioning ribosomes are either attached to ER forming RER, or present as clusters attached along the length of a single m-RNA template forming a free polyribosome. The proportion of free unattached ribosomes (monosomes) is usually very low (fig. 5.31).

Cd^{2+} affected the overall distribution of the ribosomes in amoebae (e.g. figs. 5.32 - 5.43). There was a noticeable increase in the free unattached ribosomes (monosomes) in all the Cd treated amoebae. At the same time there was an increase in the smooth folds of membranes (ER or SER). Thus it appears that an appreciable proportion of the ribosomes became detached from the RER leaving folds of smooth membranes. Similar observations were found in the liver and kidney of Cd treated rats (Hoffmann 1975; Nishizumi 1972), rabbits (Stowe et al 1972) and mouse (Gamulin et al 1977).

The increase in SER may reflect a detoxification process carried out by the cell. The ability of Cd^{2+} to detach ribosomes from RER has been demonstrated in vitro in Paramecium aurelia (Resiner et al 1975). Amoebae treated with the carcinogen N-methyl-N-nitrosourethane (MNU) or methylmercury have also been shown to demonstrate a clear increase in the proportion of the detached (free) ribosomes (Ord 1979).

In the nucleus of A. proteus there are clusters of spiral structures, these are called nuclear helices (Pappas 1956), (fig. 5.44 b, c). The nuclear helices are usually associated with the nucleoli. Eventually the individual helices become detached from the cluster and find their way to the cytoplasm through the nucleopores in the honey-comb nuclear membrane. It has been suggested that once the helix is in the cytoplasm (cytoplasmic helix) its spiral shape becomes unravelled forming a polyribosome (Minassian & Bell 1976a,b). In all the Cd treated amoebae no changes were observed in the nuclear helices, (figs. 5.45 - 50). However, in some Cd^{2+} treated amoebae a few of these helices retained their spiral structure after entering the cytoplasm; i.e. the cytoplasmic helix retained the spiral structure of a nuclear helix (figs. 5.18 a; 5.35; and 5.38.).

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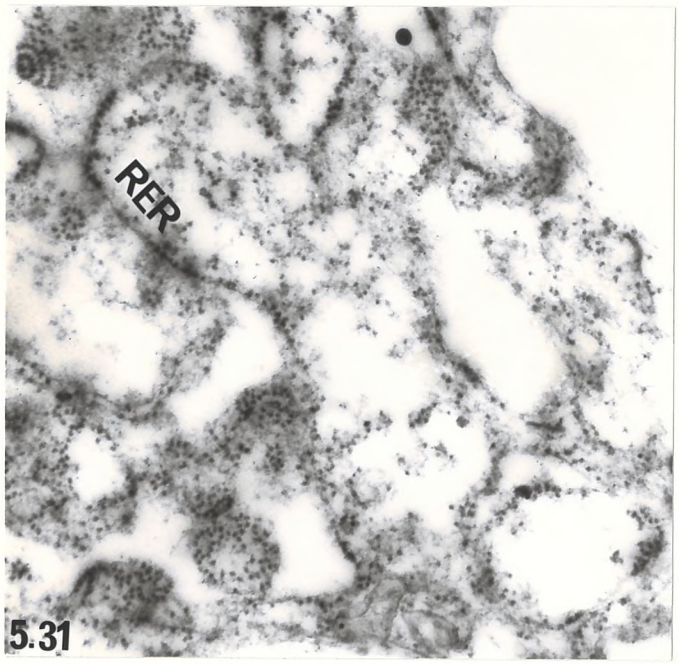
Changes in the distribution of ribosomes and RER in Cd^{2+} treated amoebae.

Fig. 5.31. Control amoeba, almost all ribosomes are attached either to endoplasmic membranes forming RER or to mRNA template forming polysomes, (polyribosomes) Magnification 30000 x.

Fig. 5.32. A large proportion of free unattached ribosomes (R) is observed in the cytoplasm of amoeba treated with 2×10^{-4}M Cd^{2+} for 1 h. Notice the helices of granular texture (arrows). Magnification 50000 x.



5.31

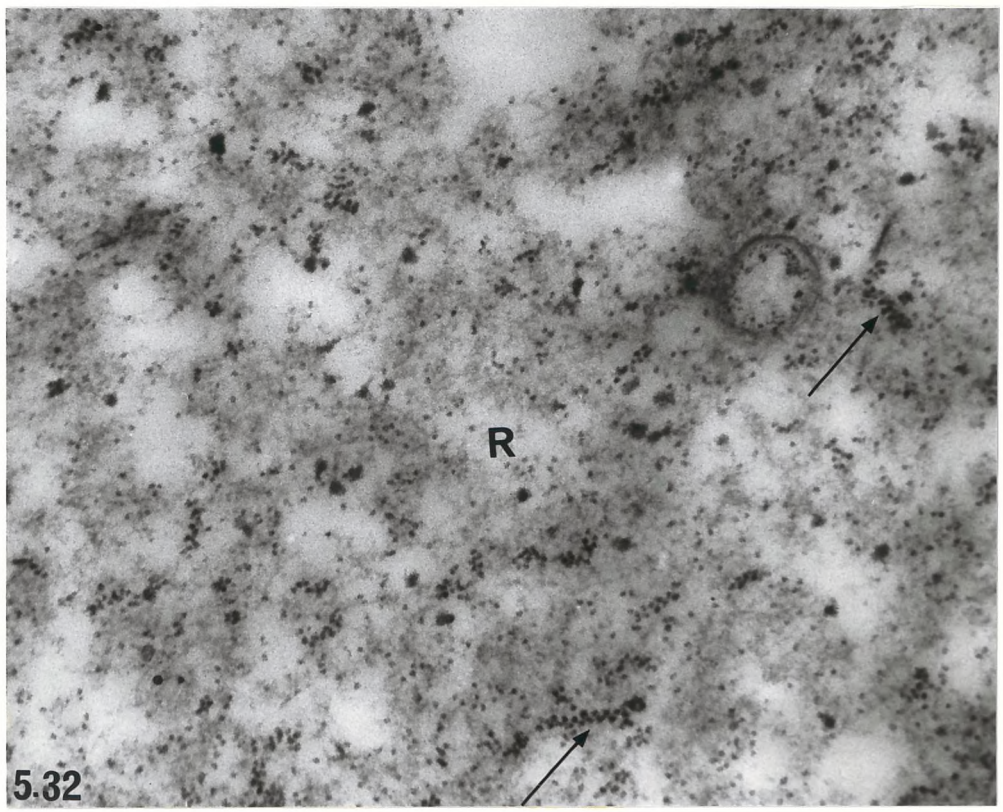


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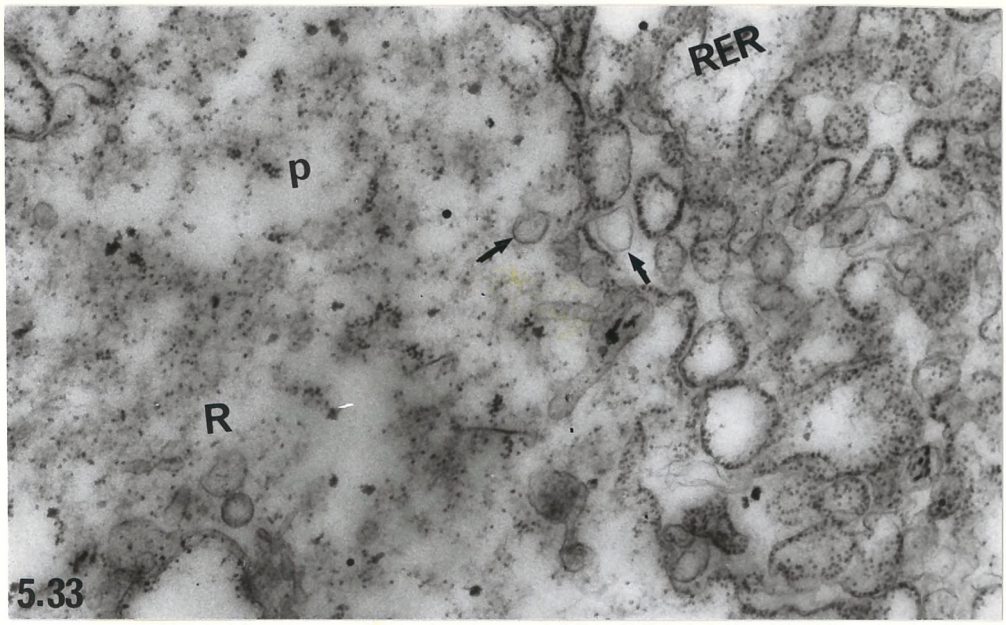
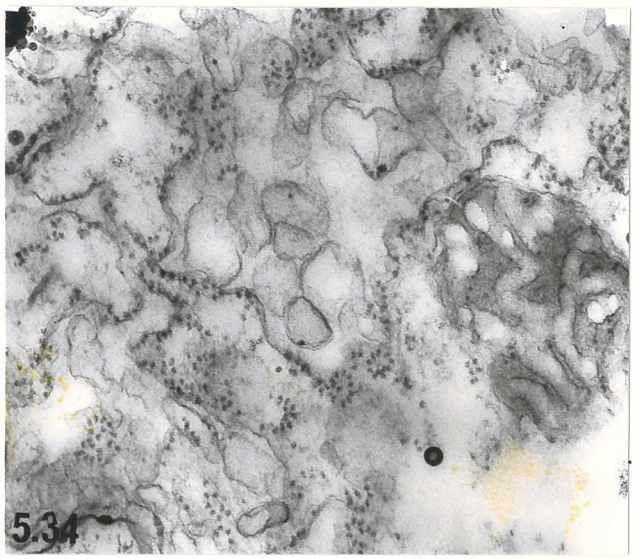
Fig. 5.33. Another field of RER, polyribosomes (P) and free detached ribosomes (R) from amoeba treated with 2×10^{-4}M Cd^{2+} for 1 h. Notice the denuded RER which appear as folds of smooth membranes (arrows). Magnification 30000 x.

Fig. 5.34. Treatment of amoebae with 1×10^{-4}M Cd^{2+} for 1 h caused detachment of large number of ribosomes from RER. This micrograph shows RER from which ribosomes have become detached leaving folds of smooth membranes or endoplasmic reticulum. Magnification 40000 x.

Fig. 5.35. Amoebae treated with 1×10^{-4}M Cd^{2+} for 2 h, showing a marked increase in the free detached ribosomes (R). Only few free polyribosomes can still be seen in the cytoplasm. Notice the smooth helices (arrows) still retaining their spiral structure. Magnification 40000 x.


RER
p
R
5.33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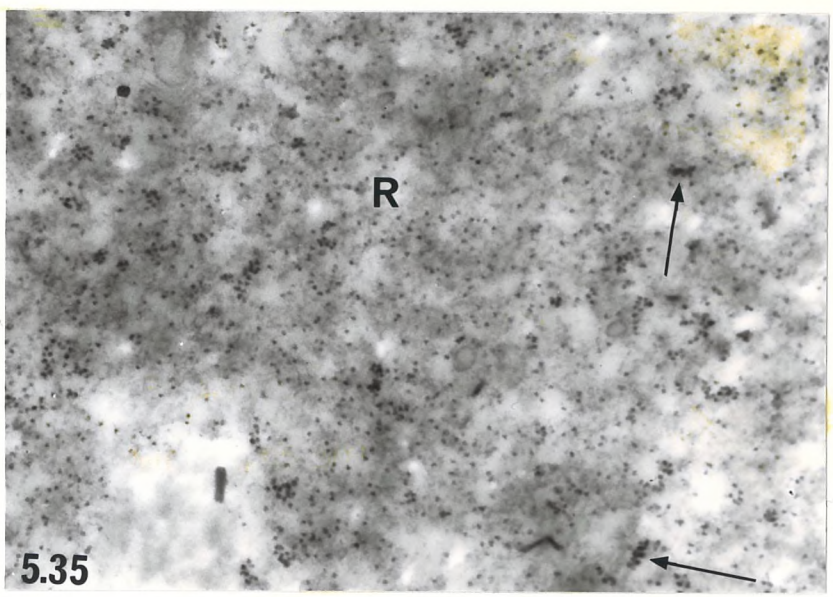
R
5.35

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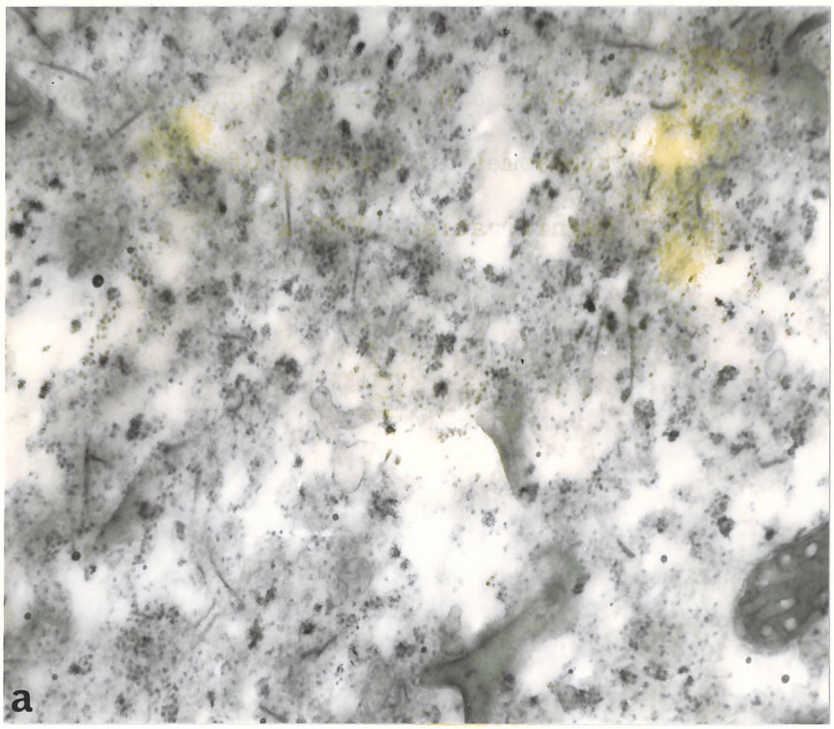
Fig. 5.36. Amoebae treated with 10^{-5}M Cd^{2+} for three days.

(a) Showing a high proportion of free detached ribosomes in a region of free polyribosomes Magnification 30000 x.

(b) Showing RER, some have become denuded from ribosomes and appear as folds of smooth membranes (arrows). Lipid droplets (L) and endosymbionts (e). Magnification 30000 x.

5.36.



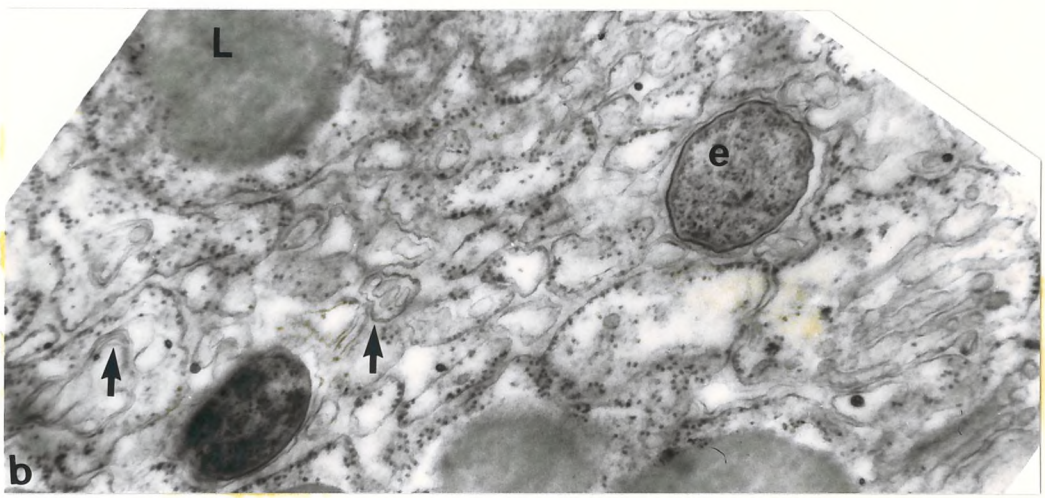
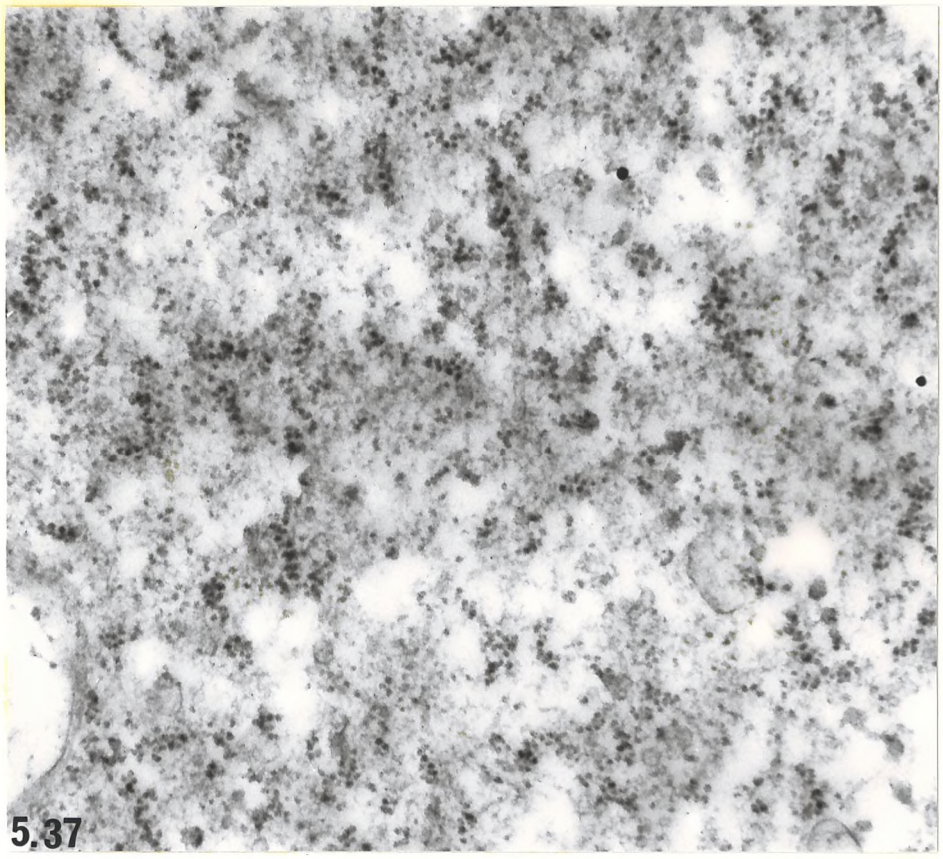
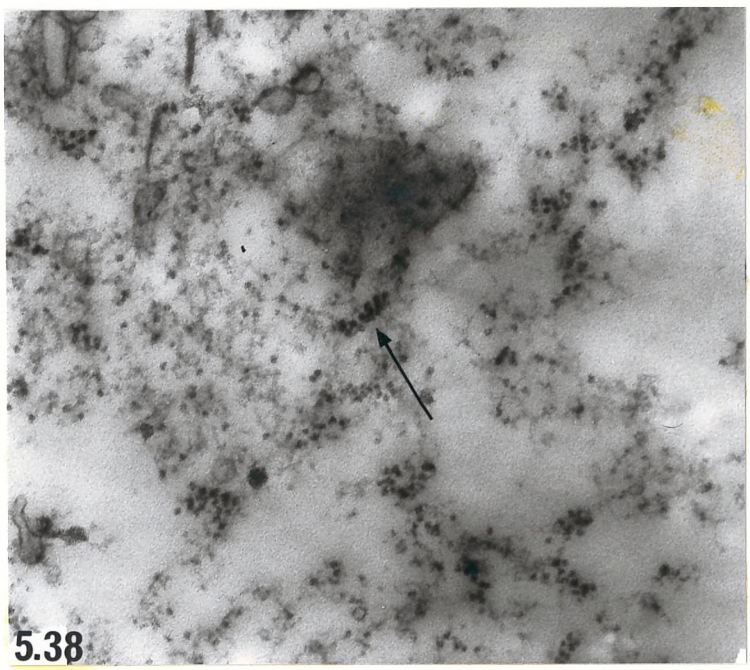


Fig. 5.37. A region of free polysomes from amoebae treated with 10^{-6}M Cd^{2+} for 6 days. Notice the presence of a high proportion of free detached ribosomes, however, this proportion does not appear to be as high as those shown in figs. 5.32, 5.35. Magnification 40000 x.

Fig. 5.38. Amoeba treated with 10^{-7}M Cd^{2+} for one month. Again there is a marked increase in the free detached ribosomes. Notice the smooth helix which is still retaining its spiral structure. Magnification 40000 x.



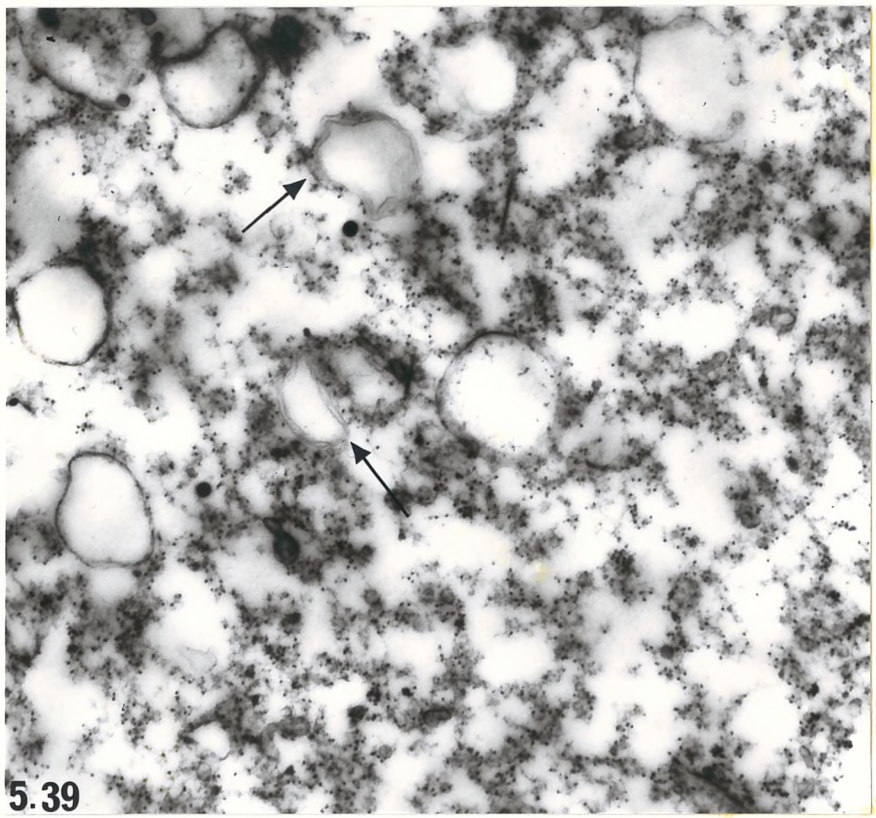
5.37



5.38

Fig. 5.39. Amoeba treated with 10^{-7}M Cd^{2+} for two months, showing laminated folds of smooth membranes (arrows) and free detached ribosomes. Magnification 25000 x.

Fig. 5.40. Amoeba treated with 10^{-7}M Cd^{2+} for nine months (Cd-adapted amoeba) showing RER and ER (possibly SER) in proportions near to those of the control amoeba. Magnification 40000 x.



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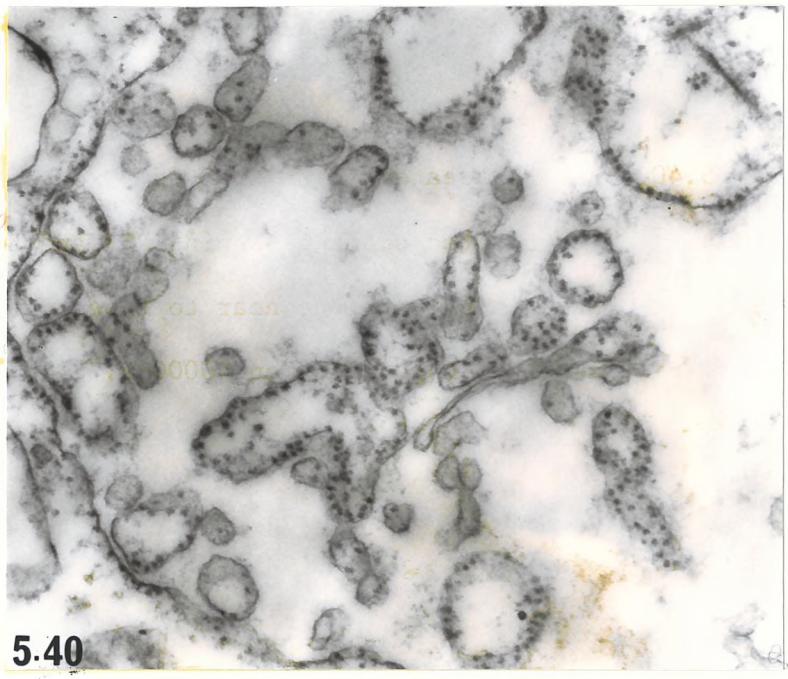

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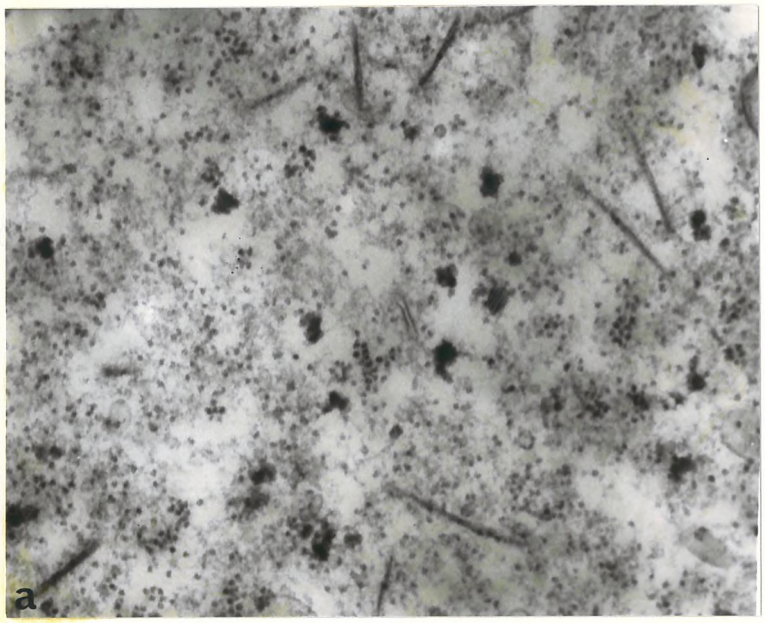
Fig. 5.41. Amoebae treated with 10^{-8}M Cd^{2+} for one month.

(a) Compared to control there is a marked increase in the free detached ribosomes and only few free polyribosomes. Magnification 40000 x.

(b) Showing RER, free polyribosomes (P) and some free detached ribosomes in an overall distribution near to that of the control amoebae. Also shown are folds of smooth membranes (long arrow), endosymbionts (e) and possibly a remnant of a golgi body (short arrow). Magnification 20000 x.

5.41.



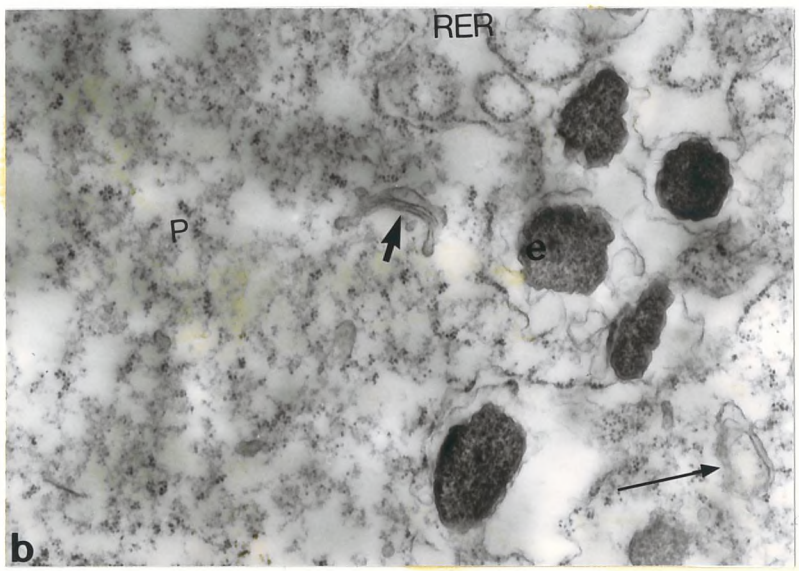
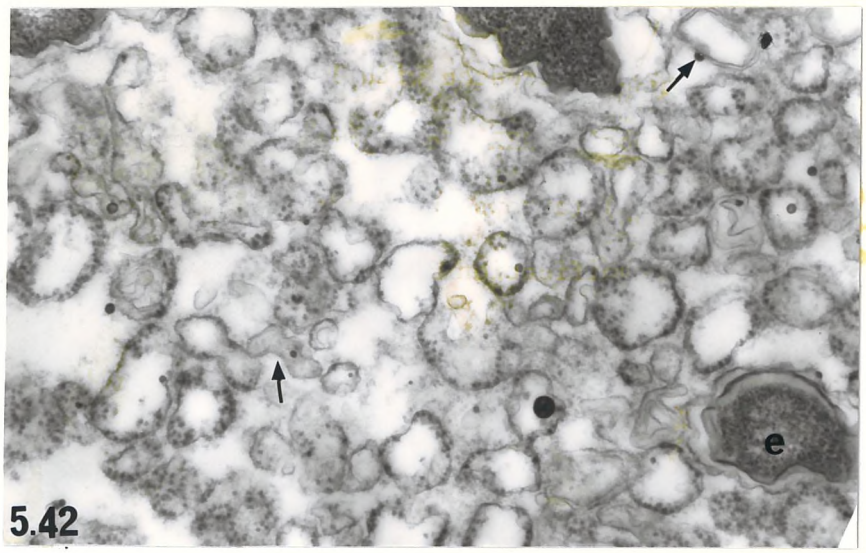
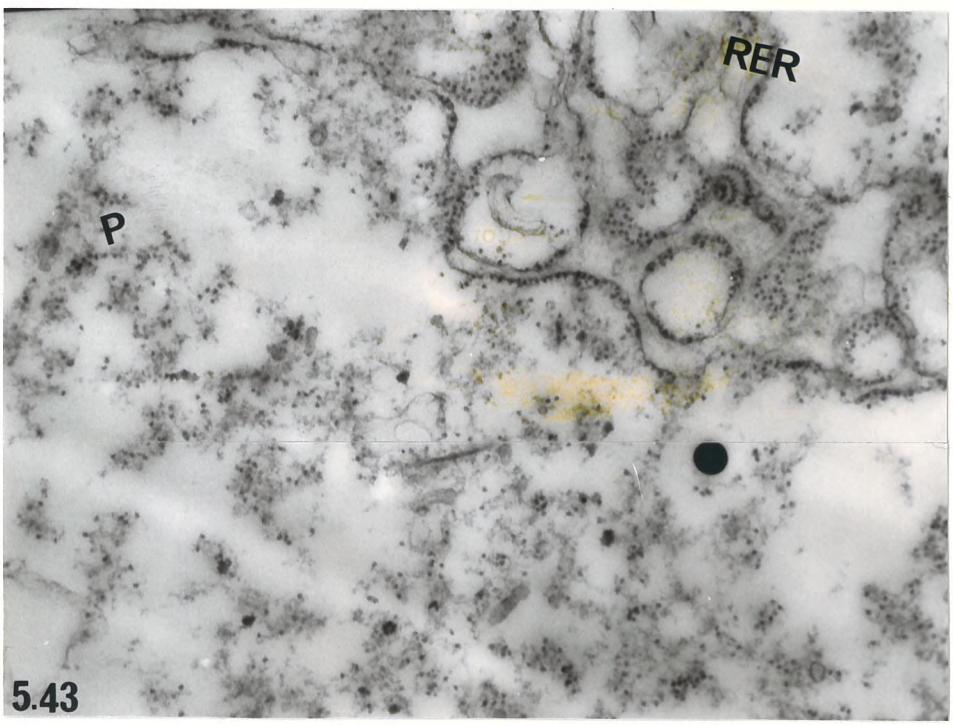


Fig. 5.42. Amoeba treated with 10^{-8}M Cd^{2+} for two months, showing RER, folds of smooth membranes (or denuded RER) (arrows) and endosymbionts (e). Magnification 30000 x.

Fig. 5.43. Amoeba treated with 10^{-8}M Cd^{2+} for eleven months (Cd-adapted amoeba) showing RER, free polyribosomes (P) and very few free unattached ribosomes. The general distribution of ribosomes is similar to that in the control amoeba. Magnification 30000 x.



5.42



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Nucleus of control and Cd^{2+} treated amoebae.

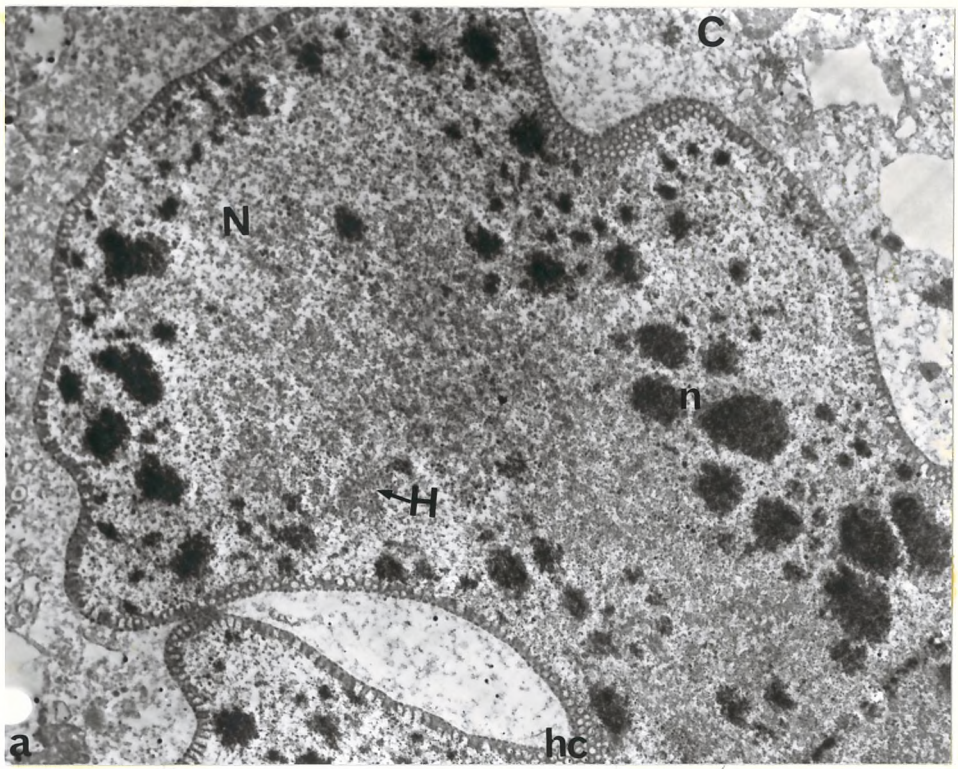
Fig. 5.44. Nucleus of a control amoeba (N).

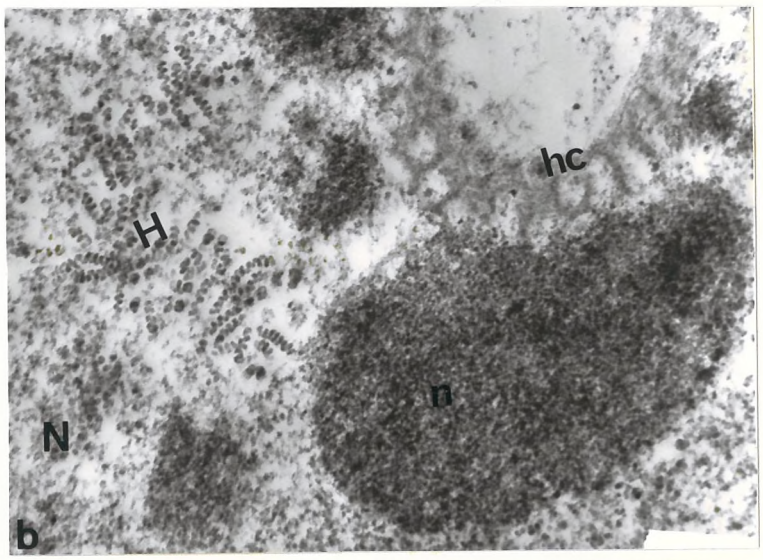
(a) Showing the honey-comb structure of the nuclear membrane (hc), the nucleoli (n) and clusters of nuclear helices (H). Magnification 5000 x.

(b) The nuclear helices (H) clustered near the nucleoli (n) and the nuclear membrane (hc) Magnification 30000 x.

(c) A nuclear helix (arrow) is shown leaving the nucleus (N) to the cytoplasm (C) through a nucleopore in the nuclear membrane (hc). Magnification 40000 x.

5.44.





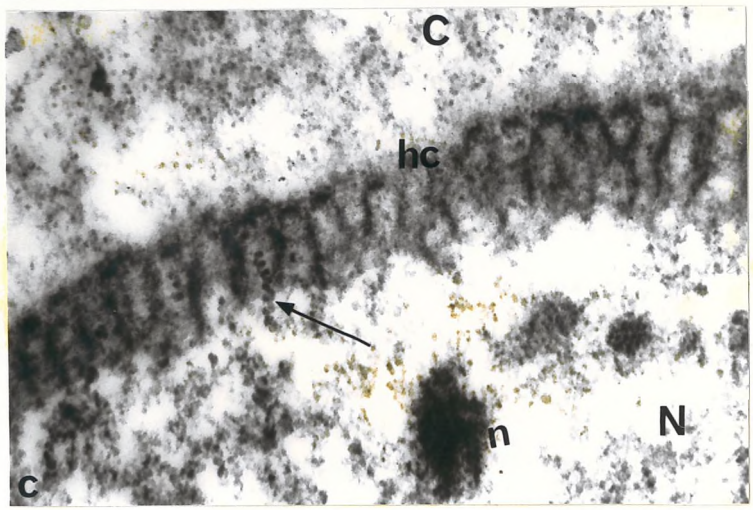


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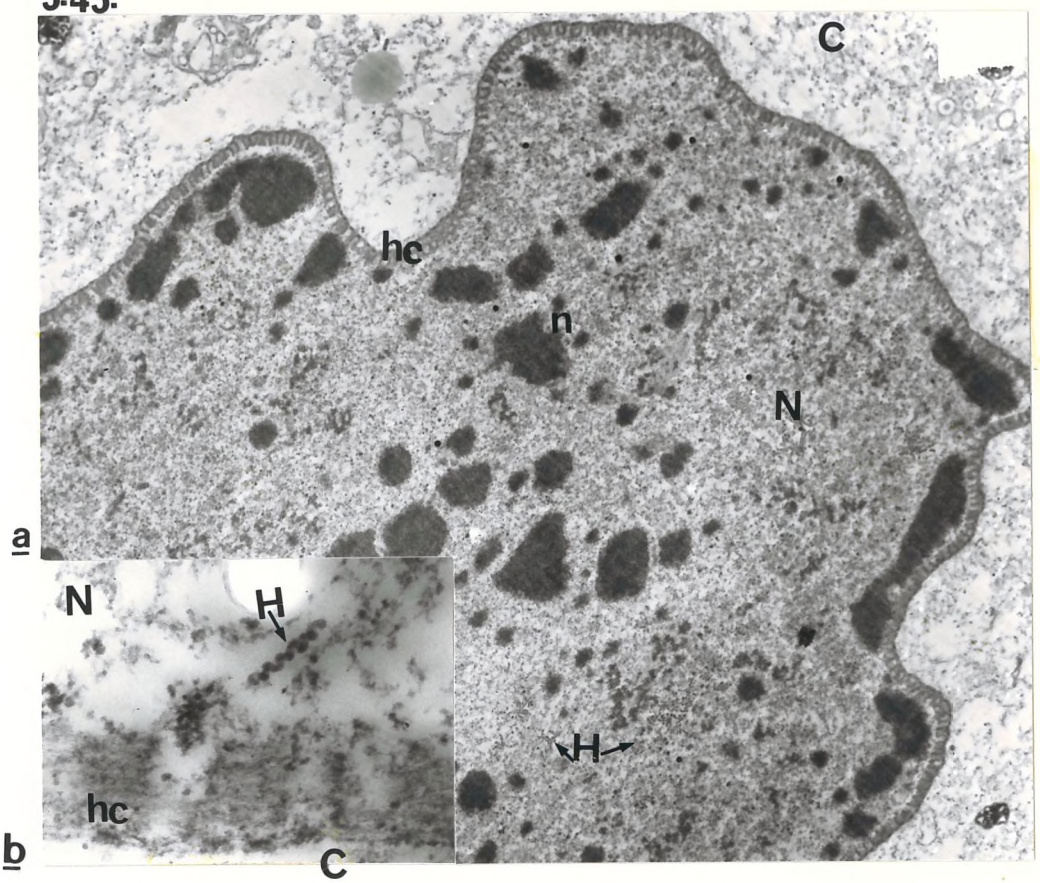
Fig. 5.45. Nucleus of an amoeba treated with 2 x 10^{-4}M Cd^{2+} for 1 h, showing the honey-comb nuclear membrane (hc), cluster of nuclear helices (H) and nucleoli (n). Magnification 6000 x. The inset lower left shows a nuclear helix (H) leaving the nucleus (N) to the cytoplasm (C) through a nucleopore in the nuclear membrane (hc). Magnification 40000 x.

Fig. 5.46. Amoeba treated with 10^{-5}M Cd^{2+} for 3 days.

(a) Showing a nuclear helix (short arrow) leaving the nucleus to cytoplasm through the nuclear membrane (hc). Also shown are mitochondria (M_1) and large lipid droplets (L). Notice a helix of a smooth spiral structure in the cytoplasm (long arrow). Magnification 15000 x.

(b) A part of the nucleus (N) showing clusters of nuclear helices (H), nucleoli (n) and the nuclear membrane (hc). Notice the presence of a a "vacuole-like" structure within the nucleoli (arrows).

5.45.



5.46.

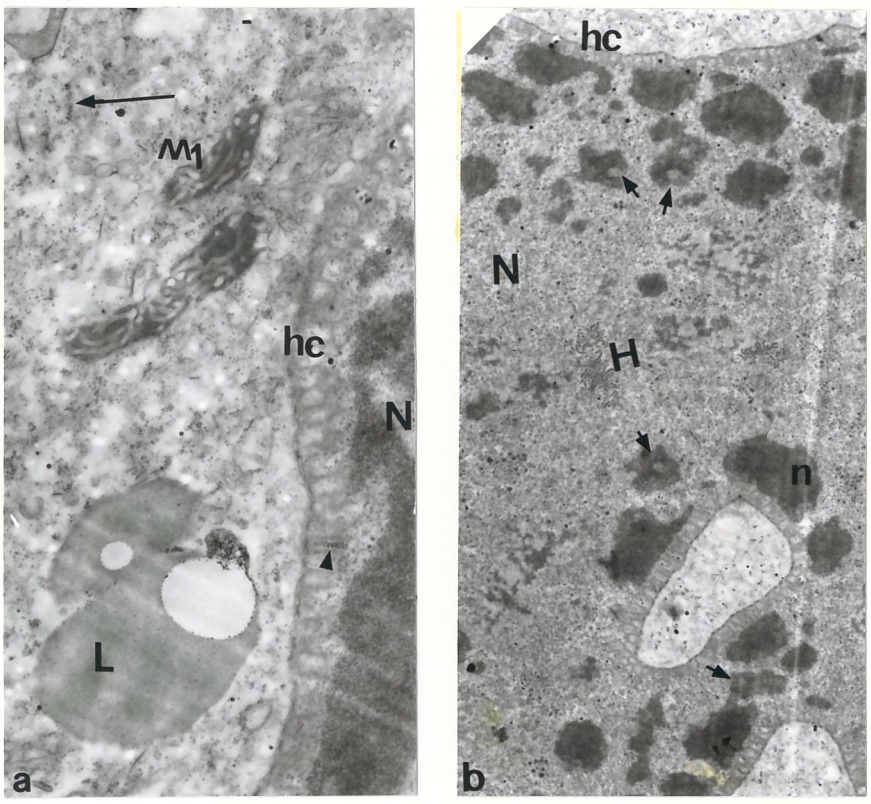
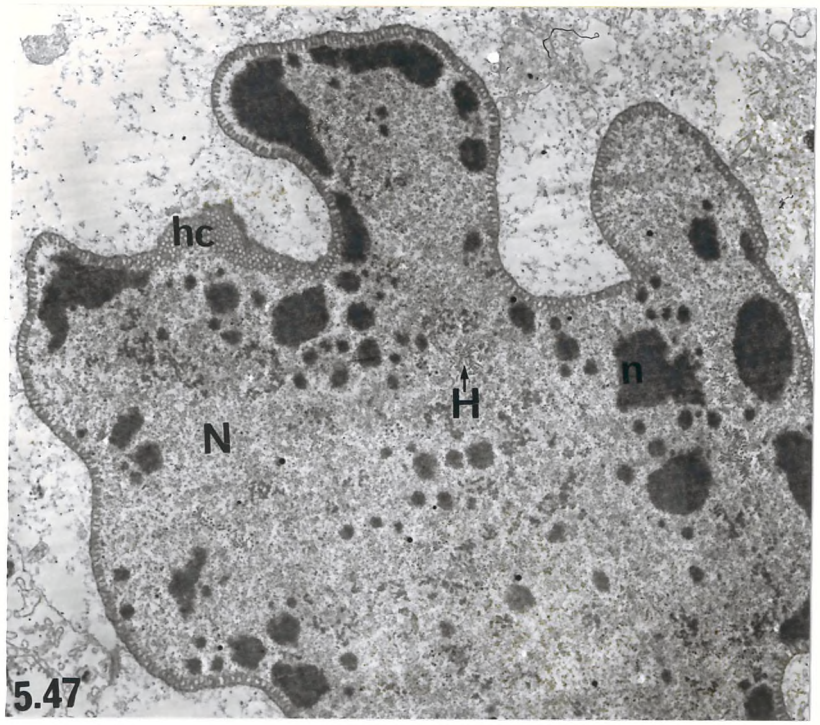


Fig. 5.47. Nucleus from an amoeba treated with 10^{-7}M Cd^{2+} for one month showing the honey-comb nuclear membrane (hc), the nucleoli (n) and clusters of nuclear helices (H). Magnification 5000 x.

Fig. 5.48. Part of a nucleus from an amoeba treated with 10^{-7}M Cd^{2+} for nine months (Cd-adapted amoeba) showing clusters of nuclear helices (H), nucleoli (n) and the honey-comb nuclear membrane (hc). Magnification 25000 x.



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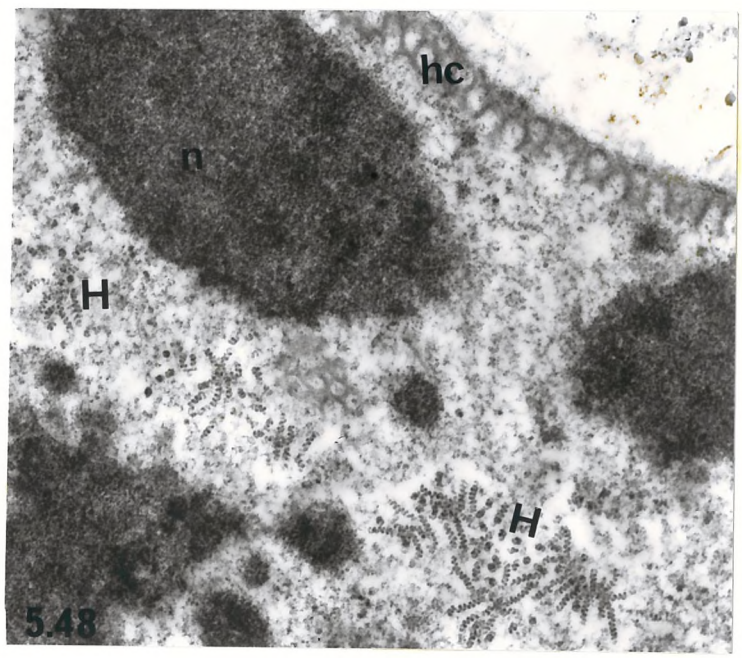

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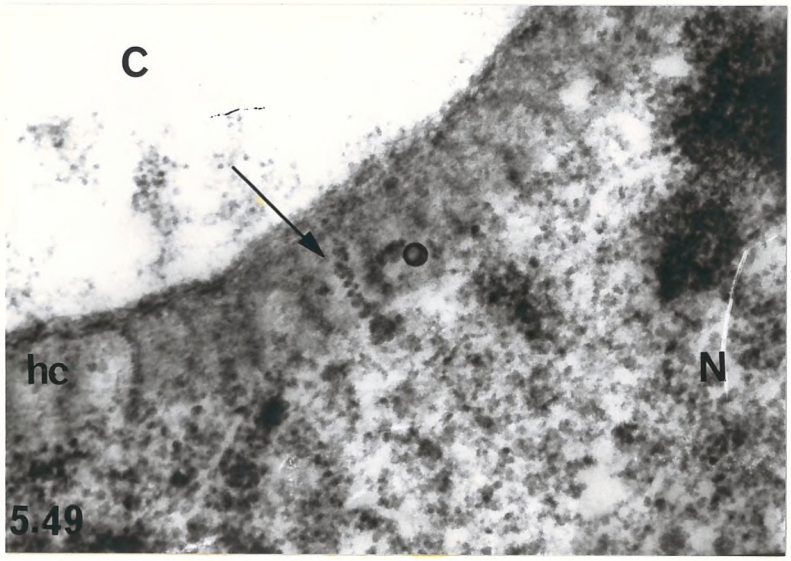
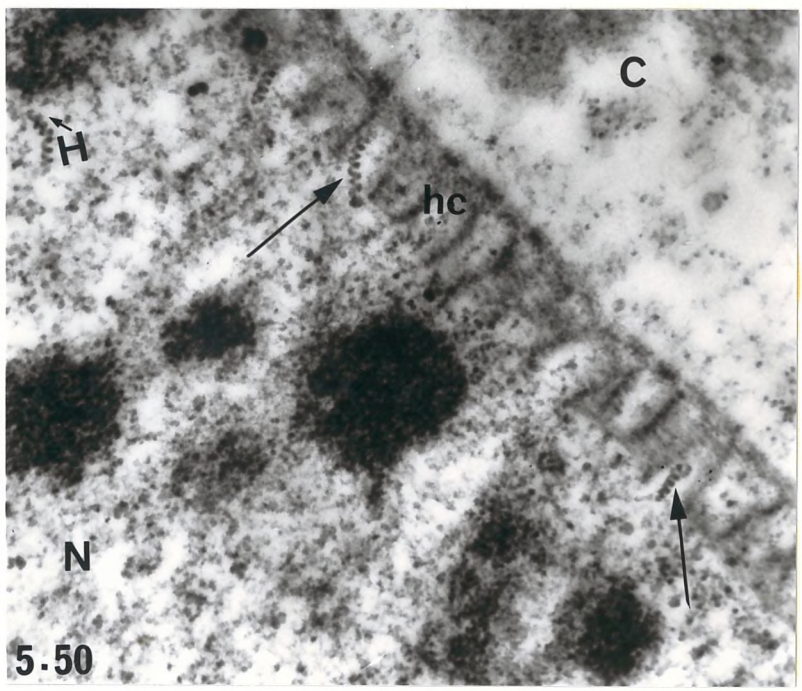
Fig. 5.49. Part of a nucleus from an amoeba treated with 10^{-8}M Cd^{2+} for one month showing a nuclear helix (arrow) leaving the nucleus (N) to the cytoplasm (C) nuclear membrane (hc).
Magnification 40000 x.

Fig. 5.50. Nucleus from an amoeba treated with 10^{-8}M Cd^{2+} for two months showing several helices (arrows) (H) finding exit via the nuclear membrane (hc) to the cytoplasm (C). Magnification 30000 x.


C
O
hc
N
5.49


H
C
hc
N
5.50

5.7. Summary.

The ultrastructural studies have demonstrated that Cd given at a wide range of concentrations and over a wide range of exposure times caused extensive damage to the integral structure of the mitochondria. A similar mitochondrial damage was demonstrated in a wide variety of Cd treated organisms.

Cd treatment also affected the overall distribution of the ribosomes. ~~This was indicated by the appreciable increase in the monosomes.~~ This was indicated by the appreciable increase in the monosomes and the proliferation of smooth membranes. Such change is likely to ultimately affect the protein synthesis within the cell.

Cd was also shown to affect the lipid metabolism causing the accumulation of lipid droplets in the cytoplasm. However, this last effect was only observed in amoebae treated with the intermediate doses of Cd .

Cd treatment seems to produce changes in the golgi bodies, reflected by diffuse ill-defined appearance and reduction in the number of cisternae and in some samples there was an increase in the smooth vesicles associated with the golgi body.

The ultrastructural studies have failed to reveal pronounced changes in the nucleus. However, some nuclear helices failed to loose their spiral shape while moving from the nucleus to the cytoplasm and were found as individual spiral helices in the cytoplasm.

The ultrastructural changes observed in this chapter complement the single cell and the biochemical studies carried out in the previous chapters (3 and 4). Further, the electron microscopic study has provided more information on the effect of Cd on the microsomal fraction (i.e. the mitochondria and ribosomes). It has also shown that the ultrastructural changes obtained were again dependent on both the Cd concentration and the exposure time given.

Chapter Six

General Discussion and Conclusion

6.1. Cd^{2+} toxicity in Amoeba proteus is concentration and time dependent.

In this study Cd^{2+} was found to be toxic to A. proteus even when given at the very low concentrations of 10^{-7}M and 10^{-8}M. This is primarily due to the ability of amoebae to accumulate Cd to a concentration far greater than that of their environment. Cd^{2+} toxicity was found to be dependent on both the Cd^{2+} concentration given and on the time of exposure to that concentration. This toxicity was reflected in the percentage of survivals as well as in the ultrastructural changes. The ultrastructural changes appeared considerably greater in amoebae subjected to Cd^{2+} at low concentrations (e.g. 10^{-7}M or 10^{-8}M) for long periods (1-2 months) than in amoebae subjected to high Cd^{2+} concentrations (e.g. 2×10^{-4}M or 1×10^{-4}M) for very short periods (1-2 h). With the long exposure periods to low concentrations of Cd^{2+} the percent survivals remained high while with the short exposure periods to high concentrations there was much poorer survival. Overall, this shows that a Cd dose of concentration(2x) for the time(y) does not necessarily result in the same effects as those of a Cd dose of concentration(x) for the time (2y), though the two doses may appear equivalent. This, therefore, raises some questions about the mechanism of cell death. The Cd distribution data given in table 4.2. (section 4.4.) suggest a possible explanation for these differences in cell death. Although data are only available for cells treated with a low concentration of Cd^{2+}, 10^{-8}M, the change in cellular distributions of Cd with increasing time of exposure could also apply when higher concentrations of Cd^{2+} are used. Basically the data show that over the first few

hours of exposure a relatively large proportion of $^{109}Cd^{2+}$ becomes associated with the nuclear fraction. Following this the proportion in the nuclear fraction decreases while an increased proportion becomes associated with the microsomal and cytosol fractions. Other authors have also reported this early association of high Cd^{2+} proportion with the nuclear fraction of mammalian liver cells *in vivo* and *in vitro* (Bryan & Hidalgo 1976; Yoshikawa & Suzuki 1976; and Failla et al 1979). Thus the nuclei of amoebae could be subjected to a greater Cd burden during early exposure when high concentrations of Cd are used than when exposure is to a low concentration for a much longer period. In these cells the nuclei could be the site of extensive damage by Cd which in turn could result in the observed higher lethality. Although the electron microscopic study did not reveal pronounced changes in the amoeba nucleus, this does not rule out visually undetectable damage. The interaction of Cd^{2+} with nucleic acids which can result in their damage has been investigated by several authors (e.g. Eichhorn & Shin 1968; Izatt et al 1971; Kascew et al 1976 a, b; Stoll et al 1976; Rohr & Bauchinger 1976; Sissoeff et al 1976; Nakamura 1961 b; and Premsagar & Rao 1969). Sina & Chin (1978, 1979) have investigated the ultrastructural changes caused by Cd^{2+} in the nucleus of the slime mould *Physarum polycephalum* and have suggested that Cd^{2+} causes functional and structural changes in the nucleolus through a 30 min to 4 h exposure. An autoradiographic study of Cd^{2+} distribution, preferably in synchronized amoebae could be of great value in elucidating the interaction of Cd^{2+} with the nucleus and its components.

6.2. Ultrastructural changes caused by Cd treatment.

Pronounced ultrastructural changes were observed in amoebae treated with low concentrations of Cd^{2+}, 10^{-8}M and 10^{-7}M, over relatively long periods (1 & 2 months). Treatments with intermediate doses of Cd^{2+}, 10^{-6}M and 10^{-5}M, given over shorter periods (6 and 3 days respectively) resulted in a comparatively intermediate change in the mitochondria, but a pronounced change in the lipid accumulation; this latter change indicates a Cd^{2+} interference with lipid metabolism. Although the ultrastructural changes obtained in cells exposed to the high concentrations of Cd^{2+}, 2×10^{-4}M and 1×10^{-4}M, over very short periods (1 & 2 h) were obvious, they appeared less extensive than those of the other doses.

6.2.1. Mitochondrial changes.

The mitochondria were shown to exhibit a wide range of structural changes (damages) in all the Cd treated amoebae. These damages were demonstrated in the following features:

a) Swelling of the mitochondria: the outer and inner membranes of these mitochondria appeared extended and sometimes ruptured, the matrix showed loss of density, and usually there was some loss of cristae.

b) Collapsing of the mitochondria: these mitochondria appeared to have membranes extrusions or finger like extensions. They usually had irregular (lobular) outlines. Ruptured inner and outer membranes and also vacuolation of the matrix could be seen.

c) Disintegrating or "ghost like" mitochondria: these are extensively damaged mitochondria, where only the outline or some cristae distinguish the remaining structure from the surrounding cytoplasm.

Similar mitochondrial damages caused by Cd^{2+} have been observed in other organisms. For example in yeast (Lindegren & Lindegren 1973); gill tissue of Jaera nordmani;(Bubel 1976); and in mammalian tissues (Nishizumi 1972; Hoffmann et al; Hayes et al 1976; Feader et al 1977). Several workers have explained some of the distructive effects of Cd^{2+} on the mitochondria. There are several ways by which Cd^{2+} can affect mitochondrial structure and function. For example, by uncoupling the oxidation-phosphorylation reactions, which is usually followed by swelling of the mitochondria (Jacobs et al 1956; Fluharty & Sanadi 1962; Mustafa et al 1971). Cd^{2+} can also displace essential cations, such as Ca^{2+}, Zn^{2+} and Mg^{2+}, which are known to have structural and/or functional roles in maintaining the integrity and controlling the permeability of the mitochondrial membrane (Brierley 1967; Webb 1979 b; Diamond & Kench 1974). Mitochondrial metalloenzymes (e.g. Zn-enzymes) contain -SH groups to which Cd^{2+} has very high affinity. Cd^{2+} can replace cations such as Zn^{2+} in these enzymes which results in changes in their activity, either inhibition or enhancement, (Vallee & Ulmer 1972).

6.2.2. Accumulation of lipid.

In control amoebae the function of the lipid droplets is considered to be an energy reserve (Mast & Doyle 1935). A Cd^{2+} interference with the metabolism of lipids in amoeba was indicated by the marked increase in the number and the size of these droplets when the cells were treated with the intermediate doses of Cd^{2+} (figs. 5.19 to 5.21). No such increase was observed in amoebae treated with either the high or the low ranges of Cd^{2+} doses. Thus, certain Cd^{2+} concentrations given over certain exposure times can

affect lipid metabolism in amoeba. Vallee & Ulmer (1972) have reported that Cd^{2+} can affect a large number of enzymes, some of which are involved in lipid metabolism. This would suggest a direct effect of Cd^{2+} on lipid metabolism, however, it is also possible that such lipid accumulation could be the indirect result of Cd^{2+} damage to the function of other organelles, e.g. mitochondria.

6.2.3. Ribosomal distribution.

In almost all the Cd treated amoebae examined under the electron microscope there was a noticeable increase in the free detached ribosomes (monosomes). This was usually associated with proliferation of smooth membrane. These smooth membranes may be endoplasmic reticulum (ER), the RER from which the ribosomes have become detached, or they may be newly produced smooth endoplasmic reticulum (SER), which is known to be often associated with detoxification of drugs (Quinn 1976). The detachment of ribosomes from RER and polyribosomes suggests an interference with the protein synthesizing machinery of the cell. The work carried out in mammals has demonstrated that Cd^{2+} treatment causes detachment of ribosomes from RER and polyribosomes. This was indicated by the increases in both the free detached ribosomes (monosomes) and the smooth membranes (denuded RER) (Stowe et al 1972; Nishizumi 1972; Hoffmann et al 1975; Gamulin et al 1977). Support for the interaction of Cd^{2+} with the ribosomal attachment to membranes in protozoa comes from the work of Resiner et al (1975). They demonstrated, in an *in vitro* study, that Cd^{2+} caused severe loss of ribosomal material from the homogenate obtained from *Paramecium aurelia*. This loss occurred by aggregation and resultant sedimentation of the previously membrane attached

ribosomal material. In amoeba other chemicals, such as methyl-mercury and the carcinogen N-methyl-N-nitrosourethane (MNU), were found to produce detachment of ribosomes from RER and polyribosomes (Ord 1979). Tamaoki & Miyazawa (1976), have shown in their work on Escherichia coli that sulphydryl reagents can cause the dissociation of some ribosomal subunits, indicating that these groups (-SH) are participating in holding the subunits together. Cd^{2+} is known for its high affinity to sulphydryl groups and therefore this might explain some of its effect on the ribosomal distribution.

6.2.4. The nuclear components.

In the nucleus of a control amoeba, the nuclear helices become detached from their cluster and find their exit to the cytoplasm via the nucleopore in the honey-comb structure of the nuclear membrane (fig. 5.4.4.). While going through the nucleopore each helix becomes uncoiled and loses its spiral structure. Later in the cytoplasm these uncoiled helices are believed to become ribosomes attached to a mRNA template which can either attach to ER forming RER or can remain in the cytoplasm as free polyribosomes (polysomes) (Minassian & Bell1976 b) Cd treatment did not appear to affect the detachment of the nuclear helices from their nuclear sites nor their subsequent exit to the cytoplasm via the nucleopore in the nuclear membrane (figs. 5.45; 5.46; 5.49 and 5.50). However, in some Cd^{2+} treated amoebae a small percentage of these helices retained their coiled form on passage into the cytoplasm (figs. 5.18 a; 5.35; 5.38). This may indicate a Cd interference with the uncoiling process of these helices which could in turn affect their ultimate function.

Other authors have shown Cd to cause pronounced visual changes in the nuclei of other tissues and organisms. For example, in rat liver and kidney, Hoffmann et al (1975) have demonstrated changes in the nucleoli, granulation of the chromatin material and dilation of the nuclear envelope. In rabbits, Stowe et al (1972), have reported nuclear degeneration in the kidney cortex region. Sina and Chin (1978, 1979), have reported structural and functional changes in the nucleoli of the slime mould Physarum polycephalum. Although in the nuclei of Cd treated amoebae no pronounced visual changes were obtained, in some samples (e.g. fig. 5.46 b) some of the nucleoli appeared to have a "vacuole like" structure. This, however, was only found in relatively few samples.

6.3. Adaptation of amoebae to low concentrations of Cd .

Amoebae given prolonged treatments with low concentrations of Cd^{2+}, 10^{-8}M and 10^{-7}M over 11 and 9 months respectively, showed much less ultrastructural damage than was observed after 1 and 2 months. In general the morphology of the various organelles appeared little different from those of their control counterparts. This suggests that the amoebae gradually became adapted or resistant to the low concentrations of Cd . In such "Cd-adapted" amoebae the mitochondria returned to their normal morphology. The three forms of mitochondria normally found in the control amoeba, i.e. dark light and intermediate (Flickinger 1968 a), were all present. This adaptation of the amoeba with a return of normal mitochondria contrasts with that of the Cd-adapted yeast strain, where the yeast became respiratory deficient, and possessed few and greatly modified

mitochondria (Lindegren & Lindegren 1973). The nature of Cd-adaptation in amoebae remains to be investigated. Other organisms have also been shown capable of becoming physiologically and/or genetically adapted to Cd . For example bacteria (Novick & Roth 1968; Kondo et al 1974; Tynecka & Zylinska 1974; Mitra et al 1975; Chopra 1971, 1975), yeast (Nakamura 1961 a, b; Nakamura & Ashida 1959; Nakamura 1963) and fungi (Ashida 1965; Cole et al 1968).

The only experiment carried out on the Cd-adapted amoebae was to investigate the pattern of $^{109}Cd^{2+}$ uptake (section 4.2.1.). From the results obtained it was found that there was a difference in the pattern of $^{109}Cd^{2+}$ uptake by the Cd-adapted and the control amoebae. In the control there was a biphasic pattern of $^{109}Cd^{2+}$ uptake, whereas, in the Cd-adapted amoebae there was a continuous and slow uptake of $^{109}Cd^{2+}$ over the same exposure period (Figs. 4.9. 4. 2. a and b). This pattern of $^{109}Cd^{2+}$ uptake by the Cd-adapted amoebae suggests that either the $^{109}Cd^{2+}$ taken up was replacing released unlabelled Cd^{2+}, or that the cells were still accumulating more Cd^{2+} from the medium at a very slow rate.

In the retention experiment (section 4.3.) it was shown that some of the $^{109}Cd^{2+}$ taken up by the cells was released. Therefore, it is possible that some of the $^{109}Cd^{2+}$ taken up by the Cd-adapted cells could be replacing released unlabelled Cd^{2+}. Further Cd-adapted cells recently divided before the uptake experiment could accumulate more $^{109}Cd^{2+}$.

6.4. Uptake, retention, and distribution of $^{109}Cd^{2+}$ in A. proteus.

In control amoebae the initial rapid uptake of $^{109}Cd^{2+}$ was followed by a "plateau". During this period the cells appeared to undergo certain adjustments which enabled them to resume further uptake of $^{109}Cd^{2+}$, though at a much slower rate. After this, i.e. on day 3 in Fig. 4.1., another plateau was reached and no further uptake was recored. The first uptake pause or "plateau" is of particular interest since it seems probably that during this interval changes must be taking place within the cell.

A biphasic pattern of Cd^{2+} uptake has been reported in other completely different living systems. For example in mammalian liver cells, there was a rapid uptake of Cd^{2+} followed by a plateau after which the accumulation of Cd^{2+} into the liver was continued when more Cd^{2+} was administered (Kapoor et al 1961; Shaikh & Smith 1976). Here the first rapid uptake was reflected by a temporary association of Cd^{2+} with high MW protein, followed by its release and reincorporation into newly produced metallothionein. Once metallothionein production had been induced the liver cells were capable of accumulating more Cd^{2+}. This Cd^{2+} became readily incorporated into metallothionein, apparently without having to bind first to a high MW protein (Webb 1979 a; Squibb & Cousins 1974).

Amoebae, like tissue culture cell lines and other unicellular and many multicellular organisms, was shown capable of accumulating Cd^{2+} to concentrations much higher than those of the surrounding media. On transfer and subsequent culture in Cd^{2+} free medium, about 50% of this accumulated Cd^{2+} was retained, apparently firmly bound within the cell(Fig. 4.3). A similar finding was reported for the

bacterium Staphylococcus aureus, where about 40% of the accumulated Cd^{2+} was retained (Chopra 1971;(Table 4.1. Fig 4.3.)

The $^{109}Cd^{2+}$ distibution within the different subcellular fractions in amoebae over a 7 days interval, during which the cells were in the $^{109}Cd^{2+}$ treatment medium for the first 2 days and then removed to MCS for the subsequent 5 days (table 4.2. section 4.4.) shows a surprising resemblance to the intracellular distribution of Cd^{2+} reported for rat tissues, including liver, kidney, intestine, pancreas, and testis (Kapoor et al 1961; Bryan & Hidalgo 1976; Sabbioni et al 1978, cited by Webb 1979; and Chen et al 1974), mouse liver (Yoshikawa & Suzuki 1976) and rat liver parenchymal cells in primary monolayer culture (Failla et al 1979). Thus despite the vast phyletic difference between these systems and amoeba the cellular responses to Cd show a marked resemblance. On the other hand, in some tumour cells the Cd intracellular distribution is markedly different from that of ordinary tissues. Heath and Webb (1967) and Webb et al (1972) have investigated the Cd distribution within the Cd induced primary rhabdomyosarcomata in rats. They found that most of the Cd was associated with the nuclear fraction (81%) a much smaller amount was associated with the mitochondrial fraction, very little or none in the microsomal fraction and about 15% in the cytosol fraction.

In E. coli the intracellular distribution of Cd was shown to be different in Cd-accomodated cells from that in non-accomodated cells (Mitra et al 1975).

Coombs (1979) listed the percentages of Cd distributed within the subcellular fractions obtained from different tissue homogenates of the following marine invertebrates: the whole soft tissue from the oyster Crassostrea gigas, the kidney of the mussel Mytilus edulis, the liver and the kidney of the sea-lion Zalophus californianus californianus. The highest proportions of Cd^{2+} were again associated with the cytosol fraction (45%, 80%, 45-58%, 60-68% respectively). Howard & Nickless (1977) and Noel-Lambot (1978), have reported that the cytosol fractions obtained from Littorina littorea, Patella vulgata and Purpura lapillus contained 55-64%, 50-79%, and 23-91% respectively of the total Cd in their soft tissue homogenates. In all these marine invertebrates, the Cd^{2+} found in the cytosol fraction was associated with low MW Cd-binding proteins similar to the vertebrate metallothioneins. However, in contrast, in the red marine alga Porphyra umbilicalis the highest proportion of Cd^{2+} was found to be associated with the nuclear fraction (Maclean & Williamson 1977 sited by Coombs 1979).

6.5. Soluble Cd-binding proteins in Amoeba proteus.

The gel-separation of the amoebae cytosol fraction has revealed two different protein peaks in which high levels of $^{109}Cd^{2+}$ counts were recovered, a high MW Cd-binding protein >45,000 MW and a low MW Cd-binding protein of 10,000-12,000 MW. The size of both peaks was found to increase in step with each increase in exposure time. However, the size of peak I was always greater than that of peak II. Similar protein peaks were obtained from the gel separation of cytosol fractions of mammalian tissues and of mammalian cell lines

in tissue culture (Winge & Rajagopalan 1972; Rudd & Herschman 1978, 1979; Hidalgo et al 1978). It is of interest that the Cd distribution between the two protein peaks obtained from the Cd-resistant A9 cell line, an enzyme deficient mutant line of mouse fibroblasts (Rugstad & Norseth 1978) were identical to that of amoebae.

6.6. The association of cysteine with the Cd-binding protein of peaks I and II.

In amoebae as in other systems, e.g. mouse tumour cells (Hidalgo et al 1978), and mouse testis (Gunn et al 1968 b) added cysteine did not only increase the total uptake of Cd^{2+} by the cells, but it also affected the final distribution of Cd^{2+} between the two protein peaks, I and II. In the presence of cysteine the increase in $^{109}Cd^{2+}$ associated with the protein of peak II was several fold greater than that associated with the protein of peak I, (Fig4.7) Furthermore, when control amoebae were treated with ^{35}S-cysteine alone, only one major peak of cysteine counts was obtained. This peak corresponded with ^{109}Cd peak I in fraction numbers. When the amoebae were treated with both unlabelled Cd^{2+} and labelled ^{35}S-cysteine simultaneoulsy, two ^{35}S peaks - corresponding in fraction numbers to those of ^{109}Cd peaks I and II - were obtained (Fig. 4.8) These results indicate that in the presence of Cd^{2+}, a marked increase in the production of a Cd-binding protein of low MW 10,000-12,000 (peak II) occurs. These findings do not rule out the possibility of a very small quantity of pre-existing metal binding protein being present at the position of peak II in the control amoebae. This is possible as small quantities of essential metal ions such as Zn^{2+}

can gain access to amoebe through the live food. Workers using mammalian systems could not rule out the possibility of pre-existing metallothioneins prior to Cd treatments, which then induced the production of much greater quantitities of Cd-thionein (Winge & Rajagophlan 1972; Bryan and Hidalgo 1976; Webb 1979 a).

The low MW Cd-binding protein of peak II appears richer in its cysteine content than that of peak I. This is deduced from the large size of the radioactive peak II (^{35}S or ^{109}Cd) of Fig. 4.7 & 4.8), when compared with its protein concentration, which was too low to be measured by the UV spectrometer used (Fig. 2.1.). Although the size of the radioactive peak I (^{35}S or ^{109}Cd) Fig. 4.7. & 4.8) was lager than peak II, the UV absorbance readings indicate a high concentration of protein at peak I compared with that of peak II. Furthermore, when labelled $^{109}Cd^{2+}$ was used together with unlabelled cysteine the increase in the size of the radioactive (^{109}Cd) peaks was markedly greater for peak II than for peak I (Fig 4.7). Nevertheless, the amino acid composition and the metal binding sites on the Cd-binding proteins of peaks I and II are yet to be identified. The use of an amino acid analyzer would provide useful confirmation of the cysteine content in both peaks. Also the use of a protein synthesis inhibitor in combinations with Cd and/ or cysteine can further elucidate the production of the Cd-binding protein at peak II.

The present results on amoebae regarding the production of Cd-binding proteins and the influence of cysteine are therefore in agreement with the mammalian findings as is the reservation which must be born in mind regarding the possibility of a small amount of

pre-existing metalloproteins. Reports in the literature have already indicated that the production of metalloproteins is not exclusive to vertebrates. Similar low MW Cd-binding proteins have been separated from a variety of invertebrates, e.g. the crabs Scylla serrata and Cancer magister the shrimp Acetes sibogae, the chiton Cryptochiton stelleri (Olafson et al 1979 a, b), the mussel Mytilus edulis (Noel-Lambot 1975), the limpet Patella vulgata, the dog-whelk Purpura lapillus (Noel-Lambot 1978) and the blue green alga Anacystis nidulans (Maclean et al 1972). It appears that the ability to produce low MW Cd-binding proteins in response to Cd^{2+} treatment is not limited to a single group of animals.

Differences between metallothioneins produced by different tissues of the same animal are known to exist (Webb 1979 a) for example liver Cd-thionein differs slightly from kidney Cd-thionein in the proportions of metal and amino acids (see tables 6.2 and 6.3 in Webb 1979 a). Therefore, differences in the characteristics of those low MW Cd-binding proteins obtained from vastly different organisms can also be expected. For example the low MW (7400 Dalton) Cd-binding protein separated from the American oyster Crassostrea virgnica was found to contain some aromatic amino acids (2.6% tyrosine and 3.5% phenylalanine) which are known to be absent from mammalian metallothioneins. Also in contrast to mammalian metallothioneins this Cd-binding protein contains relatively little cysteine (8%) (Ridlington & Fowler 1979).

6.7. Pretreatment with a low dose of Cd could protect against a subsequent higher dose of Cd.

Pretreatment of amoebae with a low dose of Cd resulted in a short-lived protection against a subsequent high dose of Cd (LD_{80}). This protection was maximal at day 1 after the pretreatment, following which its effect rapidly disappeared, i.e. the percent of survivals dropped back to near control level (fig. 3.3.). This short-lived protection cannot be attributed simply to the low-MW Cd-binding protein produced by the amoebae in response to the Cd pretreatment, since the protection was lost long before the Cd-binding protein disappeared from the cell. The nature of this short-lived protection is still to be elucidated.

In mammals many authors have considered that one of metallothioneins functions is to protect the animal or the tissue from the toxicity of subsequent doses of Cd or other similarly toxic cations (e.g. Piscator 1964; Terhaar et al 1965; Nordberg 1974; Friberg et al 1971; Magos & Webb 1976; Leber & Miya 1976; Webb 1975; Webb 1979 a). On the other hand the administration of Cd-thionein (e.g. hepatic Cd-thionein) was shown to be more toxic than an equivalent dose of free Cd^{2+} (Nordberg et al 1975; Webb & Etienne 1976). Fowler & Nordberg (1978) have also investigated the renal ultrastructural changes caused by Cd-thionein. It was therefore, concluded by Webb & Verschoyle (1976) that the mere presence of Cd-thionein (or Zn-thionein) in itself cannot confer the protection against Cd^{2+} (Webb 1979 c).

The wide variation in the percent survivals of amoebae obtained for each pretreatment/main treatment experiment was not unexpected. Amoebae workers know well that it is extremely difficult to provide different batches of amoebae at different times which have identical physiological and cultural states. Several workers have reported changes in, and the deterioration of, amoebae cultures for no obvious reasons. A number of difficult to control factors can affect the condition of amoeba culture. For example, the condition and the quantity of the live food supplied per culture, the length of time during which the amoeba cultures were fed on Tetrahymena alone i.e. the time interval between the removal of amoebae from their stock (wheat) cultures and the experimentation time. It has been reported that amoebae grown in wheat (stock) cultures have wider physiological variations than amoebae kept in MCS and fed on Tetrahymena (Griffin 1960). In the present work it was observed that more variations in the results were obtained when the amoebae used were very recently removed from the stock (wheat) cultures. Therefore, though the amoebae used were all kept for at least two weeks in MCS + Tetrahymena prior to experimentation, variations in the states of different cultures could not be completely avoided.

6.8. Conclusion

This work has shown that Cd is toxic to amoebae and that it is accumulated within the cell at concentrations far greater than those of the surrounding media. It has also shown that Cd^{2+} binds to both high MW and low MW proteins. The characteristics of the latter are in most ways similar to the metalloproteins and Cd-binding proteins of other living systems. It is clear that to obtain an

understanding of how a toxic chemical is affecting a cell it is important to apply several complementary methods of investigation. The present study has combined a single cell study where light and electron microscopic observations were made and a mass culture study where biochemical analyses were carried out. Hopefully, this study may help to stress the importance of investigating the toxicity of Cd^{2+} and other heavy metal pollutants using a wide variety of organisms; i.e. those which are likely to come into direct contact with such pollutants in their own environments, as well as systems used to detect possible human suceptability.

While it is logical initially to study the toxic effects of heavy metal ions individually it is realized that under environmental conditions organisms like amoeba can be subjected to many different heavy metal ions in combination as well as to a mixture of other pollutants. Thus studies of the effects of heavy metal ions in combination, where their effects may be synergistic rather than simply additive, could be a valuable area for further research. In Cd^{2+} polluted environments once the metal ions have been taken up by single celled organisms they can pass to higher organisms through food chains. In this way higher animals may gain access to Cd^{2+} already bound in an organic form, for example, organisms feeding on Cd^{2+} exposed amoebae can become exposed to Cd-binding protein produced within the amoebae. Since, at least in mammals, exposure to Cd-thionein can be more toxic than an equivalent dose of free Cd^{2+} (Webb & Etienne 1976; Nordberg et al 1975), such magnification of toxicity could present serious problems.

6.9. Areas for further investigation.

During the course of the present study a number of potentially worthwhile areas for continued or extended research have become apparent.

1) Investigation of the composition of the amoeba Cd-binding proteins of peak I and II using an amino acid analyser. This can provide information on the amino acid content (especially those with -SH groups) of these proteins. Also, if quantifiable amounts of peak II Cd-binding protein could be obtained, it would be possible to estimate the number of Cd^{2+} ions bound per molecule of protein. This would enable further comparisons to be made with the mammalian Cd-thionein for which these data are already known.

2) The study of $Cd^{}$ distribution within the treated amoebae could be further refined by carrying out an electron microscopy autoraiographic study. This is a feasible project as has been demonstrated by Berlin & Ullberg (1963) and Murakami et al (1975) for mammalian kidney. Of particular interest would be the course of uptake and early distribution of Cd^{2+} in amoebae, since the cellular fractionation results indicated that the nucleus was an initial site of accumulation. This is known to be the case in mammalian liver cells (Bryan & Hidalgo 1976; Kapoor et al 1961). It would also be of interest to find out whether the mitochondria, which can become very damaged in appearance, are sites of high Cd^{2+} accumulation.

3) Nuclear transfer experiments. The large size of A. proteus and its nucleus renders it a suitable organism for carrying out nuclear transfer experiments. By transferring nuclei from Cd treated amoebae to enucleated control amoebae, and vice versa, more information on the relative effects of Cd on the nucleus and cytoplasm could be obtained.

4) Microinjection experiments. Mammalian Cd-thionein was found to be 7 to 8 times more toxic than an equivalent dose of free Cd when injected into mammals (Webb & Etienne 1976). The effect of Cd-binding proteins obtained from amoebae and other sources could be investigated by quantitative microinjection into amoebae.

5) Does Selenium protect against Cd toxicity in A. proteus? It is known that selenium protects against Cd toxicity in mammals (Gunn et al 1968 a, b, c; Chen et al 1975, and Prohaska et al 1977). It would be of interest to determine whether this also occurrs in amoebae and if so to investigate the mechanism of such protection.

6) The effect of chelating agents on Cd toxicity in A. proteus. There was an increase in toxicity when chelating agents such as nitrilotriacetic acid and sodiumtripolyphosphate ($Na_5P_3O_{10}$) were injected subcutaneously into mice together with Cd^{2+} compared with animals given Cd alone, yet there was a reduction in the Cd^{2+} level accumulated in their liver tissue. On the other hand, when each of the chelating agents was given orally in combination with Cd there was no difference between the toxicities (Engstrom & Nordberg 1978). Carrying out such a study at the cellular and the

ultrastructural level in an organism such as A. proteus could provide useful information on the toxic interaction between chelating agents and Cd .

7) What are the toxic effects of other heavy metal ions in amoebae, e.g. Pb^{2+}, Hg^{2+} and Cu^{2+}, when given separately or in combination with Cd^{2+}? What is the effect of relatively high levels of cations such as Mg^{2+} and Ca^{2+} on Cd^{2+} toxicity in amoebae?

8) In the present study it has been shown that amoebae cultured in the continuous presence of low concentrations of Cd^{2+} (10^{-8}M and 10^{-7}M) appear to become adapted to such concentrations of Cd^{2+}. It would be most interesting to find whether these adapted amoebae respond to higher doses of Cd in the same manner as normal amoebae, and whether they can gradually be moved to higher and higher concentrations of Cd . If so, this mechanism could be investigated using both microsurgery and biochemical studies.

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