EFFECTS OF HEAVY METALS AND ORGANIC MICROPOLLUTANTS IN THE WATER OF THE WESTERN SCHELDT ON REPRODUCTION OF SEA MUSSEL, SEASTAR, SQUIRT AND/OR SHRIMP.

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Chapter 1. MICROPOLLUTANTS IN MARINE ORGANISMS

To estimate the damage and to predict the danger of industrial pollution, modern toxicological research must involve a wide range of marine organisms. A great number of recent studies are dealing with accumulation, immobilization and metabolism of toxic substances discharged into the marine environment, but the list of their deleterious effects on marine organisms is far from being complete.

Cadmium in *Mytilus edulis*

The uptake, accumulation and excretion of Cd by *M. edulis* have been studied in detail. The uptake and accumulation rates of Cd have been found to depend on different conditions, such as exposure time, external Cd concentration, food availability, experimental system (e.g., through-flow or semi-static). The rate of excretion is 18 times slower than that of uptake. The final tissue distribution is decreasing in order: kidney > viscera > gills > mantle > muscle, foot. (George and Coombs, 1977; Jansen and Schölz, 1979; Poulsen *et al.*, 1982; Nolan and Duke, 1983).

Cadmium is believed to penetrate the cell membrane by one or more mechanisms: (1) passive diffusion, which may be facilitated by Cd binding to intracellular ligands (Carpene and George, 1981); (2) or pinocytosis of Cd in a chelated form, since chelating produces a doubling in the rate of Cd uptake (George and Coombs, 1977); (3) or possible use of metal ion pomps. George (1984) has proposed a mechanism for trans-membrane transport of Cd which involves binding of Cd to membrane phospholipids and removal of Cd at the inner membrane face by formation a complex with soluble intracellular binding ligand. As the available intracellular ligands are saturated with Cd, the uptake will slow down. Excess of Cd is detoxified by the induced synthesis of metallothionein. Duke and Nolan (1983) have suggested that intracellular binding ligands not only transfer Cd to MT, but may transport Cd into the circulation system and effect a transfer to MT in different tissues.

Metallothionein

MT is a low molecular weight protein which plays a major role in metabolism and toxicity of Cd. The main function of MT with regard to Cd is protection against long-term toxicity. Under normal conditions of chronic low-level exposure, the basally synthesized MT is probably sufficient to chelate Cd ions. Under unusual conditions of sudden high-level exposure, the induction of MT

gene transcription at the cellular level has been postulated by Cherian and Nordberg (1983).

Hamer (1986) has reviewed the recent studies of MT on molecular biological level. It has been demonstrated that heavy metal regulation occurs largely if not exclusively at the level of transcription initiation. Experiments utilizing cloned MT gene hybridization probes have indicated that substantial variations in MT synthesis can be caused by gene amplification and methylation. MT gene transcription is also induced in some mammalian cells by glucocorticoids, interferon and stress conditions. MT gene expression can be altered by cellular differentiation and development.

The amino acid sequence and structure of mammalian MT have been determined, revealing several metal binding places and high cysteine content (Elinder and Nordberg, 1985).

Noël-Lambot (1975) has found that Cd is associated with low molecular weight protein in the homogenate of Cd-treated *M. edulis*. George *et al.* (1979) have isolated Cd binding proteins with Mr 10 Kd from *M. edulis*. The incorporation of ^{35}S cysteine into 22 Kd copper-rich protein fraction was stimulated by Cd exposure (80 ppb, 48 h) of *M. galloprovincialis* (Viarengo *et al.*, 1980). Amino acid analysis of the metal-binding proteins from the tissues of the metal-exposed mussels has indicated that these proteins belong to the MT class (George *et al.*, 1979; Viarengo *et al.*, 1984). The amount of MT is greatest in kidney and hepatopancreas of *M. edulis* (Nolan and Duke, 1983). Amount of MT is found to increase when Cd body burden *M. edulis* is augmented (Köhler and Rijsgard, 1982). The experiments with purified lysosomes of mussel kidney have demonstrated the presence of MT in secondary lysosomes, but not in tertiary lysosomes, although the tertiary lysosomes contain significant concentrations of Cd (George, 1983a). These tertiary lysosomes have been suggested to act as a store for essential metals as Zn and Cu required for metallo-enzyme synthesis in cells where MT is not induced (George, 1983b).

In experiments with Cd-exposed (100 and 300 ppb) oysters, the "spill over" of Cd from MT fraction into high molecular weight fraction and low peptide-amino fraction occurred at 300 ppb Cd, indicating that the capacity of detoxifying system was overwhelmed (Engel, 1983). In *M. edulis*, the effect to spill over seemed to occur at 100 ppb Cd (critical concentration). It appears that biochemical analysis of the Cd_{mt}/Cd_{hmw} (defined as ratio between amounts of MT-associated Cd and high molecular weight protein-associated Cd) relationship in tissue such as hepatopancreas can provide a sensitive index of toxicity status of *M. edulis* with respect to Cd, with value decreasing as toxicity increases. This approach also may be valid for other aquatic invertebrate groups (Nolan and Duke, 1983).

Effects of Cd on *Mytilus edulis*

In *Mytilus edulis*, body burden up to 150 ppm Cd caused no effects on either clearance, ingestion, assimilation or growth (Poulsen *et al.*, 1982). It has been shown that only acute intoxication of *M. edulis* with 500 ppb Cd causes severe histological changes in the gill and mid-gut gland (Jansen and Jansen, 1983). Cd-induced histological changes in the gill of *M. edulis* have been found at 10 ppm Cd. Gills filaments parted from each other after breaking off the interfilamentar junction. Later the epithelium broke up and the inner parts of the filaments disappeared completely (Sunila, 1981). The ultrastructural studies (SEM and TEM) of *M. edulis* exposed to 4 ppm Cd confirmed the histological changes in the gill tissue (Sunila and Lindström, 1985). Chronic histopatologic effects of short-term Cd (1-8 ppm, 24 h) exposure have been studied in the gills of *M. edulis*, showing that morphological damage of the gill tissue persisted over a period of one year (Sunila, 1986).

The effect of heavy metals on phagocytosis by molluscan hemocytes (American oyster) have been investigated. The exposure of the isolated hemocytes to 1 and 5 ppm Cd for two hours resulted in no alteration of phagocytic activity of molluscan hemocytes (Cheung and Sullivan, 1984).

Effects of Cd and other heavy metals on reproduction of *Mytilus edulis*

Maung-Myint and Tyler (1982) have investigated the effects of temperature, nutritive and metal stressors on the reproductive biology of *M. edulis*. Their experimental observations indicated that gametogenic cycle is adversely modified by the combined effects of temperature, nutritive and sublethal metal-induced stress. Cd (50 ppb) was the least toxic of three metals (Cd, Cu, Zn) studied and suppressed the gametogenesis only in the initial stages of gonadal development. Cu was the most toxic metal, suppressing both the growth of young oocytes and vitellogenesis in larger oocytes.

Akberali and Earnshow (1985) have demonstrated that copper stimulates the respiration of *M. edulis* unfertilized egg. They have proposed a working model of Cu action, suggesting that Cu has an uncoupling effect on mitochondrial respiration, possibly by initiating potassium ion uptake which will consume energy and hence stimulate egg respiration. The stimulation of unfertilized egg respiration prior to fertilization by Cu will produce significant perturbations in the metabolite pool and will presumably lead to decrease in the total substrate reserve which could well affect egg development upon fertilization.

Cu- and Cd-induced histological changes in the mantles of *M. edulis* have been observed. Acute exposure to 2 and 8 ppm Cd for 24 h resulted into disor-

ganization of male follicles, while female follicles did not react to exposure. The cytoplasm of the ova have been shown to vacuolisate only after 14 days of exposure (Sunila, 1984).

Early embryo-larval stages of bivalve molluscs have been proposed as very convenient organisms for biological testing of potentially toxic substances, including heavy metals, because of their high susceptibility in comparison with adult organisms and possibility of measuring the deformation and other sublethal parameters in populations of uniform size and age (Calabrese *et al.*, 1977; Martin *et al.*, 1981).

Pavicic *et al.* (1985) have studied the heavy metal tolerance of the developing veliger of *M. galloprovinclalls* in relation to induced synthesis of MT. It has been found that 2.75 ppm Cd induced the highest level of MT. The results of this study also have shown that each of three selected metals exhibited a different potency for inducing MT synthesis in the decreasing order Cd > Zn > Hg, indicating a possible correlation with the decline of tolerance in the same order.

The response surface method was used to study the effects of temperature, salinity and Cd contamination on the development of the fertilized eggs of *M. edulls* from the Western Baltic Sea to the veliger stage as well as on growth and cumulative mortality of veliger population. The trochophora stage proved to be the most sensitive to the factors studied. A significant influence of Cd on different life functions was found from concentrations of about 50 ppb on. Cd only slightly influenced various temperature-dependent life functions, but strongly modified those depending on salinity. The development optimum was shifted to higher salinities with increasing Cd concentration of medium, while that of survival and growth was shifted to lower salinities. These factor interactions modified the tolerance limits (Lehnberg and Theede, 1979).

Cadmium in crustaceans

Cd was shown to be more toxic to decapod crustacean, *Palaemon elegans*, than Cr. Low concentrations of both metals were found to affect the metabolism producing usually an increase of the respiratory rates. Comparing the respiratory reaction of animals from two areas exposed to Cd (area I - 0.1 ppb Cd, area II - 0.6 ppb Cd: different parts of Saronicos gulf), became obvious that in all tested concentrations the animals of area II presented lower increases in oxygen consumption than animals from the area I. This could be considered as an indication of increased tolerance of the animals from the area II to Cd (Moraïtou-Apostolopolou *et al.*, 1982)

The studies on the population dynamics of *Gammarus tigrinus* (crustacean, amphipod) have revealed that Cd burden depends on temperature and length of the animal: high temperature are correlated with small Cd burden and juveniles carry a higher Cd burden than adults, perhaps as a consequence of different food (Hackstein *et al.*, 1986).

Two species of mysid shrimps were exposed to Cd in a continuous-flow bioassay system to determine the effect on survival and reproductive success. The 96 h LC_{50} was 110 ppb for both species. At 10 ppb Cd the series of morphological aberrations were observed in both species at the onset of sexual maturity. In this study the observed no effect concentration was 5.1 ppb Cd. The following reproductive parameters were determined to be the best indicators of pollutant stress: sexual maturation time, the first appearance of eggs in the brood sacs, first brood release, the number of broods released per female and average number of young per brood. This study demonstrated (1) that acute and chronic responses of the mysid shrimps to Cd toxicity were essentially identical; (2) the interaction of both acute and chronic toxicological responses with the environmental variables salinity and temperature; (3) that mechanism of Cd toxicity is a complex phenomen potentially involving physiological processes of a celetal calcification and osmoregulation (Gentile *et al.*, 1982).

A theoretical compartment model for the uptake of Cd and Zn by fresh water crayfish was constructed, and included a factorally designed experiment to study the relative importance of food and water as uptake factors for ^{109}Cd and ^{65}Zn. Two uptake factors were first order independent and additive. Rate constants for uptake from 5 ppb and 10 ppb Cd were 0.84 and 0.83 µg/day. Crayfish reached a steady state concentration of about 12.5 ppm dry weight (Giesy *et al.*, 1980).

The disposition of Cd and Zn in *Pandalus montadni* has been studied. Cd uptake rates in various tissues of shrimps exposed to 37 ppb Cd for 14 days ranged from 0.002 to 0.06 ppm dry weight/h. During depuration for 57 days, the levels of Cd in most tissues decreased slightly but continued to rise in hepatopancreas indicating the redistribution of Cd. In presence of Zn, Cd concentration was doubled in hepatopancreas, depressed by one-third in the carapax and did not change in the other tissues (Ray *et al.*, 1980).

The LC_{50} values of 4 heavy metals were determined for the tropical grass shirmp by the method of static bioassay. The 96 h LC_{50} values (ppm) determined by probability-logarithm transformation were found to be 1.6 for Cu, 6 for Cr, 16 for Zn. Since all shrimps tested at lethal concentrations of Cd died before 96 h, it was impossible to determine the 96 h LC_{50}; the 48 LC_{50} value was 6 ppm Cd. These results indicated that the order of sensitivity of heavy

metal tests to the shrimp was Cd > Cu > Cr > Zn. The LC_{50} of these metals (obtained form different literature sources) for crustaceans under different conditions (temperature, salinity) were summarized in Table 2 (Chung, 1980).

The acute toxic response of species pairs (phytoplankters, mysid shrimps, copepodes and fishes) tested simultaneously was determined for three toxicants (sodium lauryl sulfate, Cd and Lannate). Mysids were found to be the most sensitive to Cd and Lannate, and algae were most sensitive to sodium lauryl sulfate (Robrts *et al.*, 1982).

Laboratory soft bottom microcosms, containing sediment with natural populations of meio- and microfauna and amphipod *Pontoporia affinis*, were used to investigate chronic effects of Cd on a typical Baltic Sea benthic community. In the first experiment, *P. affinis* was exposed to different concentrations of Cd (0, 6.3, 41 and 127 ppb). After 265 days of exposure, the sediment contained about 7600 times x 6.3 ppb Cd, and *P. affinis* contained about 1,300 times x 6.3 ppb Cd. The effects on the embryogenesis were detected after 105 days of exposure in all Cd concentrations tested. In the second experiment, *P. affinis* was exposed to 0, 6.5 and 46 ppb Cd for 460 days. At the lowest concentration (6.5 ppb), the prolonged exposure did not increase the sediment concentration of Cd (about 700 times x 6.5 ppb) which indicated a steady state, while Cd concentration in *P. affinis* reached about 3,500 times x 6.5 ppb after 460 d. In the first experiment, the mortality of juveniles was significantly increased at 51 and 1127 ppb Cd after 265 days of exposure. In the second experiment, the juveniles matured sexually and reproduced normally in all microcosms, but their offspring died in all microcosms with added Cd. This occurred at Cd concentrations not uncommon in polluted marine areas. The effects are serious in comparison to those found in conventional short-term laboratory experiments with the same Cd concentrations, and they have demonstrated the importance of long-term experiments in ecotoxicology (Sundelin, 1983).

The burrowing marine shrimps (*Callianasa australlensis*) were exposed to Cd-contamined water and sediment for 56 days. The concentrations of Cd ranged from 0.6 to 63 ppb for water at a rate commensurable with increases in Cd concentration in water and the duration of the experiment. Cd concentration in sediment had no effect on Cd uptake by the shrimp. The shrimp dry weight decreased with increasing Cd concentrations in water and duration of exposure, but was not affected by the concentration of Cd in sediment (Ahsanullah *et al.*, 1984).

Blue crabs (*Callinectes sapidus*) were exposed to either 100 ppb Cd in water or fed with Cd-enriched oysters for 14 days. The patterns of uptake showed that in short-term exposure the route of exposure determined the tissue distri-

bution. Crabs exposed to Cd in the water had an order of magnitude more Cd in the gills than in hepatopancreas. Conversely, the levels of Cd in the hepatopancreas were greater than in the gill when Cd was presented in food. A single Cd-binding protein was produced in the gills and hepatopancreas in response to Cd exposure through food or water (Engel, 1983).

Effects of Cd on reproduction of marine invertebrates and Ascidians

Oocyte maturation of the starfish, fertilization and embryogenesis of the sea urchin and the development of amphioxus and brine shrimp were used to assay the effects of several metals (Cd, Cu, Pb, Cr, Hg, Ni and Zn) and agrichemicals (triphenyl tin acetate, triphenyl tin fluoride and sodium pentachlorophenate) frequently found in the marine environment. While the brine shrimp embryos were tolerant to metals and agrichemicals used, the sea urchins and amphioxus showed a differential response to the metal pollutants. Starfish oocyte maturation process was affected by agrichemicals. The result of this study have indicated that no one single organism, or its embryonic form, or a particular stage of development, can be used as "indicator" for a particular pollutant. However, the use of lower forms of marine organisms can be useful collectively for environmental investigations and management of waste disposal (Lee and Xu, 1984).

The sensitivity of swimming behavior and predator-escape responses of nauplii of the estuarine copepod (*Eurytemora affinis*) to sublethal doses of Cd and Cu have been investigated. Behavior was generally altered at metal doses below those affecting growth rates or survival of copepods. Naupliar swimming velocity was affected by Cd: swimmingspeeds were reduced after 24 h at 130 ppb and development was slowed after 48 h at 116 ppb. The LC_{50} was > 120 ppb Cd. Molting abnormalities were observed at and above 75 ppb Cd. The abnormalities consisted of twisted or missing caudal spine on both living and dead nauplii. Nauplii exposed to Cu for 24 h were observed to be generally hyperactive, a condition which could increase their encounter frequency with predator. Reduced numbers of escape responses of nauplii to a simulated predator, another indication of increased vulnerability to predation, were observed after 48 h of Cu-exposure (Sullivan *et al.*, 1983).

Cd accumulation and ultrastructural alteration in oogenesis of the prawn, *Palaemon serratus*, have been studied. After 44 h of exposure, the gonads accumulated Cd in proportion to the Cd (50 ppm) in the surrounding medium. The only change that was observed in the oocytes of specimens exposed to 50 ppm Cd was in mitochondria. The mitochondria shape was changed, the cristae be-

came wollen, expecially during the the late stage of vitellogenesis. Within the mitochondria the dielectronic material was observed, without a limiting membrane. All these alterations indicated that mitochondria did not perform their normal function and therefore the energy level could be decreased (Papathanassiou, 1986).

A standard screening test with *Artemia* early nauplii (ARC-test) is now used in a number of laboratories as a reference test with marine pollutants. *Artemia* has a world wide role in rapid screening tests for and regulatory purposes, having the advantages of low cost, sensitivity, practicality and a well-known biology. This role is presented in detail, and further directions in research and in application of *Artemia* in water pollution control are discussed (Persoon and Wells, 1985).

A flow-through hatching and cold-storage system has been developed to accumulated and maintain large homogeneous populations of instar I nauplii of *Artemia* at 4°C. Intermediate development of nauplii transcending instar I to IV was characterized after the onset of 25°C incubation by measuring changes in drinking activity, body length, body water volume, DNA and protein levels. Development related differentials in naupliar vulnerability were shown by comparing of LC_{50}s estimated for Cd, Hg and sodium azide. With Cd and Hg, the LC_{50}c were found to decrease as nauplii aged and developed. Developing nauplii were differentially vulnerable to Cd and Hg (Sleet and Brendel, 1985).

Post-gastrula and early larval development of *Artemia* present some useful advantages for studies of developmental aspects of environmental toxicology. Toxic effects can be analyzed in terms of specific molecular events. Therefore, *Artemia* larvae was used to study the effects of Cd and Zn on the larval development. The results demonstrated that pre-nauplius larvae prior to hatching were much more sensitive to Cd than were the hatched nauplius larvae. At 11.1 ppb, Cd retarded development and hatching of larvae. Higher concentration blocked hatching almost completely and thus were lethal. However, the larvae arrested at the emergence stage survived for 24 h or more before succumbing to the effect of Cd, and during this period the potentially lethal effect and reversible if the larvae were placed in Cd-free medium. The effects of Zn paralleled those of Cd, although Zn was somewhat less toxic than Cd at equal concentrations (Bagshaw *et al.*, 1986).

The gametes and embryos of three sea urchin species were exposed to $CdCl_2$ at concentrations ranging from 10^{-8} to 10^{-3} M. The embryotoxic action of Cd was inversely related to salinity and to Ca concentration. Cd-exposed larvae displayed similar abnormalities if Cd was present throughout development or only after hatching, while pre-hatching exposure produced no developmental

defects. The pretreatment of sperm or eggs did not affect the ensuing development of embryos, both for acutely toxic levels (up to 10^{-2} M), and for prolonged exposure in relatively low Cd levels. The fertilization rate was differently affected depending on whether sperm or eggs were pretreated (Pagano *et al.*, 1982).

The effects of chronic Cd exposure (0.1 - 5 ppm) on gametogenesis of mature sea urchins and on the development of their offspring were examined. At 5 ppm Cd, test animals died within several hours; at 1 ppm Cd within several days; at 0.5 ppm within one month. Cd induced obvious anomalies in oocytes at 0.5 and 1 ppm. Long-term exposure of adults to 0.1 ppm Cd produced neither changes in external appearance nor any obvious alterations in gonads. However, this exposure resulted in the production of animalous sex cells and in inviable offspring (Kristoforova *et al.*, 1984).

The effect of Cd on the development of Ascidian eggs (of *Clona intestinalis*, *Ascidia malaca* and *Ascidia aspersa*) has been studied. The Cd concentrations ranged from 0.01 ppb to 1110 ppm. The toxicity of Cd was found to depend not only on concentration, but also on time of exposure. The results of this study have shown that Cd, in certain concentrations, is toxic to Ascidian eggs and produces noticeable modifications and abnormalities in development. Treatment of unfertilized eggs of *C. intestinalis* with Cd concentrations higher than 1 ppm was found to affect the embryogenesis. Eggs at two blastomere stage developed normally after exposure to 1 and 10 ppb Cd. The dechorionated eggs at the same stage appeared to be more sensitive to Cd treatment. Their exposure to 1 ppb Cd resulted in development of abnormal larvae (*A. malaca*) or in production of tail buds with pigment spots (*A. aspersa*) or in complete cessation of development after first divisions (*C. intestinalis*). The fertility of sperm was not affected by concentrations which were toxic to eggs (100 and 1,000 ppm) (Carollo and Camicatti, 1983).

PCBs in marine organisms

Polychlorinated biphenyls are a group of compounds which have found a wide variety of industrial uses. Because of their resistance to chemical and biological break down, PCBs become a persistent contaminant in both terrestrial and aquatic ecosystems. They are characterized by low solubility in water and consequent concentration in lipid-rich body tissues.

Show and Connell (1980) have determined the concentrations of PCBs in water, sediments, fishes, crustaceans, molluscs, polychaets and birds from the Brisbane River estuary (Australia). No relationship was found between trophic

class and PCBs accumulation, although the birds (highest trophic level) exhibited the highest muscle tissue concentration of PCBs (15.7 ppm, wet weight). In *Mytilus corscus*, the PCB concentration was 0.25 ppm, wet weight.

Uptake of PCBs from sediment by *Nereis virens* and *Crangon septemspinosa* has been studied. The concentration of PCB, as aroclor 1254, accumulated from sandy sediment worm was directly related to PCB concentration in sediment and to exposure time, and was inversely related to animal size. There was no obvious excretion of PCB by *N. virens* during 26 days' post exposure. Accumulation of PCB by shrimp was also directly related to PCB concentration in sediment and inversely related to animal size. Concentration factors at 32 days ranged from 10.8 to 3.8 for worms of 0.69 and 4.7 g, respectively; and from 3.5 to 1.9 for shrimps of 0.1 and 2.9 g, respectively (McLeese *et al.*, 1980).

The effects of feeding, egg laying and fecal pellet production on the elimination of PCBs from the marine copepod *Acartia tonsa* were studied in a series of experiments. Copepods were exposed to ^{14}C-labelled Aroclor 1254 and allowed to depurate in clean seawater. Copepods fed during depuration eliminated PCB more rapidly than unfed copepods, whether or not the original PCB exposure medium had contained food. The concentration of PCB in eggs (up to 407 ppm, dry weight) exceeded four times that in the female that produced these eggs. Females eliminated PCBs twice as rapidly as males, indicating that egg production is an important route for PCB elimination (McManus *et al.*, 1983).

The scope for growth in resident *Mytilus edulis* from San Francisco Bay was significantly regatively correlated with increased environmental concentrations of the following trace metals (Cr, Cu, Hg, Ag, Al, Zn) and higher molecular weight chlorinated organic compounds (total chlordanes, dieldrin). The environmental gradient of pollutants was measured as a body burden of transplanted mussels *Mytilus californianus* PCB concentration of transplanted mussels showed a marked increase: 27 ppb in mussels from reference place and 1.5 ppm in mussels along pollution gradient (Martin *et al.*, 1984).

The effect of pentachlorophenol (PCP) (0.1 - 0.8 ppm) on the activity of some basic enzymes (glucose-6-phosphate dehydrogenase, glutamate oxaloacetate transaminase and glutamate pyruvate transimase) and on the production echinochrome in sea urchin developing eggs were evident and measurable. The enzyme activities, except G6P-DH, and production of echinochrome were negatively correlated with PCP concentration. The toxic effect of PCP was clearly manifested in disturbed morphogenesis and lowered survival (Ozretic and Krajnovic-Ozretic, 1985).

Recently, the kinetics of individual PCB compounds in different marine organisms have been studied by Boon (1986). Concentrations of PCBs were deter-

mined in *Macoma baltica*, *Arenicola marina* and *Crangon crangon*. Notwithstanding the different feeding habits, the PCB patterns were highly similar in all samples of *M. baltica* (mixed suspension and deposit feeder) and *A. marina* (sediment feeder). Samples of *C.crangon* (carnivorous) showed a pattern with lower contribution of PCB congeners which have structural features, decreasing the resistance to enzyme mediated metabolism, *i.e.*, these congeners possess vicinal protons and lack a 4,4' chlorine substitution. PCB concentrations were approximately similar when expressed per unit of lipids in all species investigated, if the samples derived from the same area of the Dutch Wadden Sea. Therefore, the effects of different feeding habits and effects of size or age were not observed. To investigate the processes regulating PCBs concentrations in different tissues and organs, juvenile soles *Solea solea* were injected with technical PCB mixture Clopen A 40. Concentrations of PCBs in organs were found to depend mainly on organ lipid content. The elimination rates of PCB component were shown to depend on the number and position of chorine atoms at the biphenyl skeleton. The elimination rates were similar in all organs except brain where rates were faster for all PCB components and gall-bladder where high PCB concentrations were initially observed, but which decreased rapidly. The PCB-injected fishes had a decreased growth rate and slower increase of lipids in liver. The kinetics of PCB components also were studied in relation to lipid metabolism under different conditions: in fed fish or under starvation. Under condition of growth and lipid enrichment (fed fish) in some organs and tisues of soles, the lipid-based concentrations of many PCB components decreased in organs, irrespective of lipid metabolism and despite the presence of large amounts of PCBs in the diet. In starving soles, the kinetics of PCBs were related to PCBs in solution and changes in lipid metabolism. Analyses were made of brain, liver and "remainder" of the fish. The PCB concentrations on lipid basis increased with starvation due to changing lipid content and composition of some organs. Despite the increase in concentration, the absolute amount of PCBs in remainder decreased with increasing starvation due to the decrease in lipid content of the tissues (Boon, 1986).

Boon (1986) has suggested that benthic invertebrates that use glycogen as their main energy-depot, are the most suitable tools for environmental monitoring of lipophilic compounds. The concentrations of such compounds should be expressed on the basis of extractable lipids. As some representatives of these compounds are strong inducers of MFO system, the measurement of MFO activity might serve as a useful stress indicator for the additive effects of PCBs and other lipophilic pollutants.

MFO system in *Mytilus edulis*

Cytochrome P-450 dependent mixed function oxygenases (monooxygenases) are involved in the transformation, of a wide range of organic compounds including many foreign non-biological compounds or xenobiotics to polar metabolites. The MFO system enzymes are frequently associated with endoplasmatic reticulum (microsomes) and the membrane of nuclear envelope. MFO system requires NADPH and molecular oxygen as substrates and is essentially composed of cytochrome P-450 reductase and cytochrome P-450. Other enzyme of generally cytosolic or microsomal location are also involved. Other electron carriers are: NADH-cytochrom c reductase. Other enzymes of xenobiotic metabolism are: UDP-glucoronyltransferase, glutathione s-tranferases, superoxide dismutase and nitroreductases.

MFO system oxidizes foreign compounds by aromatic hydroxylation, N-dealkylation, O-dealkylation or epoxidation. In the course of such metabolic transformations, reactive (electrophilic) intermediates can be formed, some of which are carcinogens, mutagens or other cytotoxic agents. The MFO systems must be therefore viewed as components of a detoxication/toxication system, as some of the metabolites may be more biologically damaging than the parent compounds.

MFOs are present with relatively low activity in normal animals, where their role seems to be to degradate endogenous lypophilic substrates such as steroid hormones, however, if the organism is stressed by exposure to various foreign compounds, among which are environmental contaminants (DDTs, PCBs or aromatic hydrocarbons), the MFO activity will increase dramatically, apparently to enhance the degradation and clearance of the offending compounds.

NAD(P)H-producing enzymes (glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase and NADPH-dependent isocitrate dehydrogenase) may be also responsive to the presence of organic xenobiotics and therefore offered potential as a specific index of pollution stress.

Moore *et al.* (1980) have found an enhancement of NADH neotetrazolium reductase, glucose-6-phosphate dehydrogenase and microsomal hexose-6-phosphate dehydrogenase in *M. edulis* after experimental treatment with aromatic hydrocarbons. They have proposed that induction of MFO systems in *M. edulis* and other bivalves may have some potential for the monitoring of biological response to organic contaminants. However, the additional information is still required on the effects of season, nutrition, gametogenesis and temperature on the detoxication system.

A survey of the tissue and subcellular distribution of some enzyme activities of MFO system and benzo(a)pyrene (BP) metabolism in *M. edulis* has been car-

ried out. MFO activities and cytochromes were localized in the microsomes. Cytochrome P-450 was present only in the digestive gland and the P-450-associated activities were highest in this tissue. In contrast cytochrome b5 was also present in the gills and mantle. The activities were higher in the digestive gland of female mussels than of males. It has been concluded that a cytochrome P-450 mediated MFO system is present in mussels (Livingstone and Farrar, 1984).

Responses of the cytochrome P-450 MFO system to diesel oil were studied in *M. edulis* and in the periwinkle *Littorina littorea*. Cytochrome P-450 and b5, and NADH-neotetrazolium and NADH-cytochrome c reductase activities were elevated in digestive gland microsomes in long-term exposed molluscs (Livingstone *et al.*, 1985).

Benzo(a)pyrene oxidation and microsomal enzyme activity have been investigated in the *M. edulis* and other bivalves from the Western North Atlantic. Analysis of subcellular fraction revealed a complement of microsomal electron transport components including reductases and heme proteins in several organs of the three bivalve species *M. edulis*, *Macrocallista maculata* and *Aracca zebra*. The levels of these components and the rates of BP metabolism were highest in the digestive gland, and were very similar between species. In *M. edulis*, there was a suggested seasonal variation in BP metabolism (Stegeman, 1985).

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Chapter 2. SURVEYING EXPERIMENTS

2.1 Introduction

There is much recent concern about the increasing concentrations of the toxic substances (heavy metals, PCBs, PAH) in the estuarine and coastal marine environments, resulting from increased industrial activity. A great number of recent studies are dealing with accumulation, immobilization and metabolism of the micropollutants discharged into environment, but the list of their deleterious effects on the marine organisms is far from being complete. The studies of the pollution effects on the marine ecosystems are even more fragmentary and incomplete. The knowledge of the contaminant effects on the reproduction and energy metabolism of representative organisms is essentially lacking. To estimate the damage and to predict the danger for the marine ecosystems, the toxicological research must pinpoint the "key" organisms, their sensitivity to pollutants and possible effects of the latter on the reproduction and energy metabolism.

The present study concerns with the effects of heavy metals and organic micropollutants (PCBs) in the water of the Western Scheldt on the reproduction of the sea mussel, sea star, sea squirt and/or shrimp. These marine invertebrates have been chosen as typical representatives of the ecosystem of Dutch coastal and estuarine waters. Furthermore, *Mytilus edulis* and *Asterias rubens* have been the objects of the research at our department for a number of years. The fundamental studies on the regulation of the anaerobic energy metabolism in *M. edulis* and reproductive physiology in *A. rubens* can provide an excellent basis for toxicological research. Both *M. edulis* and *A. rubens* have an annual reproduction cycle. In contrast, shrimp and sea squirt are known to reproduce several times a year. Sea squirts are hermaphrodites. The latter may be of interest for the investigation of contaminant effects on the reproduction. Comparing to the above-mentioned sessile animals, the shrimp possesses a higher metabolic rate and, thus, may be more vulnerable to pollutants. The economical importance of the shrimp is also taken into consideration.

Mussels and some species of crustaceans have been extensively used in toxicological studies during last decade. The high capacity of mussels to accumulate cadmium and other heavy metals is used for monitoring the pollution of marine environment. However, the toxic effects in *M. edulis* have been demonstrated only at high metal concentrations (Sunila, 1981; Sunila and Lindström, 1986). Body burdensup to 150 ppm Cd caused no measurable effects on either clearance, ingestion, assimilation or growth in *M. edulis* (Poulsen *et al.*, 1982). The MT synthesis has been demonstrated in mussels and crustaceans

(Viarengo *et al.*, 1980; Nolan and Duke, 1983; Engel, 1983). The mechanism of cadmium toxicity in crustaceans (*Mysidopsis*) is suggested to be a complex phenomen, potentially involving physiological processes of the skeletal calcification and osmoregulation (Gentile *et al.*, 1982).

A few data are available for sea stars and sea squirts. Recently, Voogt *et al.* (1986) have found that cadmium exposure affected steroid metabolism and steroid levels in the sea star *A. rubens*. Since steroids are involved in the regulation of gametogenesis, this proces may be disturbed. The toxic effect of cadmium on the development of the Ascidian eggs (of *Ciona intestinalis*, *Ascidia malaca* and *Ascidia aspersa*) has been demonstrated at cadmium concentrations, ranging from 10^{-5} to 10^{3} ppm (Carollo and Canicatti, 1983). We could not find any studies on the accumulation and distribution of cadmium or MT synthesis in the sea stars and squirts.

The primary goal of the first research stage has been to assess the sensitivity of the above-mentioned invertebrates to contaminants. The toxic effects have to be easily detectable at contaminant concentrations not uncommon in polluted marine areas.

The basic idea of the experimental approach was to examine the changes in animal survival under the conditions of natural stress as a consequence of the pollutant accumulation. This approach becomes of a special importance since the organisms in the estuary such as Western Scheldt are subjected to the large fluctuations in salinity, temperature, submersion and oxygen availability. Within certain tolerance limits, organisms have evolved the strategies to survive unfavorable environmental variables (natural stresses). As example of similar strategies in the marine invertebrates, the anaerobic metabolism in mussel and osmoregulation in shrimp can be mentioned. An alteration in tolerance limits may be considered as an indication that contaminant interferes with the basic metabolic processes, underlying the survival strategy. In assessing these metabolic processes, the biochemical (general and/or specific) indices of the pollution stress can be discovered and developed.

For squirts, another approach was chosen because little is known about the natural-stress tolerance of these animals. Gametogenesis is a continuous process in ascidians and gonad development can be studied easily by lightmicroscopy. Therefore, we have chosen gonad development as a parameter for sensitivity of squirts to toxicants.

The program of our surveying research was worked out in cooperation with H. Herwig and A. De Zwaan. This program was introduced and accepted at a meeting held in November 1986.

In surveying experiments, animals (mussels, shrimps, sea stars and squirts)

were initially exposed to cadmium for different period of time. Subsequently, the survival of the animals was examined under the conditions of natural stress (exept for squirts).

2.2 Materials and Methods

Animals

Mussels, *Mytilus edulis*, were collected in the Eastern Scheldt in January 1987. The mean shell length was 4.9 ± 0.3 cm.

Shrimps, *Crangon crangon*, were caught in the Eastern Scheldt in January 1987.

Sea squirts, *Molgula manhattensis* and *Ascidiella aspersa*, were collected in the Eastern Scheldt in October 1986 and in January 1987, respectively.

Sea stars, *Asterias rubens*, were caught in thech Wadden Sea in 1986.

After transportation to Utrecht, animals were kept in aquaria with recirculating sea water at 12°C. Salinity of the sea water was 28°/$_{oo}$. Sea stars and shrimps were fed with mussels. Sea squirts and mussels received no food.

Exposure system

The animals were exposed to approximately 50 ppb Cd in glass aquaria (volume of 80 l) to which sea water and metal solution were supplied with pomps at rates 1 l/h and 10 ml/h, respectively. The sea water temperature was 12°C. Cadmium was added as $CdCl_2.H_2O$ (Merck, no.2011). The water concentration of Cd was measured twice a week by AAS. Actual concentrations amounted to 47 ± 3 ppb (mussels), 51 ± 11 ppb (sea stars), 47 ± 8 ppb (shrimps) and 49 ± 7 ppb (sea squirts). During exposure the sea stars and shrimps were fed, whereas mussels and sea squirts received no food. Animals were exposed in the period January - April, 1987.

Metal analysis

After different periods of time (1, 2, 4 or 6 weeks), animals were examined for Cd content. The excised tissues were frozen (-20°C) overnight and lyophilized for 48 h. The dry tissues were decomposed by heating in 65% (w/v) nitric acid (Merck, no. 456) at 90°C for 2 h, using teflon (PTFE) bombs placed in a sandbath. Small amounts of tissue, up to 300 mg dry wt, were decomposed in 17

cm^3-bombs with 3.5 ml nitric acid. Larger fractions were decomposed in 60 cm^3-bombs with 7.0 ml nitric acid. After decomposition, 65% nitric acid was added to a final volume of 5 ml. The Cd concentration was determined by atomic absorption spectrophotometry. In *M. edulis*, Cd content was determined per total animal. In *A. rubens*, Cd content was examined either in soft parts (tissue homogenate) and skin, or in four organs: caeca, gonads, stomach and skin. In *C. crangon*, Cd concentration was determined in the soft parts (flesh) and carapax. In *A. aspersa*, Cd content was examined in whole body and tunic.

Anoxic test

Mussels

After Cd-exposure, groups of 20 mussels were subjected to anoxia. Prior to aerial exposure, mussels were kept in clean sea water for 3 h to remove the adherent cadmium. Mussels were exposed to air at 18°C. The survival of the animals was assessed daily. Death symptoms were considered to be a specific smell and open shell due to the absence of any muscle activity.

Sea stars

After Cd-exposure, sea stars were transferred into the oxygen free sea water. Sea water was made anoxic by bubbling with N_2 for 30 min. Sea stars were kept individually in closed jars (volume of 2.5 l) in 2 l of sea water at 8°C. The survival was recorded daily. The cease of any podium acitivity was regarded as the death criterium. Sea stars were not fed during anoxic test.

Salinity test

Salinity test was carried out in the glass jars (2.5 l of volume) at 12°C. Two shrimps were placed in 2 l of sea water. Lowered salinities were obtained by mixing the sea water with copper free tap water. After two weeks of Cd-exposure, shrimps were directly transferred from 28‰.S to 14‰.S. The salinity was decreased by 50% in 24 h steps. Mortality was recorded every 24 h. The death symptoms were the immobility and the opal colour of the animals. Shrimps were fed during this experiment.

Histology

Prior to the histological procedure (as well as prior to metal analysis), sea squirts were kept in "clean" sea water for 3 h to eliminate the adherent cad-

mium. For light microscopical analysis, the whole bodies (without tunic) were fixed in bouin's fluid, embedded in paraffin and sectioned at 5 µm. Sections were stained with haemaluin-eosin.

For light microscopic visualization of metal by a silverprecipitation method, the samples were pretreated in sulphide solution (0.1% Na_2S in 0.1 M phosphate buffer, pH 7.4) for 10 min at room temperature. The samples were fixed in 1% glutaraldehyde for 3 h, dehydrated in ethanol and embedded in paraffin. Sections of 5 µm were developed in solution, containing 50 ml 20% gum arabic, 10 ml citrate buffer, pH 3.5, 15 ml 16% hydroquinone and 15 ml 0.75% $AgNO_3$, for 30 min in complete darkness. After development, sections were counterstained with Alcian Blue. Control sections were prepared, omitting the sulphide pretreatment, in order to correct the aspecific silver precipitation.

2.3 Results and Discussion

Sea mussel *Mytilus edulis*

The time-dependent accumulation of cadmium is shown in Fig. 1.1 for the whole animal. Accumulation proceeded linearly, reaching the value of 72 µg Cd/g dry wt after 6 weeks. Between the 6th and 8th week, the accumulation rate decreased, reaching a Cd concentration of 81 µg Cd/g dry wt after 8 weeks.

Prior to anoxia surviving test we have carried out the preliminary experiments to study the effect of temperature on the anoxic survival of *M. edulis*. Fig. 1.2 shows that LT_{50} of anoxic survival strongly depends on temperature. LT_{50} at 12°C (21 days) is more than 3 times higher than LT_{50} at 21°C (6.5 days). We have chosen the temperature of 18°C for the anoxia surviving test. The LT_{50} at 18°C is approximately 9 days which allows to assess the anoxia tolerance of the animals within limited experimental time.

The results of the anoxic test of Cd exposed mussels are shown in Figs.2.3-2.6. Two weeks of Cd-exposure caused a significant decrease in the survival of the exposed mussels ($p<0.05$). LT_{50}'s of exposed and control group were 9.5 and 10.7 days respectively. The shift of the survival curve towards diminished anoxia survival became more pronounced after 4 weeks of Cd-exposure (Fig.4). LT_{50} of the exposed group was 7.6 days; whereas LT_{50} of the control group remained unchanged (10.5 days). After 6 weeks of Cd-exposure, the initial death rate was higher in the exposed group, but no significant differences in mortality were observed at the advanced anoxia stage (Fig.2.5)

The LT_{50} of the exposed group was 8 days, whereas that of the control group decreased to 9.5 days. This shift of the survival curve could be ascribed to the deteriorated condition of the control mussels, since over 50% of animals had thin, exhausted mantles and several mussels were invaded by parasites. Eight weeks of Cd-exposure affected the survival only at the advanced anoxia stage, while no changes in mortality were found during the first week of anoxia (Fig. 2.6). The LT_{50} of the exposed and control groups were 7.8 and 9.3 days, respectively. Cadmium content of the mussels was assessed individually with respect to anoxia duration, however, no correlation between the body burden and survival time was found. In Fig.2.10 the effect of Cd-exposure time on the anoxia survival LT_{50} is shown.

In studies on *M. edulis* De Zwaan (1986) has found that mussels can exploit different pathways at low and high output mode of anaerobic metabolism which is reflected in the accumulation of either succinate and propionate or mainly pyruvate derivates (lactate and opines). The ratio of the two end product spectra is correlated to ATP turnover rate and can be used to measure changes in the energy utilization rate. Low output mode, (environmental) anaerobiosis, is a main strategy to survive the anaerobic conditions by energy conservation, whereas high output mode, (exercise) anaerobiosis, augments the energy production when respiration alone is not sufficient to meet tue energy demand. De Zwaan *et al.* (1985) have pointed to the fact that stress in general (*e.g.*, pollution) causes an elevated metabolic rate. It was shown that forced shell closure by a rubber band caused an elevated metabolic rate ($\dot{M}ATP$ from 0.014 to 0.06 µmol/min.gram) which corresponded with an increase in the strombine + octopine/succinate + propionate ratio (from 0.49 to 1.77). The increased energy expenditure will make mussels more vulnerable to anoxia, thus shortening the survival of the animals.

Our results have provided a direct evidence that short-term exposure to Cd (ca. 50 ppb) significantly decreases the survival time of the exposed mussels. Furthermore, field experiments (De Zwaan *et al.*, 1958) have revealed that the ratio of end products appears to be a usefull stress index for "coarse" discrimination of sites for the presence of bioavailable contaminant, while propionate formation correlated even with a contaminant gradient. The results of laboratory and field experiments clearly indicate a relationship between the content of contaminant within the tissues and basic metabolic processes. However, further research is required to elaborate the biochemical indices of pollution stressors and to reveal the mechanism of pollutant action on the metabolic pathways.

Sea star *Asterias rubens*

Time-dependent accumulation of cadmium was studied in *A. rubens*. Fig. 7A shows that accumulation of cadmium in the total soft parts of the sea star proceeded linear-like, reaching the value of 7.7 µg Cd/g dry wt after 4 weeks of exposure. During the first 2 weeks the accumulation rate was almost 3 times lower in the skin, thereafter, it increased slightly, resulting in 3.4 µg Cd/g dry wt after 4 weeks of exposure (Fig. 1.7B).

Fig. 1.8 shows the organ distribution of cadmium after 4 weeks of exposure. The highest Cd concentration was found in caeca, whereas the gonads contained the lowest Cd concentration. The high Cd contents in caeca and stomach of *A. rubens* correspond with the available data on the bioaccumulation of PCBs in the intestine of Asterioidea species (Picer and Picer 1986) and suggest a major role in the uptake of toxicant for these organs.

Organ distribution of cadmium in *A. rubens* was studied in presence or absence of food during 4 weeks of exposure to 50 ppb Cd. The results are summarized in Fig. 9AB. In soft parts, caeca and skin no considerable differences in Cd content were found with respect to food availability. Cd concentration in stomach was higher in the fed animals, whereas Cd content was increased in the gonads of the animals kept under starvation. These results imply that uptake of Cd largely occurred from the water. However, additional experiments must be carried out in which sea stars will be fed with Cd-exposed mussels while kept in the clean sea water.

The results also suggests that differences between organs in Cd concentrations are smaller in starved animals than in fed animals. In fed animals relatively more cadmium is taken up in the food uptaking organs (stomach, caeca). Without food uptake, Cd may be more equally distributed between organs, giving relatively higher concentrations in the gonads compared with fed animals. An increase of Cd content in gonads may also indicate an enhanced transfer of nutritive substances from caeca to gonads under starvation.

Sea stars are known to possess a certain anoxia tolerance, being able to survive under anoxic conditions a period of about 4 days at 10°C (Theede, 1973). As fluctuations of oxygen availability occur in the estuary areas, it is not unlikely that the animals are confronted with hypoxic conditions. Therefore, we have applied the anoxia stress to check the vulnerability of Cd-exposed animals.

In preliminary experiments, we have measured the survival time of sea stars subjected to anoxia at different temperatures. Fig. 1.10 shows that anoxic survival time at 12°C was comparable with that obtained from literature

(Theede, 1973). Cd-exposed and control sea stars were subjected to anoxia at 8°C. At this temperature survival time of sea stars is approximately 8 days, which is an appropriate experimental period.

As can be seen in Figs. 1.12 and 1.13, two and four weeks of exposure did not alter the anoxic survival of the sea stars. After 2 weeks of Cd-exposure, death rate was initially higher in the exposed group, but then mortality fluctuated at random in either group. LT_{50} of the Cd-exposed and control group was 8 and 6 days respectively. After 4 weeks of Cd-exposure, mortality proceeded at a similar rate in either group. LT_{50} was 11 and 14 days in the Cd-exposed and control group respectively.

At the temperature of 8°C, single animals survived anoxia for long time. This influences the mean survival time of a group, but LT_{50} is unaffected by these single long survival times. The mean LT_{50} of the four experimental groups is 10 days, which is somewhat longer than expected. Moreover, the general condition of the animals seems to be of great influence on the survival time, causing differences between groups and within groups.

The anoxia tolerance test for sea stars can only be a crude estimation of general condition. Apparently, another parameter must be applied to test the sensitivity of sea stars to toxicants. Studies on the embryonal development may provide a better basis for testing this sensitivity.

Sea squirt *Ascidiella aspersa*

Cadmium exposure of *A. aspersa* resulted in the significant accumulation of cadmium. The results are shown in Fig. 1.14. Accumulation proceeded at highest rate in the tunic, reaching a value of 24 µg Cd/g dry wt after 4 weeks. Thereafter, Cd concentration in tunic slightly decreased. Accumulation in the "body" proceeded linear-like and at low rate during 4 weeks of exposure. Whereas Cd accumulation in tunic seemed to have reached the saturation, accumulation in the body ensued at higher rate, attaining the value of 15.4 µg Cd/g dry wt after 6 weeks. In the whole animal, accumulation continued during 6 weeks, having the highest rate during first two weeks. The values for whole animal were calculated from the contents of body and tunic.

Sea squirts are known to accumulate and use for their metabolism vanadium and other heavy metals. The metals are absorbed through the branchial sac and transferred to the blood plasma, where they are accumulated in the amoebocytes, forming the morula cells (vanadocytes, metal-containing cells). Blood cells, particularly morula cells, are found throughout the tunic substance and apparently wander in there through the mantle wall or through the epithelial

walls of the tunic vessels (Goodbody, 1974). Considering the accumulation pattern of cadmium in the body and tunic of *A. aspersa*, it appears that cadmium may adopt the metabolic pathways of "physiological" heavy metals, being initially transferred into the tunic until saturation occurred and then accumulated in the body. However, further investigation is required to reveal the routes of cadmium transfer and accumulation in Ascidians.

It is worthwhile to note that Ascidian tunic contains the microfibrils of carbohydrate associated with a protein resembling collagen and elastin (Goodbody, 1974) and, therefore, the tunic tissue is difficult to decompose for cadmium analysis. We have carried out the tunic decomposition 3 times. First time, the tissue was decomposed by heating under pressure in nitric acid for 4 h at 110°C. Cd recovery was 79%. The second and third decompositions of insoluble pellet were carried out as described in Materials and Methods and resulted in 19% and 2% of Cd recovery, respectively.

To assess the sensitivity of *A. aspersa* to cadmium, we have chosen the gonad development as a parameter. At different periods of Cd-exposure time sea squirts were examined histologically. Lightmicroscopical study revealed that male and female gonads are located asymmetrically in the mantle on one side of the body. No differences in gonad development were observed with respects to Cd-exposure. Both types of gonads were scarcely present and seemed to be at an early stage of growth (results not shown).

We have applied the silver sulphide technique to visualize the free metal distribution in the Cd-exposed squirts. After 2 and 6 weeks of exposure, the metal was found in the intestine epithelium. In all Cd-exposed animals, the metal positive staining was observed in the mantle epithelia and in the food debris. However, some control animals also contained similar metal distribution. Conspicuous silver precipitates were located in the granules of the pharinx (branchial sac) and in the mantle cells of the exposed and control animals. This was silver deposit independent of the sulphidation step before fixation of the material.

Although *A. aspersa* is shown to accumulate considerable amounts of cadmium, the histolocal study has revealed the negligible quantity of the free metal in the exposed animals. These findings suggest that cadmium in present in a complex-bound form. Therefore, the study on MT in the sea squirts may be an interesting idea for future research.

Two other species, *Molgula manhattensis* and *Ciona intestinalis*, are considered for breeding under laboratory conditions. Consequently, the effects of contaminants on growth and reproduction can be more easily assessed. Moreover, *Molgula* appeared to be a more suitable animal to study the growth and

maturation of the gonads. Our histological study has revealed that *Molgula* gonads are symmetrically located in mantle on both sides of the body, and that different stages of gonad development are easy to detect. *Ciona* is known to breed successfully in aquarium under laboratory conditions. In contrast to *Molgula* and *Ascidiella*, *Ciona* has a single hermaphroditic gonad that consists of lobulated ovary, partly surrounded by a mass of branching testicular masses, and is located within the loop of the intestine (Berril, 1975).

Shrimp *Crangon crangon*

Time-dependent accumulation of cadmium in the soft parts (flesh) and carapax of *C. crangon* is shown in Fig. 15AB. After one week of exposure, mean Cd concentrations were 2.7 µg Cd/g dry wt and 1.7 µg Cd/g dry wt in the soft parts and carapax, respectively. The second week of exposure did not result in further increase of Cd concentration in the fresh, whereas carapax continued to accumulate cadmium. After two weeks, cadmium was almost equally distributed between the flesh and carapax of *C. crangon*.

We have recorded the death rate of shrimps during the Cd-exposure experiment. The mortality was high, but no significant differences were observed with respect to Cd-exposure (Fig. 16). In the storage aquaria the animals survived for about two months, indicating that our laboratory conditions were not yet suitable to sustain shrimps for longer periods of time.

The osmoregulation in *Crangon* is a well studied process (*e.g.* Spaargaren, 1971). Due to osmoregulation, shrimps can endure the wide range of salinity changes. Certain pollutants are known to affect the salinity tolerance of shrimp. Nimmo and Bahner (1974) have demonstrated that PCB-exposure significantly decreased the survival of shrimps at low salinities. Therefore, we have subjected the shrimps to the lowered salinity to check vulnerability of the Cd-exposed animals. Fig. 17 shows the survival curves of the exposed and control animals. Initially, lower mortality was observed in the control group. Equal number of animals were dead in either group after 24 h at 3.5°/$_{oo}$ of salinity. The last Cd-exposed shrimp survived 24 h longer at 3.5°/$_{oo}$ S than the control one. Considering the declined general condition of the shrimps in the storage aquaria and high death rate during Cd-exposure experiment, no conclusion can be drawn from the salinity test. Moreover, *Crangon crangon* appears only to be suitable experimental animal for long-term semi-field experiments, if shrimps can kept under proper conditions.

Another crustacean, ***Mysidopsis bahia***, has been suggested for our research. This animal has a short reproduction cycle (28 days) and is easy to sustain and

to breed in the aquarium under the lab. conditions. *Mysidopsis* is extensively used for toxicological research and the effects of cadmium and other pollutants on a number parameters, *e.g.*, reproduction, osmoregulation, growth, population dynamics and survival, have been investigated (Gentile *e al.*, 1982; Roberts *et al.*, 1982; Gentile *et al.*, 1983; Carr *et al.*, 1985). Yet, the effects of contaminants on the biochemical processes of *Mysidopsis* are not well-studied.

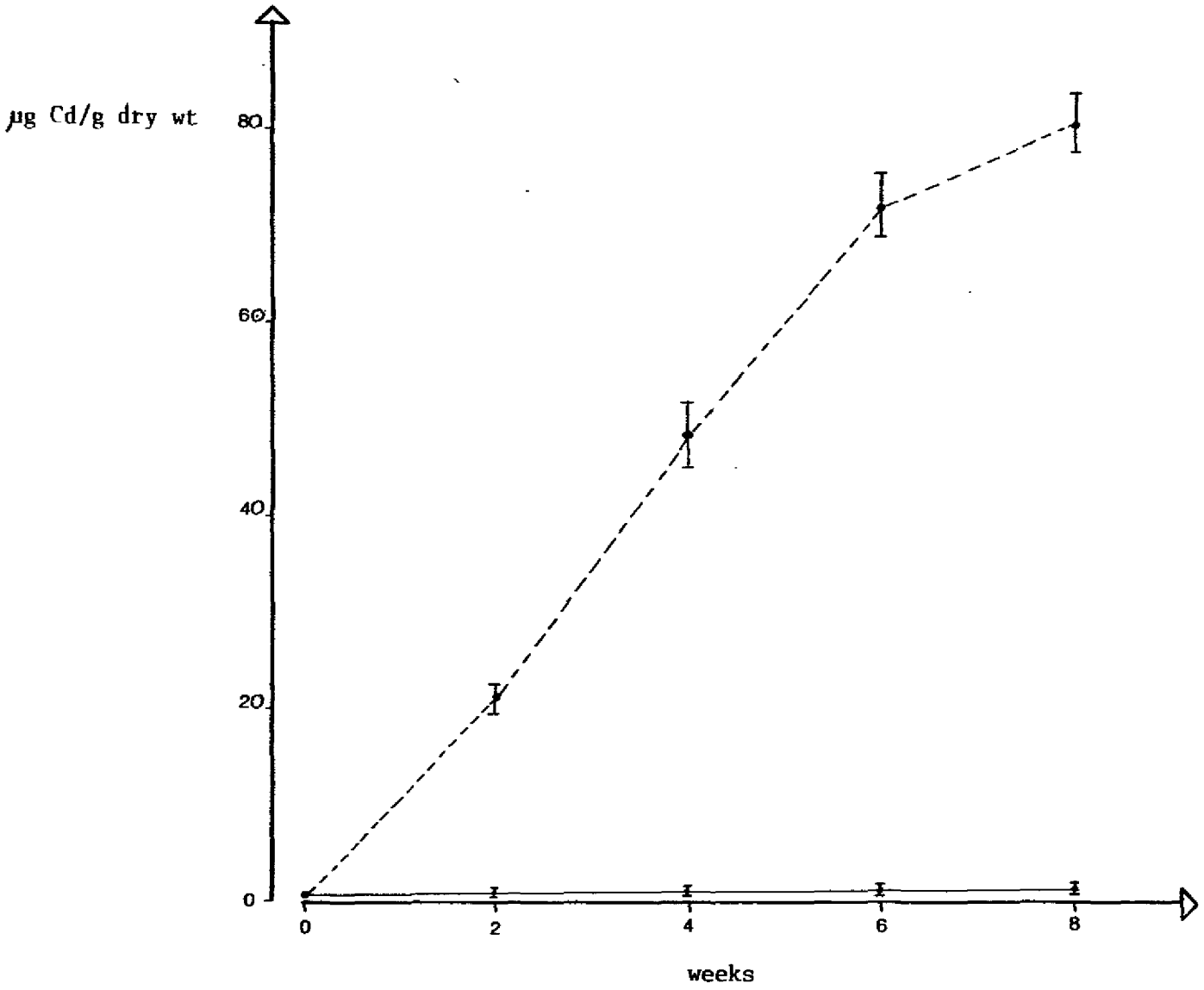

Fig.2.1. Cd concentration **vs** exposure time in whole animals,**Mytilus edulis**, exposed to 50 ppb Cd (----). Control group (——). Mean of 20 animals ± SEM.

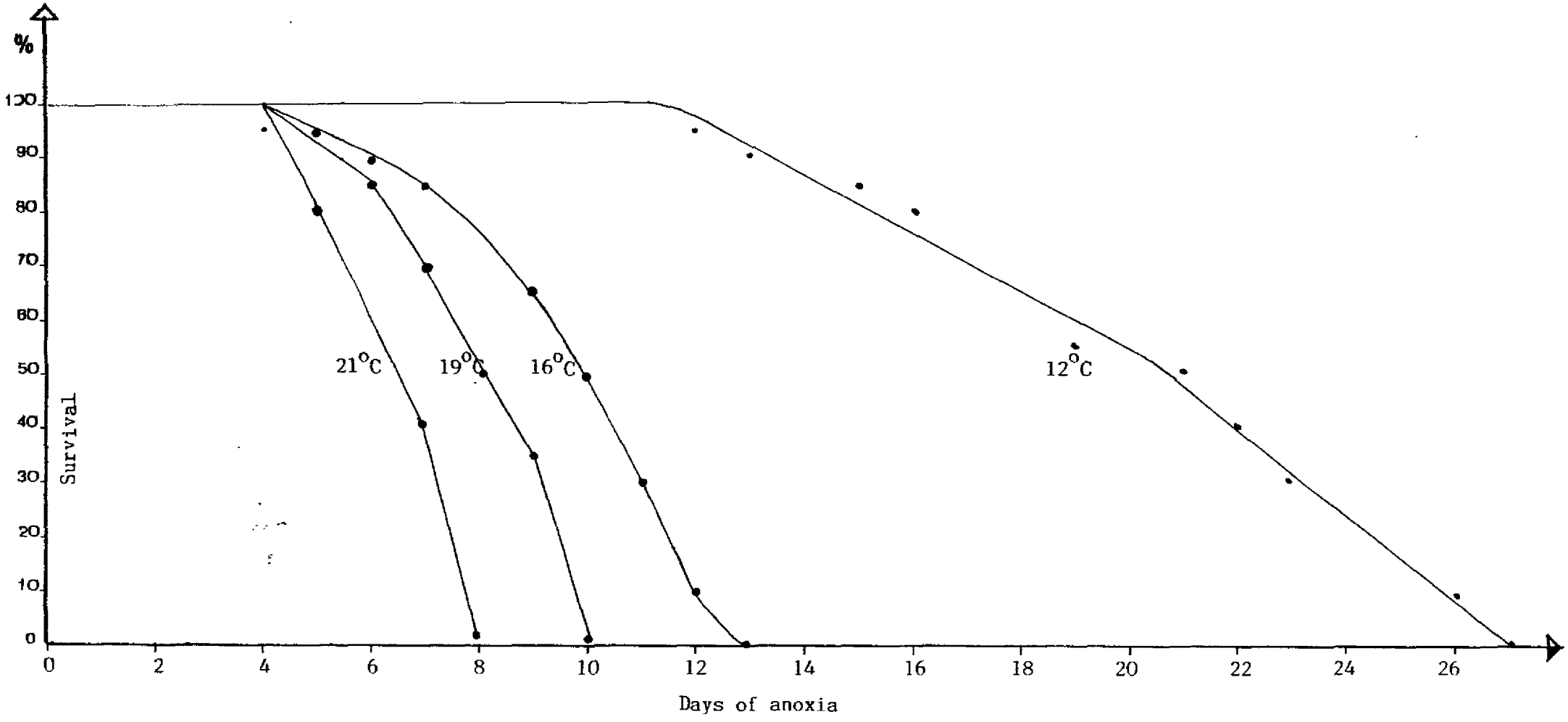

Fig.2.2. The effects of temperature on the anoxic survival of mussels.Groups consist of 20 mussels.

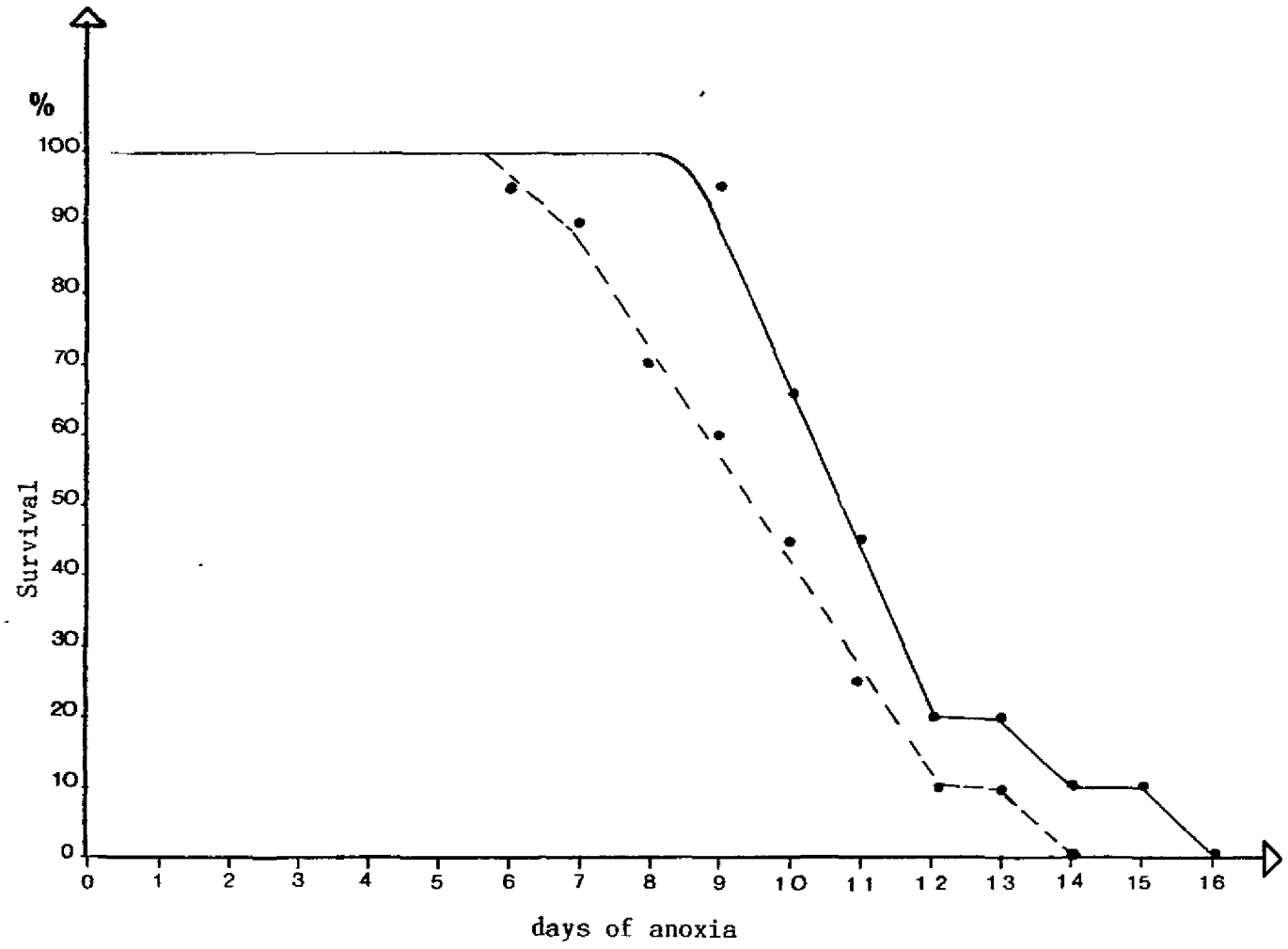

Fig.2.3. Anoxic survival of mussels after two weeks of Cd-exposure.
Exposed group (----). Control group (——).

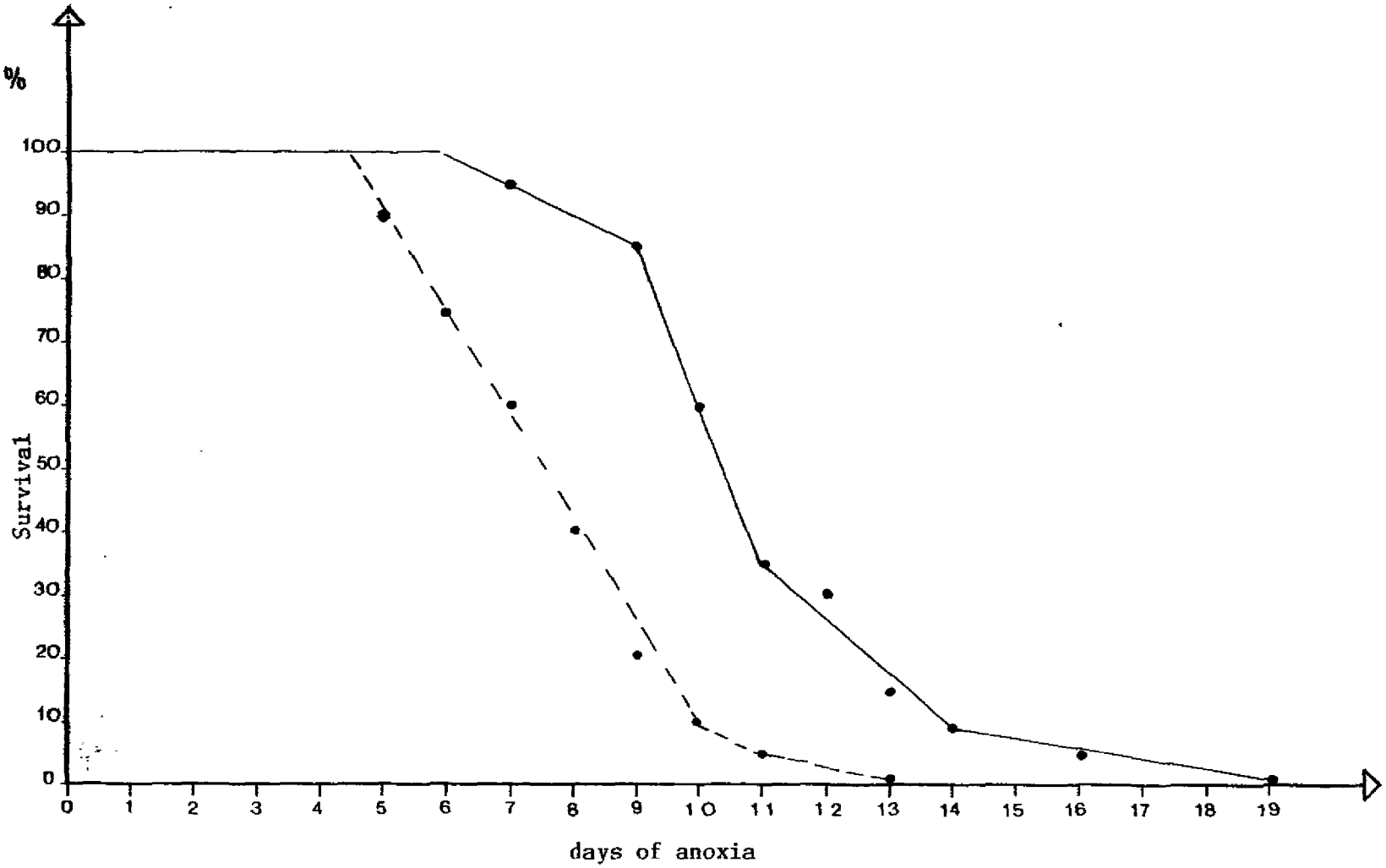

Fig.2.4. Anoxic survival of mussels **Mytilus edulis** after four weeks of Cd-exposure.
Exposed group (----). Control group (——).

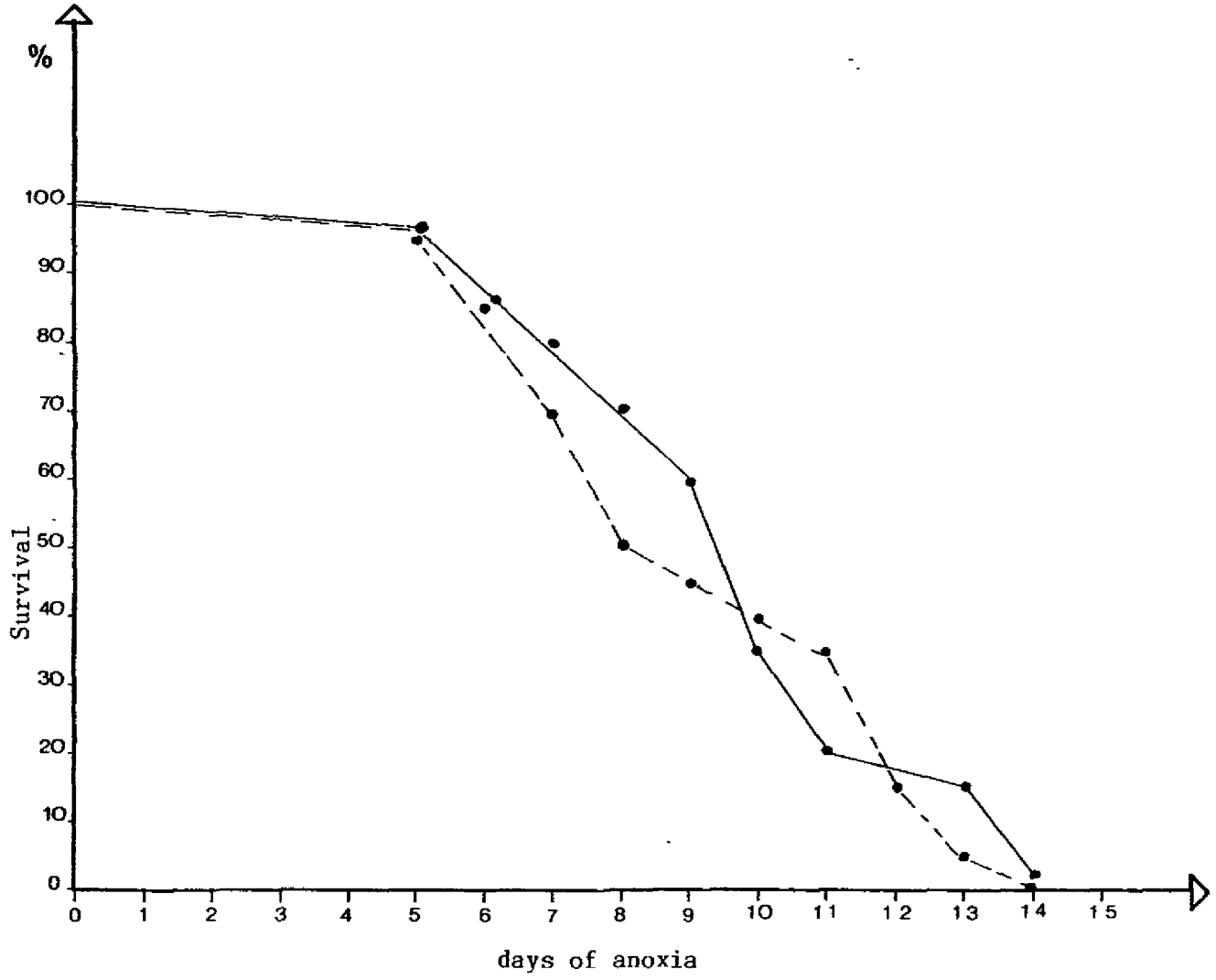

Fig.2.5. Anoxic survival of mussels **Mytilus edulis** after six weeks of Cd-exposure. Exposed group (——). Control group (——).

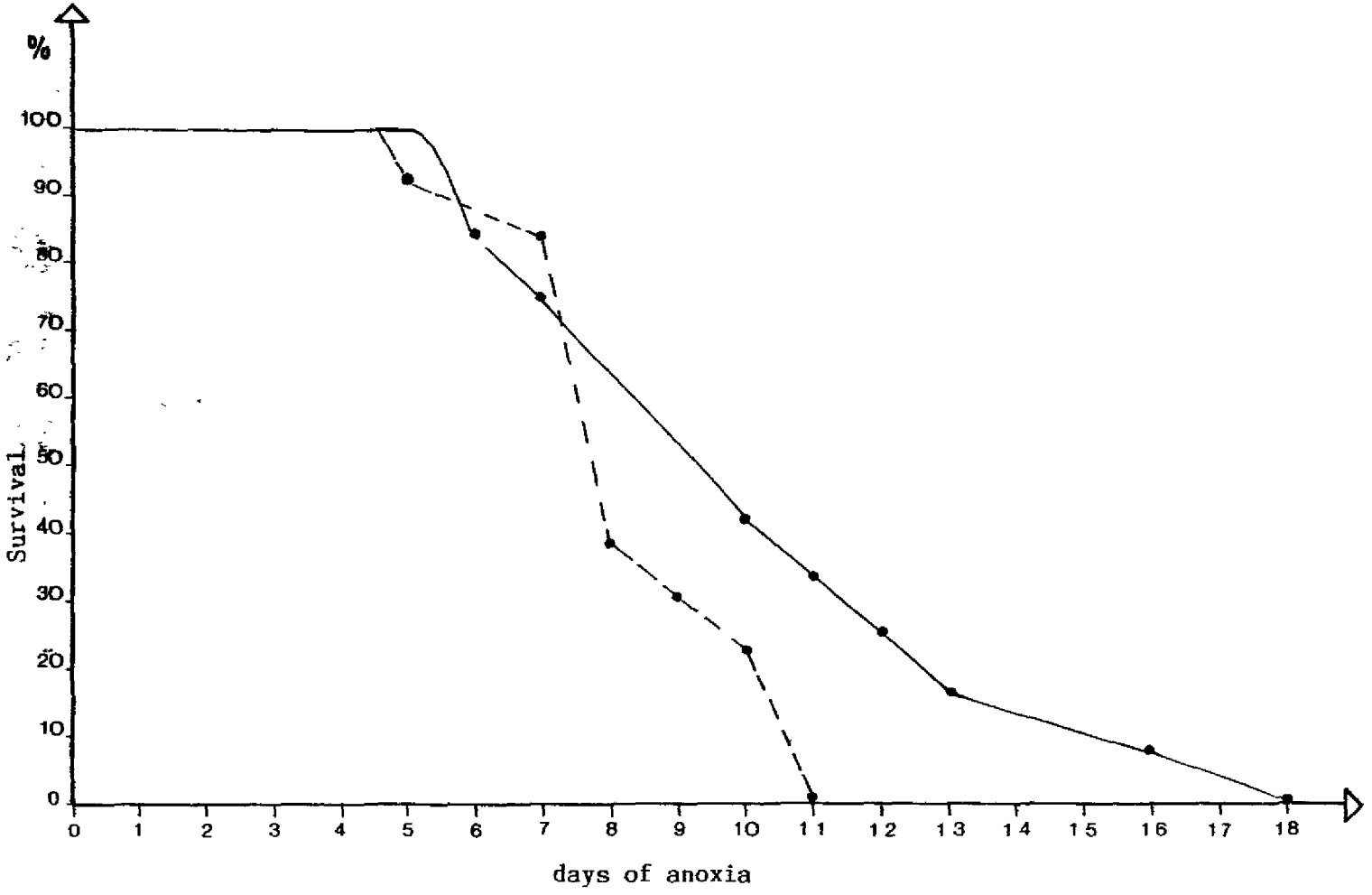

Fig.2.6. Anoxic suvival of mussels **Mytilus eulis** after eight weeks of Cd-exposure. Exposed group (----). Control group (——).

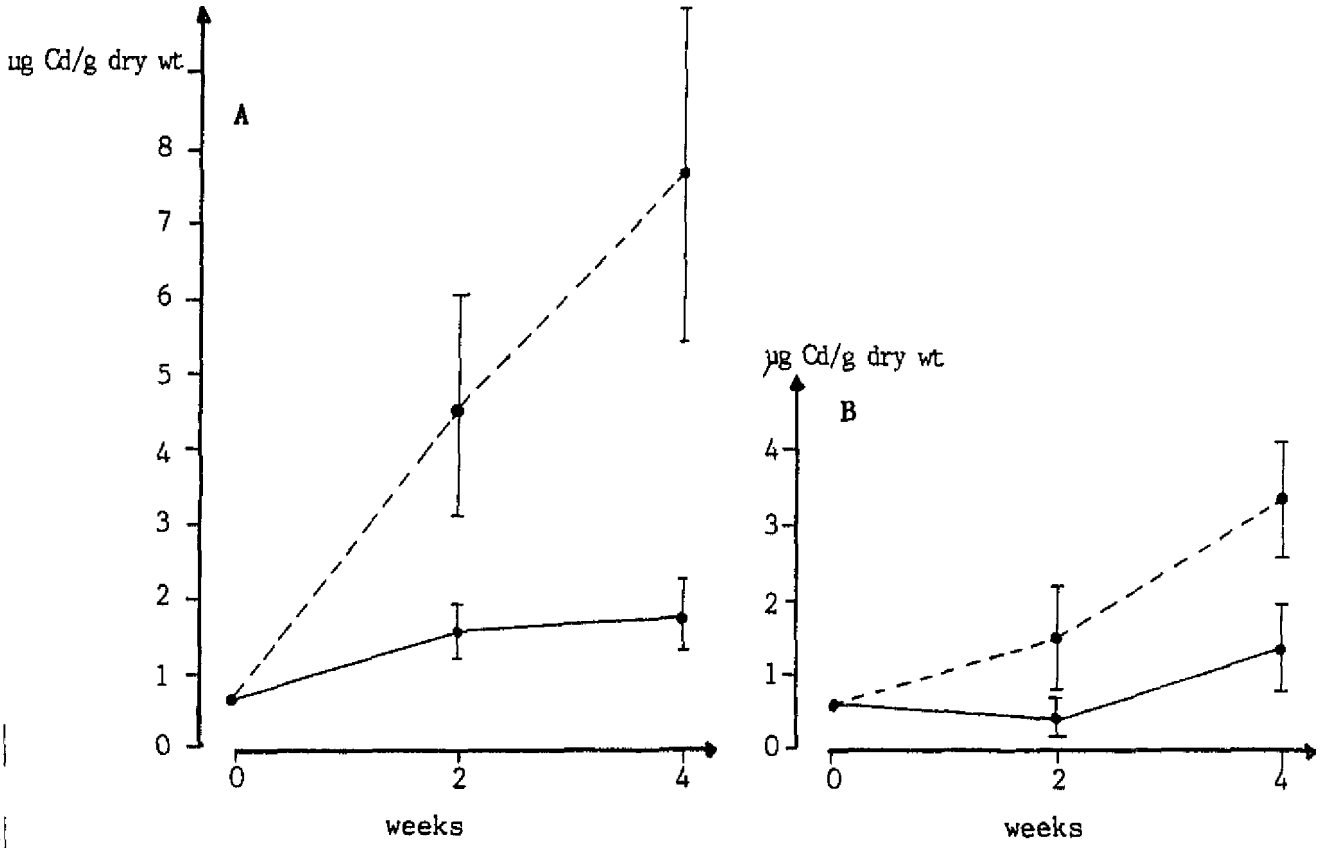

Fig.2.7. Time-dependent accumulation of Cd in sea star **Asterias rubens**. A. Cd concentration in soft parts. B. Cd concentration in skin. Exposed group (---). Control group (——). Mean of 5 animals ± SEM.

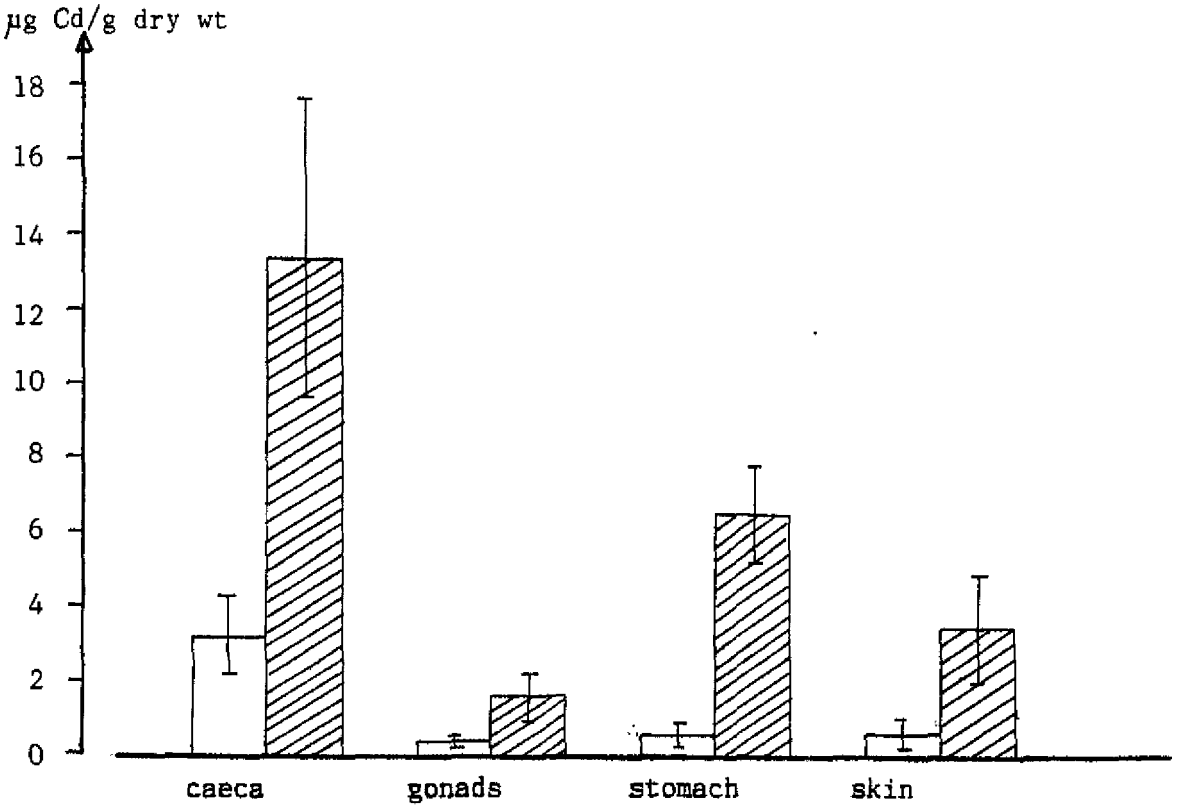

Fig.2.8. Distribution of Cd in caeca, gonads, stomach and skin of **A. rubens** exposed to 50 ppb Cd for 4 weeks. ▨ - exposed group. □ - control group. Mean of 6 animals ± SEM

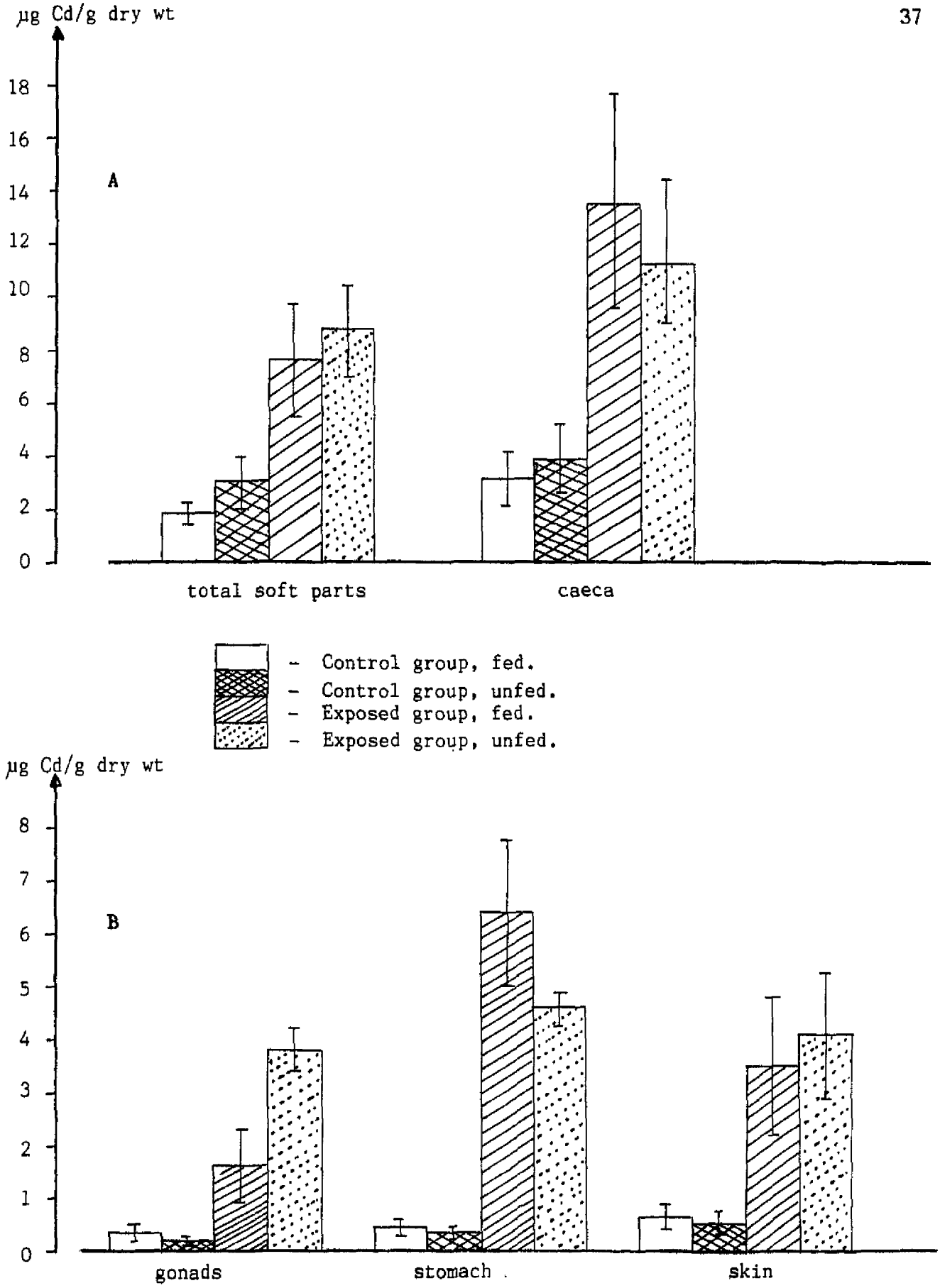

Fig.2.9. Distribution of Cd in organs of **A. rubens** exposed to 50 ppb Cd for 4 weeks in presence or absence of food. A. Total soft parts and caeca. B. Gonads, stomach and skin. Mean of 6 animals ± SEM.

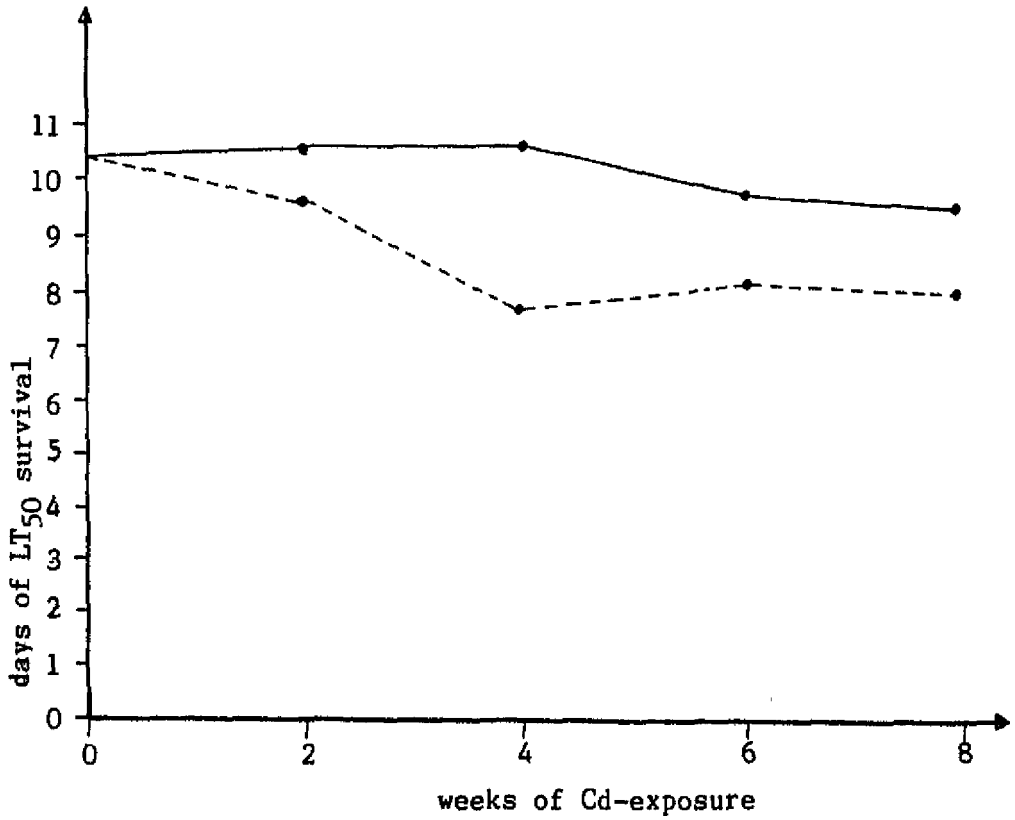

Fig.2.10. The effect of the duration of Cd-exposure on anoxia survival LT_{50} of **M.edulis**. Exposed groups (-- -). Control groups.

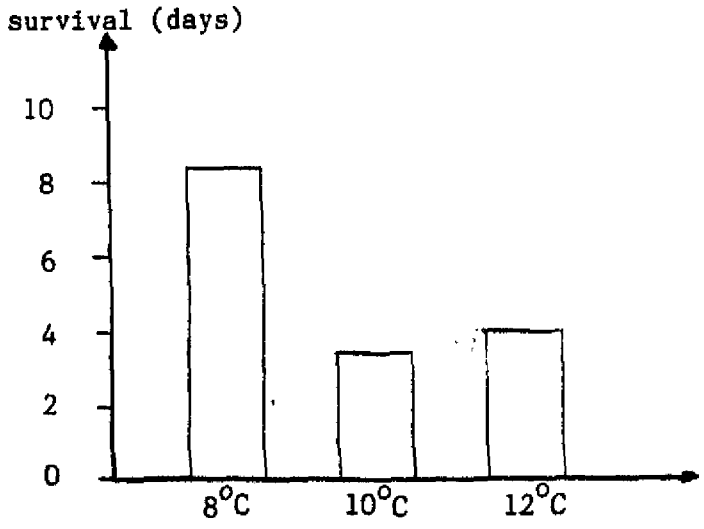

Fig.2.11. The effects of temperature on the anoxic survival of sea stars A. rubens.

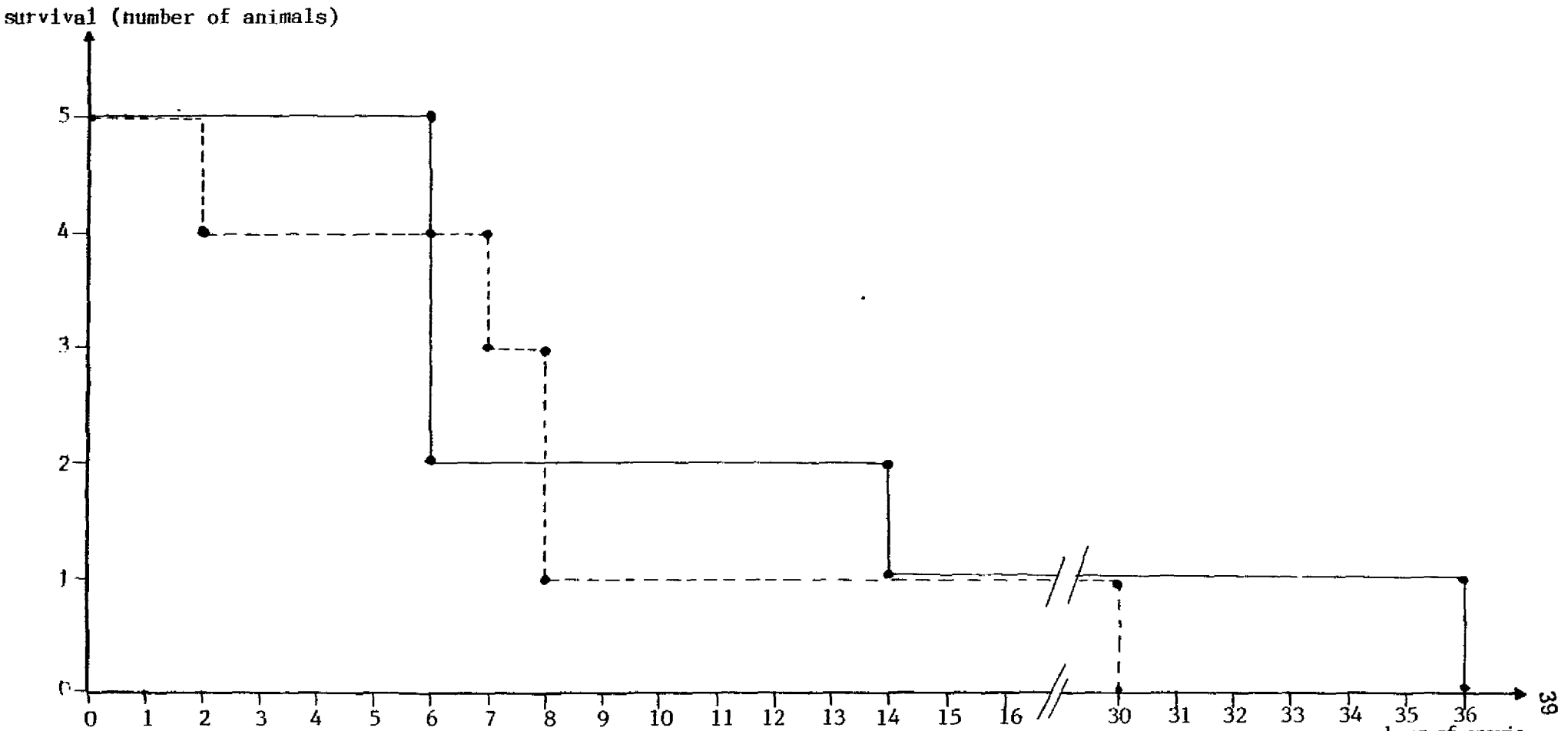

Fig.2.12. Anoxic survival of sea stars after 2 weeks of Cd-exposure. Exposed group (---). Control group (—).

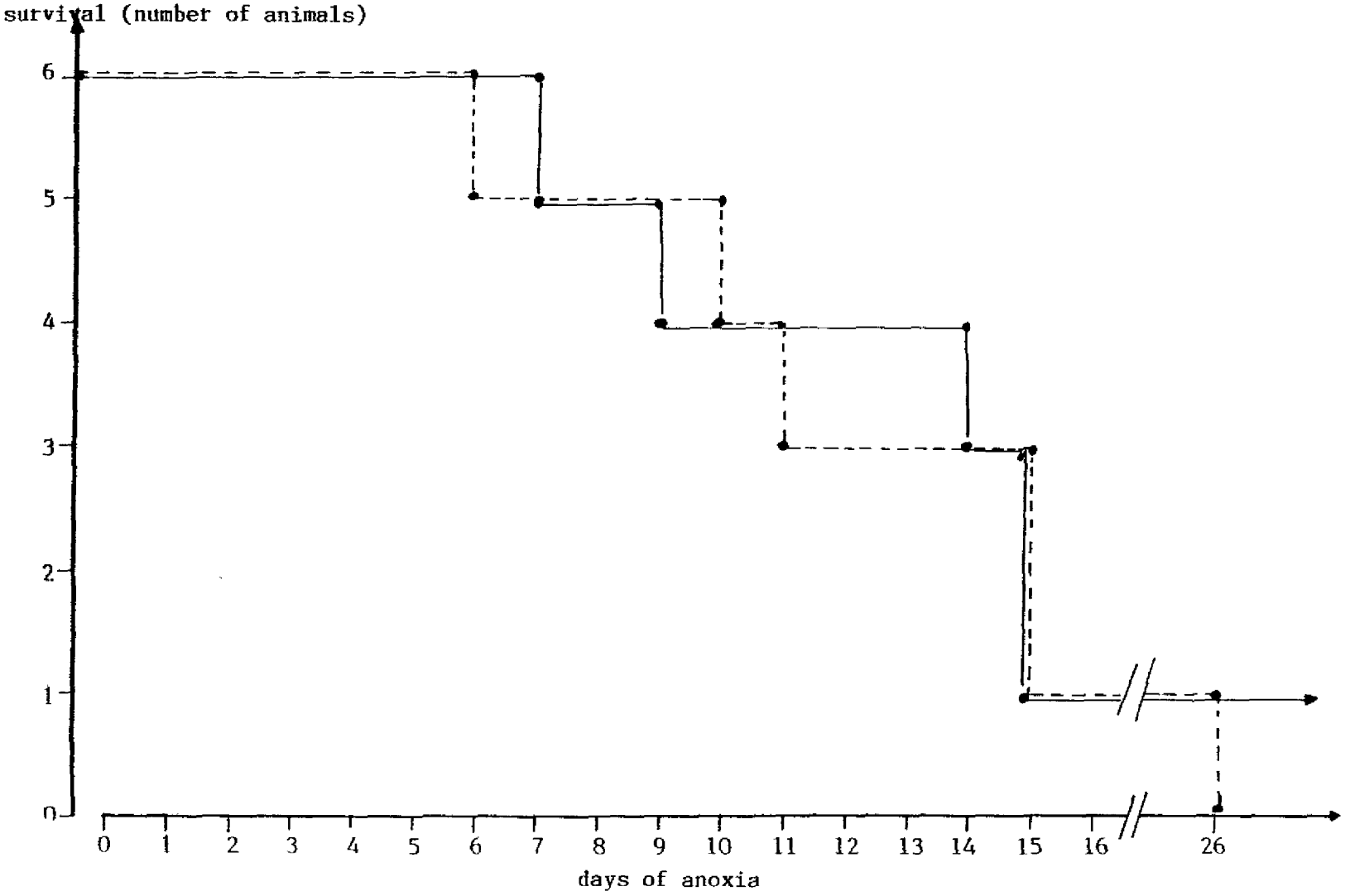

Fig.2.13. Anoxic survival of sea stars after 4 weeks of Cd exposure. Exposed group (---). Control group (——).

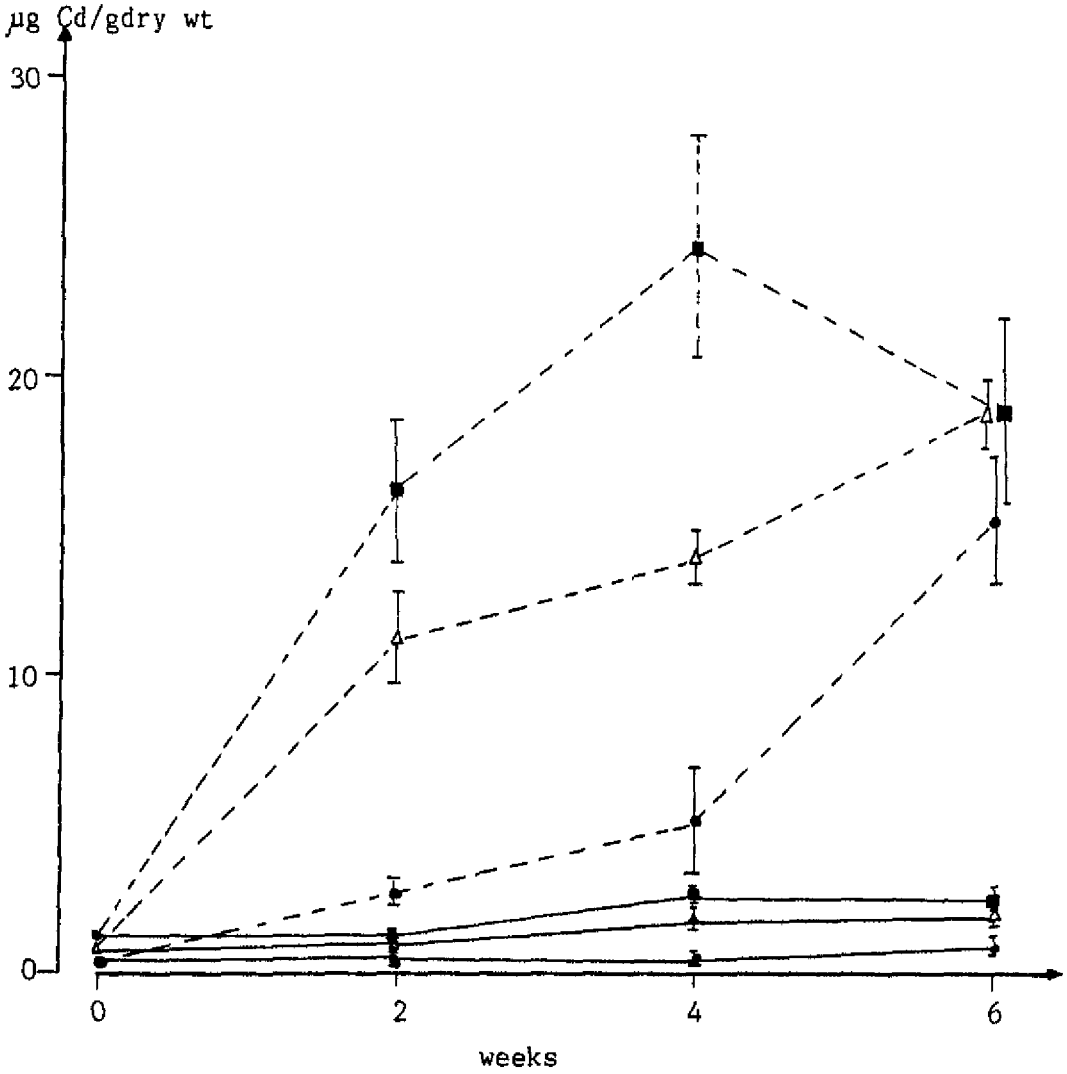

Fig.2.14. Time-dependent Cd accumulation in sea squirts **Ascidiella aspersa**. Cd concentration in tunic (-■-). Cd concentration in body (-●-). Cd concentration in whole animal (-▲-). Exposed group (---). Control group (—). Mean of 5 animals ± SEM.

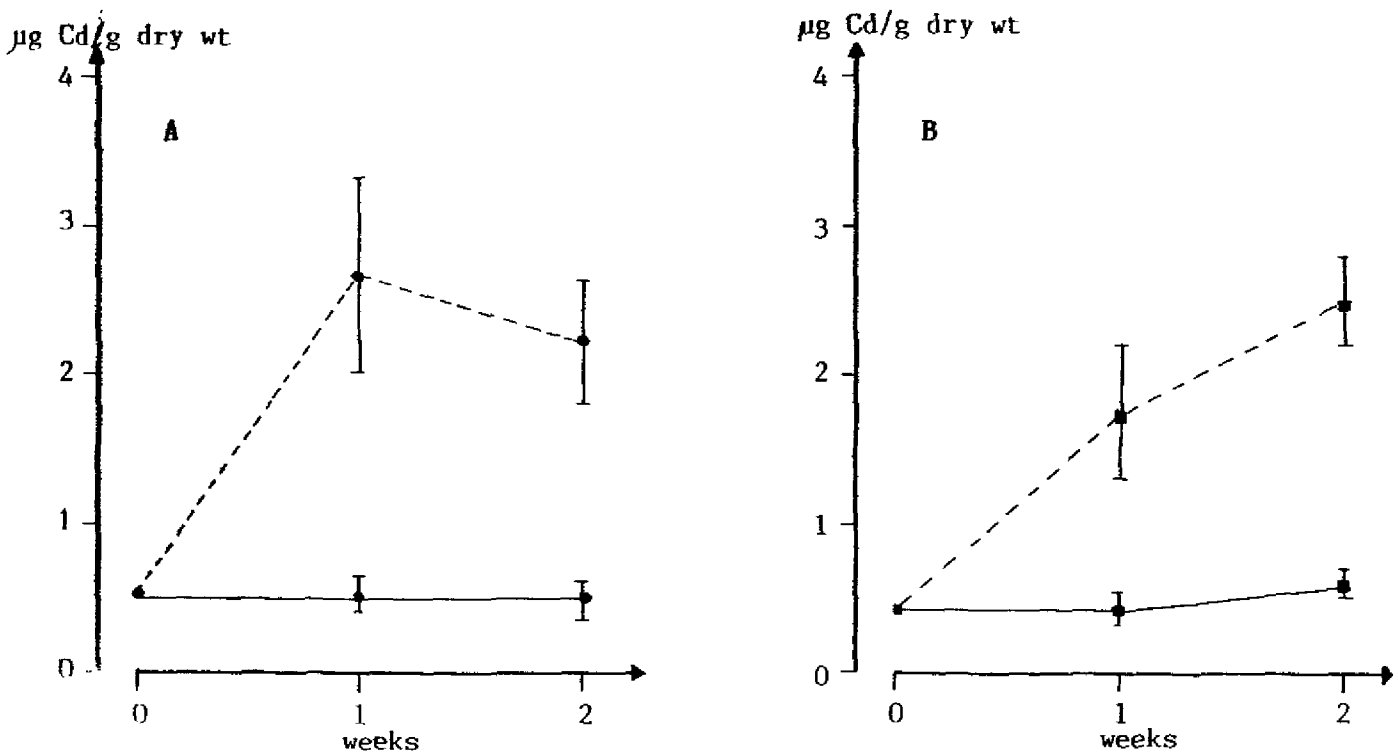

Fig.2.15. Time-dependent Cd accumulation in shrimps **Crangon crangon**. A. Cd concentration in soft parts (flesh). B. Cd concentration in carapax. Exposed group (---). Control group (——). Mean of 5 animals ± SEM.

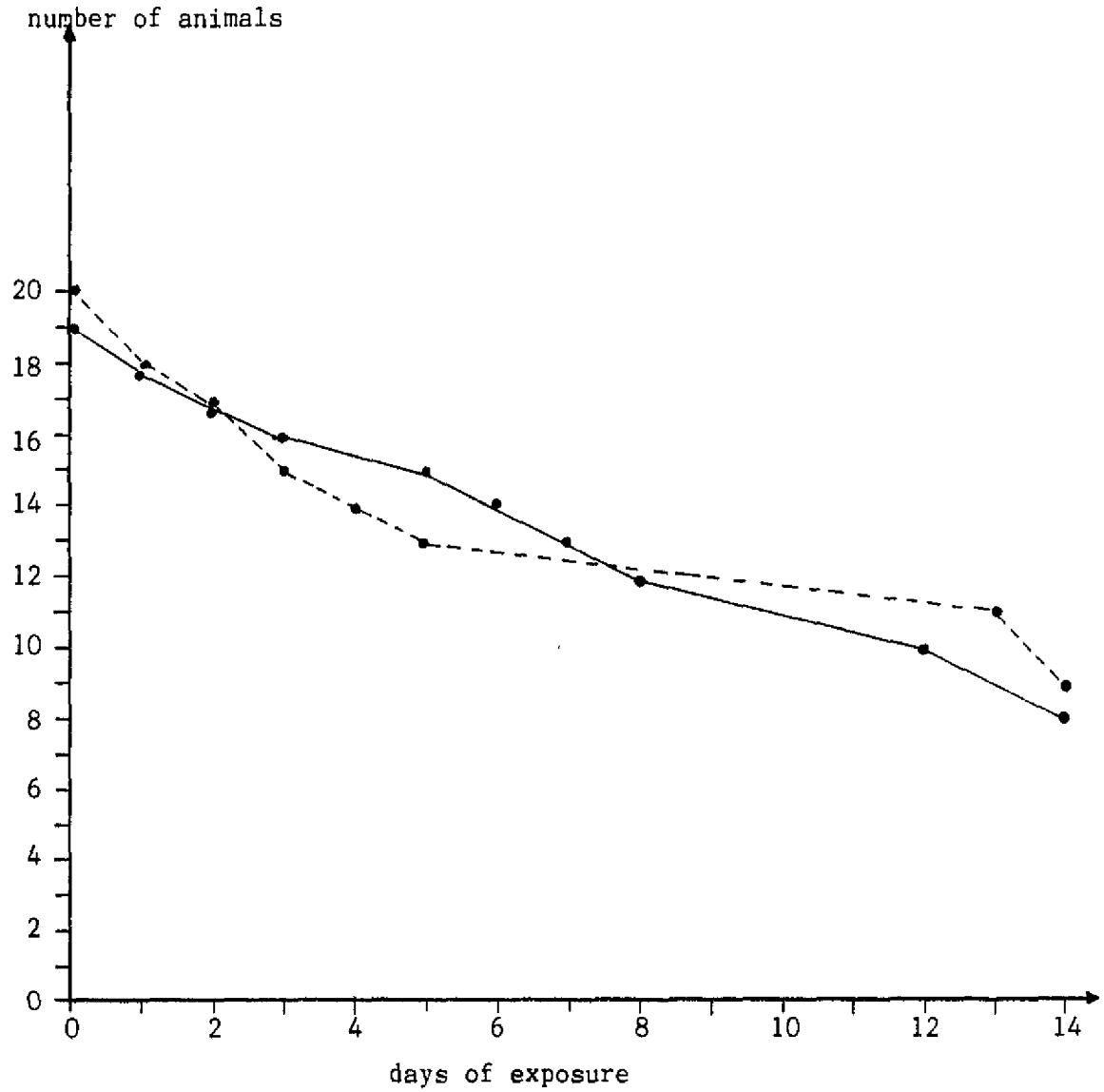

Fig.2.16. Mortality of shrimps during exposure to 50 ppb Cd.
Exposed group ((---). Control group (—).

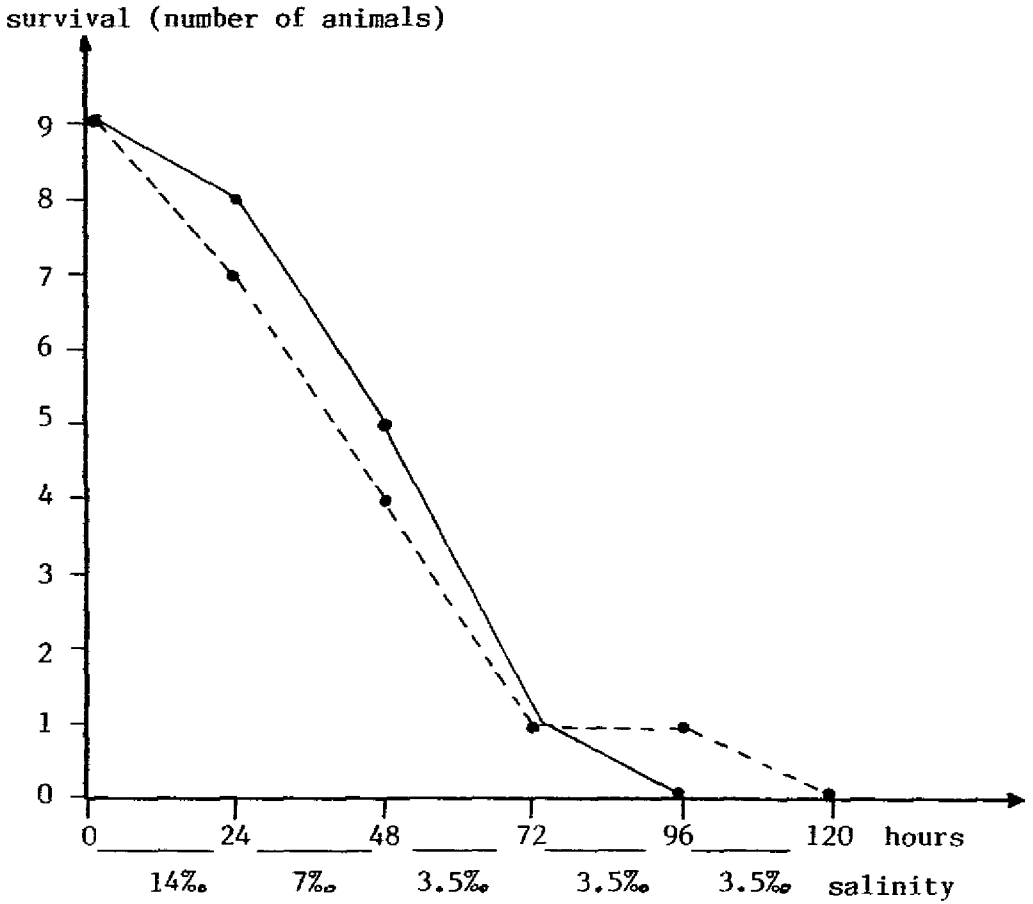

Fig. 2.17. Survival of shrimps at lowered salinities after two weeks of exposure to 50 ppb Cd. Exposed group (---). Control group (—).

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Chapter 3. EFFECTS OF CADMIUM ON THE EMBRYONAL DEVELOPMENT OF THE SEA STAR

3.1 Introduction

From the surveying experiments it was concluded that sea stars are not affected directly by cadmium in terms of a decreased anoxia-tolerance. Short term exposure to moderate Cd-concentrations seemed to leave adult sea stars unaffected, whereas significantly increased Cd-levels in tissues were found. Voogt *et al.* (1987) found that cadmium affected steroid metabolism and steroid levels of *A. rubens*. Steroids are involved in the regulation of gametogenesis, so this process may be disturbed. Reproduction has been reported to be a very vulnerable process many times. Jangoux and Vloebergh (1973) studied the sea star population near Knokke at the Belgian coast. They found that it was characterized by animals of small size and a high degree of oocyte degeneration. These finding may be due to the pollution via the Western Scheldt. In order to asses the sensitivity of *A. rubens* to pollution, effects of pollutants on reproduction must be studied. Apart from studies on the process of gametogenesis, also studies on the effects of toxicants on the embryonal development can give relevant information. Kobayashi (1984) states that echinoderms can provide an ideal material for marine ecotoxicological tests. It is easy to handle embryos, eggs and sperm in the laboratory. Studies on adult animals can be combined by studies on the embryonal development of the offspring. The eggs and embryos have been accepted internationally as appropriate test materials for bioassays. Fertilization and embryonal development of sea urchin and sea star eggs are well-studied (Giudice 1973; Gemill 1914; Couillard and Guerrier 1987) and many observations have been made on the biological effects of various agents on echinoderm embryos (Waterman 1937; Harvey 1956; Lallier 1964; Hörstadius 1973). Sea urchin eggs are widely used for toxicological testing (Wilson 1951, Pagano et *al.* 1982; Kobayashi 1984).

This chapter concerns with a study on the effects of cadmium on the embryonal development of *A. rubens*. Embryos were exposed to Cd starting just before fertilization These embryos and those obtained from Cd-exposed adult sea stars were examined during early development.

3.2 Materials and Methods

Animals

Sea stars were collected from the Eastern Scheld or the Wadden Sea in october 1986 and kept in aerated 200 l glass aquaria at 12°C (salinity 28°/$_{oo}$). The animals were fed with mussels.

Embryo test

Oocytes and spermatozoa were obtained by injecting sea stars with 1 ml 1-Methyladenine (10^{-4}M). Oocytes were kept in monolayers in filtered sea water (salinity 3.0%) at 12°C. Cd was added just before fertilization at concentrations of 0.1 and 1.0 ppm (administered as $CdCl_2$). Fertilization was achieved by adding a few drops of sperm suspension to the oocytes. To determine the effects of Cd, the success of fertilization and of blastula and gastrula formation was studied.

Exposure of adult sea stars

Adult sea stars were exposed to 100 ppb Cd for 22 days in the exposure system described earlier (Chapter 2). After 22 days the animals were transferred to clean sea water and spawning was induced. Control sea stars were kept under the same conditions in clean sea water. To raise embryos, the same procedure was followed as above. Development of embryos from exposed and unexposed parents was compared.

3.3 Results and Discussion

Normal development of the embryos of *Asterias rubens* can be characterized by a low frequency of aberrations during embryogenesis (Fig. 3.1, photographs 1-4). Gastrulation is followed by growth of embryos and further development of archenteron.

Cd did not prevent the formation of the fertilization membrane at concentrations of 0.1 and 1.0 ppm. 1-2 hours after fertilization, abnormal cleavage was observed among embryos exposed to 1 ppm Cd. After 24 hours of exposure at this concentration, the number of embryos developing normally was decreased by more than 50% (Fig. 3.2, photographs 1 and 2). Further embryotoxic effects

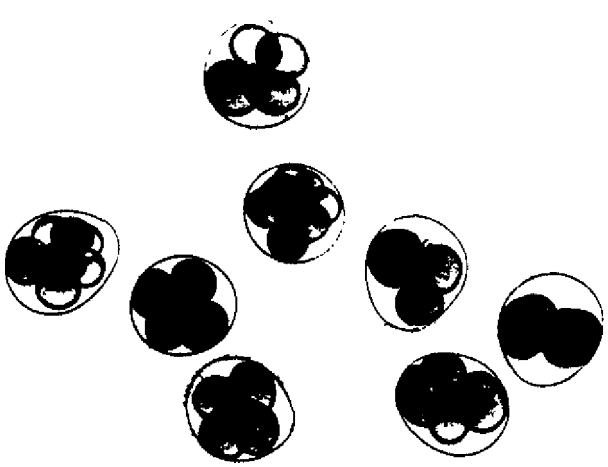
of Cd can be shown after 48 hours by the appearance of exogastrulae (Fig. 3.2, photograph 3). Gastrulation is also affected after exposing embryos to 0.1 ppm Cd, as can be seen by gastrulae with archenteron malformations (Fig. 3.2, photographs 4 and 5).

Development of embryos obtained from Cd-exposed parent sea stars is also disturbed. Aberrations, similar to those described above, occur frequently. Again, abnormal cleavage was observed after fertilization, resulting in decreased numbers of normal blastulae after 24 hours. Apart from exogastrulae also gastrulae with archenteron malformations appear after 48 hours, finally resulting in a low number of normal gastrulae.

In literature the observed aberrations at the first cleavages are called polyspermic cleavages. This phenomen, caused by penetration of more than one sperm into the oocyte at fertilization, is induced especially by heavy metals, usually over a wide range of concentrations (Clark 1936; Kobayashi 1971, 1984). Normally a block to polyspermy is established about 2 seconds after the entrance of the first sperm, thus preventing other sperms to enter the oocyte. This process is not completely understood yet, but proteins with SH-groups may be involved (Giudice 1973). Interaction between these proteins and heavy metal ions might cause delay or inhibition in establishing the block to polyspermy, thus increasing the chance that more than one sperm will enter the oocyte and cause polyspermic cleavage.

In embryology Cd is known to be a vegetalizing agent: at high Cd-concentrations (>> 1 ppm Cd) development of embryos will arrest in the blastula-stage, resulting in large, irregular shaped blastulae. In literature this Cd-effect is described usually (Giudice 1973). Apparently, when at lower Cd-concentrations gastrulation does occur, aberrations at this stage become more prominent. The archenteron formation is affected, resulting in malformations or, in the extreme, exogastrulation. Exogastrulation was also observed in the early development of the Ascidian *Ciona Intestinalis*. At the same concentration (1 ppm) 40% of the larvae showed exogastrulation, whilst at 10 ppm embryos did not develop beyond the early blastula stage (Carollo and Canicatti 1983).

The embryotoxic effects of Cd on the embryonal development of echinoderm species have been reported earlier. Pagano *et al.* (1982) investigated fertilization and larval development of three mediterannean sea urchin species during exposure to cadmium and after exposing gametes to the metal at concentrations ranging from 1 ppb to 100 ppm. The embryotoxic effects only manifested at the process of skeletal differentiation (Pluteus stage) and no abberations during early cleavages were observed. On the other hand, Nacci *et al.* (1986) have given an EC_{50}-valve of 13.9 ppm Cd for development of *Arbacia punctulata*,



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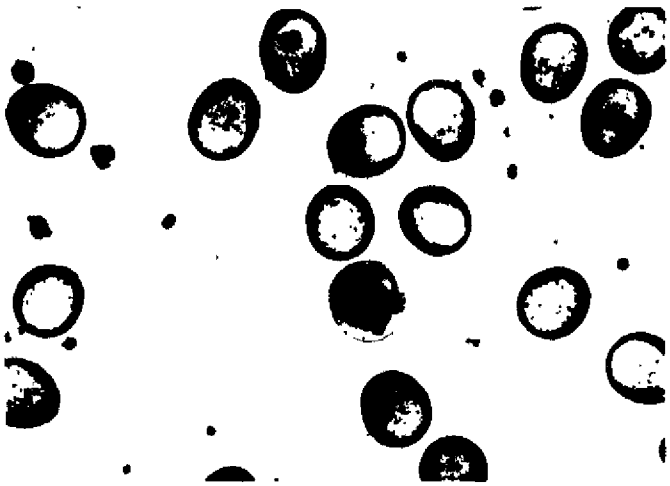

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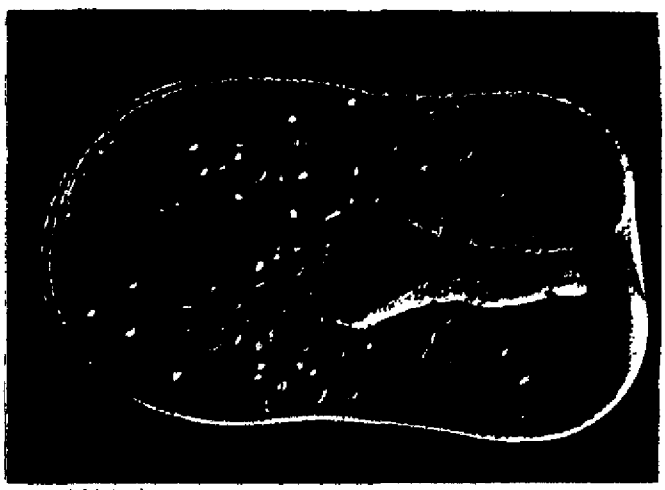

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Fig. 3.1. Developing embryos in normal sea water. 1: early cleavages, 2 h. 2: blastula stage, 24 h. 3 and 4: gastrulation stage, 48 and 72 h.

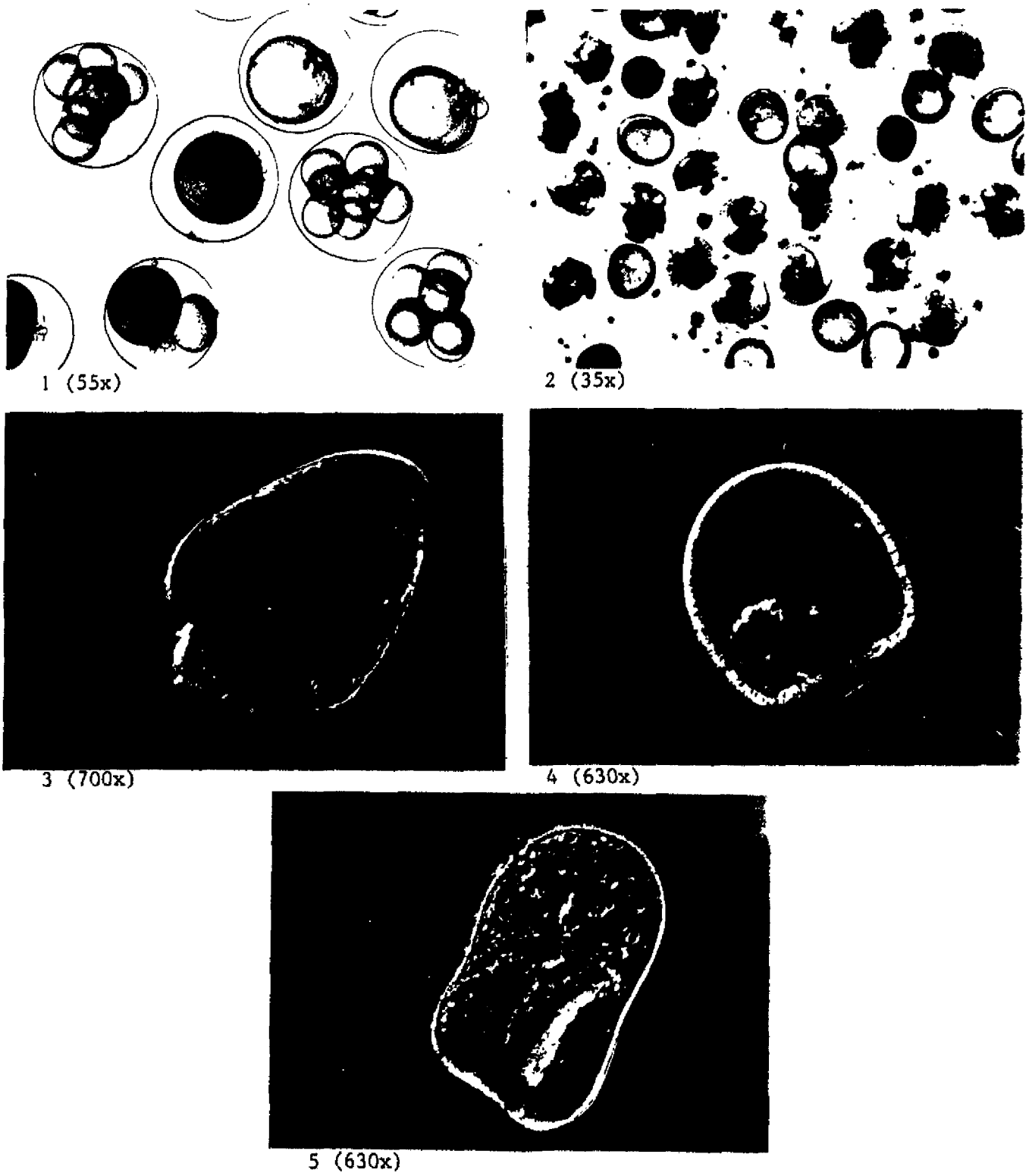

Fig. 3.2. Developing embryos exposed to 0.1 and 1.0 ppm Cd. 1: Polyspermic cleavage, 2 h (1 ppm Cd). 2: Blastula stage, 24 h (1 ppm Cd). 3: Exogastrulation, 48 h (1 ppm Cd). 4 and 5: Archenteron malformations at gastrulation stage, 48 and 72 h (0.1 ppm Cd)

estimated from an early embryo test (measurement of ^{3}H-thymidine incorporation after 2 h of exposure). Kobayashi (1977) has also demonstrated for the sea urchin *Anthocidaris crassispina* that pluteus formation is more sensitive to Cd and other pollutants than earlier stages. For the sand dollar, *Peronella japonica*, it was found in this study that metamorphosis, which in this species occurs after about 4 days, was the most sensitive stage. From this it may be concluded that larvae of some sea urchin species are less vulnerable than the larvae of *A. rubens*, but that it is well possible that later stages in the development of embryos of *A. rubens* are even more sensitive to pollutants.

Embryos, obtained from sea stars exposed to 100 ppb Cd for 22 days, show similar aberrations during early development as discussed above. Khristoforova *et al.* (1984) studied gametogenesis and early development of the offspring of Cd-exposed sea urchins *Strongylocentrotus intermedius*. After exposure of sea urchins to 0.1 ppm Cd for 1 month no morfological changes were observed in the gonads, but the gametes produced were anomalous and resulted in inviable offspring. These results correspond with the results from this study. Exposure time was rather short compared to the gametogenesis-cycle. Therefore, it is well possible that long term exposure to low Cd-concentrations will cause the same effects on the offspring of exposed sea stars.

3.4 References

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Chapter 4. SYNTHESIS OF METALLOTHIONEIN-LIKE PROTEINS IN SEA STAR

4.1 Introduction

In the second chapter of this report, Cd-accumulation in sea star was described. It was shown that accumulation occurs at highest rate in the pyloric caeca, whereas in the gonads Cd-concentrations remained relatively low. Köhler and Riisgard (1982) showed in *Mytilus edulis* an increasing amount of Cd-binding proteins (metallothioneins) when the body burden of Cd increased. Metallothionein (MT) plays a major role in the metabolism and toxicity of Cd. The term "metallothionein" is used for a class of soluble, heat-stable, metal-rich, low mol. weight proteins characterized by an unusually high cystein content and by the absence of aromatic amino acids and histine (Vallee 1979).

Metallothionein-like proteins have been demonstrated in a wide variety of organisms including vertebrates, invertebrates, plants and microorganisms (Hamer 1986). MT is also shown to be present in sea urchin embryos yet nothing is known about adults of echinoderm species. In relation to cadmium the function of MT is regarded as a detoxification by binding the Cd-ions. The biological function of MT under normal conditions is not completely understood. It is assumed however that MT serves as a storage protein for metals (zinc and possibly copper) during embryonal development and probably in adults as well.

Synthesis of MT is inducible by cadmium under conditions of elevated exposure levels (Piscator 1964; Cherian and Nordberg 1983). The tissue MT-level is mainly related to the tissue deposition of the inducing metal. In vertebrates over 90% of the accumulated Cd is bound by MT (Kägi and Nordberg 1979), whilst in *Mytilus edulis* this is approximately 60-70% (George and Prie 1979; George 1983). The remainder is mainly bound to high molecular proteins, where it can be incorporated into for example metalloenzymes and disturb metabolic activities of the enzymes. When there is a spill over of Cd, the fraction of Cd bound to MT, which represents the detoxified Cd-fraction, is low. This is likely to happen when the level of Cd-exposure is high or in initial exposure stage before synthesis of MT is induced.

In relation to Cd-accumulation in sea stars it is interesting to know if an increase in Cd-content is involved with an increase in MT in pyloric caeca and gonads. Moreover, the state of detoxification in which Cd is found in these organs, may provide a better understanding of toxic effects. These aspects of MT-synthesis in sea stars were studied by exposing animals to Cd after which cytosolic proteins of pyloric caeca and gonads were chromatographied by gel filtration and analysed for Cd-content

4.2 Materials and Methods

Animals

Sea stars *Asterias rubens* were caught in the Dutch Wadden Sea in 1986. After transportation to Utrecht, animals were kept in aquaria with circulating sea water 12°C (salinity 28°/$_{oo}$). Sea stars were fed with mussels.

Cd-exposure experiments.

In section 2.2 details are given about the exposure system used for these experiments. Sea stars were exposed to approximately 50 ppb Cd for 4 and 8 weeks or to approximately 100 ppb Cd for 3 weeks, in the period May-June 1987. After exposure, animals were taken from the aquaria and dissected. Pyloric caeca and gonadal tissue was immediately used for gel-filtration chromatography. Oocytes from exposed sea stars were obtained by injecting sea stars with 1-methyladenine to induce spawning. The oocytes were frozen, lyophilized, and used for gel-filtration chromatography.

Gel-Filtration Chromatography.

Pyloric caeca and gonadal tissue or oocyte material was cooled on ice and homogenized in 1-2 parts 50 mM Tris-HCl, pH 8.2, containing 50 mM NaCl. The homogenate was centrifuged at 27,000 g, 4°C, for 15 min (Sorwall centrifuge). The supernatant was then centrifuged at 100,000 g, 4°C for 1 hour. 1 ml aliquots of the supernatant were chromatographied on a Sephadex G75 superfine 67 x 1.35 cm column (Pharmacia Fine Chemicals, Uppsala, Sweden). Elution was performed with 50 mM Tris-HCl, pH 8.2, containing 50 mM NaCl, at 5°C and a flow rate of 4 ml/h. Samples of 1.8-2.0 ml were collected with an automatic sampler (LKB 2070 ultrorac II). The absorption of the samples was measured at 280 and 254 nm (Pye Unicam SP8-100 UV/V15 spectrophotometer). Cadmium measurements were performed by flame absorption spectrophotometry (Video 11, Instrumentation Laboratory, air/acetylene flame).

4.3 Results and Discussion

In Figs. 4.1 A-B typical elution patterns of gel-filtrations of soluble fractions (100.000 g) of homogenates of pyloric caeca from sea stars exposed to

50 ppb Cd for 4 and 8 weeks respectively. Two protein classes contain relatively high Cd-levels: high molecular weight (in the figures indicated by HM) and low molecular weight proteins. The low molecular-weight proteins have a molecular weight of approximately 10,800 D and a high A254/A280 ratio, which in combination with a high metal-content is typical for metallothioneins (MT). For sea urchin larvae, MT has been shown to be present already on basis of its characteristic properties as a small protein (6-8 kD) with extraordinarily high cysteine content, whose biosynthesis is readily induced by heavy metals (Nemen *et al.* 1984). The molecular weight of MT's usually lies between 6,000 and 10,000 D, but for *Mytilus edulis* cadmium-binding proteins > 10,000 D have been reported (George *et al.*, 1979) and for fish 10,000 and 15,000 (Yamamoto *et al.* 1878; Overnell and Coombs 1979). Viarengo *et al.* (1980) reported the existence of a copper-binding MT of 12,000 D.

In the soluble fraction of pyloric caeca of unexposed animals, also MT-like proteins containing Cd were found (Fig 4.2). Here, however, zinc and copper appeared to be the dominant metals bound by MT (results not shown).

During exposure, the amount of Cd bound to the soluble fractions increases. After 4 and 8 weeks of exposure to 50 ppb Cd, respectively 67% and 57% of this Cd was bound to MT-like proteins. The remainder is mainly bound to high molecular weight proteins. Carpene and George (1981) demonstrated that in *Mytilus edulis* after 3 months exposure to 100 ppb Cd 75% of the cytosolic Cd was bound to metallothionein and the remainder to high molecular weight proteins. Engel (1983) showed that, after exposing oysters to 100 ppb Cd for 28 days, all cadmium was bound to MT. After 28 days of exposure to 300 ppb Cd, the metal was also present in high molecular weight fractions and in the low molecular weight peptide-amino acid fraction. This spill over suggested that the capacity of the detoxification system was overwhelmed. This is also likely to occur shortly after the start of exposure, as shown for cytosol of several organs of *Mytilus edulis* exposed to 100 ppb Cd (Nolan and Duke 1983). In this study it was demonstrated that in hepatopancreas, kidney and mantle after 48 hours more than 70% of the cytosolic cadmium was bound to MT whilst after 1 hour a neglectible amount of cadmium in the MT-fraction was bound.

In the soluble fraction of gonadal tissue of female sea stars exposed to 100 ppb Cd for 3 weeks, no cadmium was found in a MT-fraction (Fig. 4.3A). High Cd contents were only found in the high molecular weight fractions. The same pattern was found for oocytes of the Cd-exposed animals, as shown in Fig 4.3B. The fraction of Cd bound to MT represents the detoxified part. Carpene *et al.* (1987) showed inhibition of pyruvate kinase in the early stage of Cd-exposure to goldfish, when most of the cytosolic Cd was found to high molecular weight proteins. This inhibition decreased as the exposure time increased

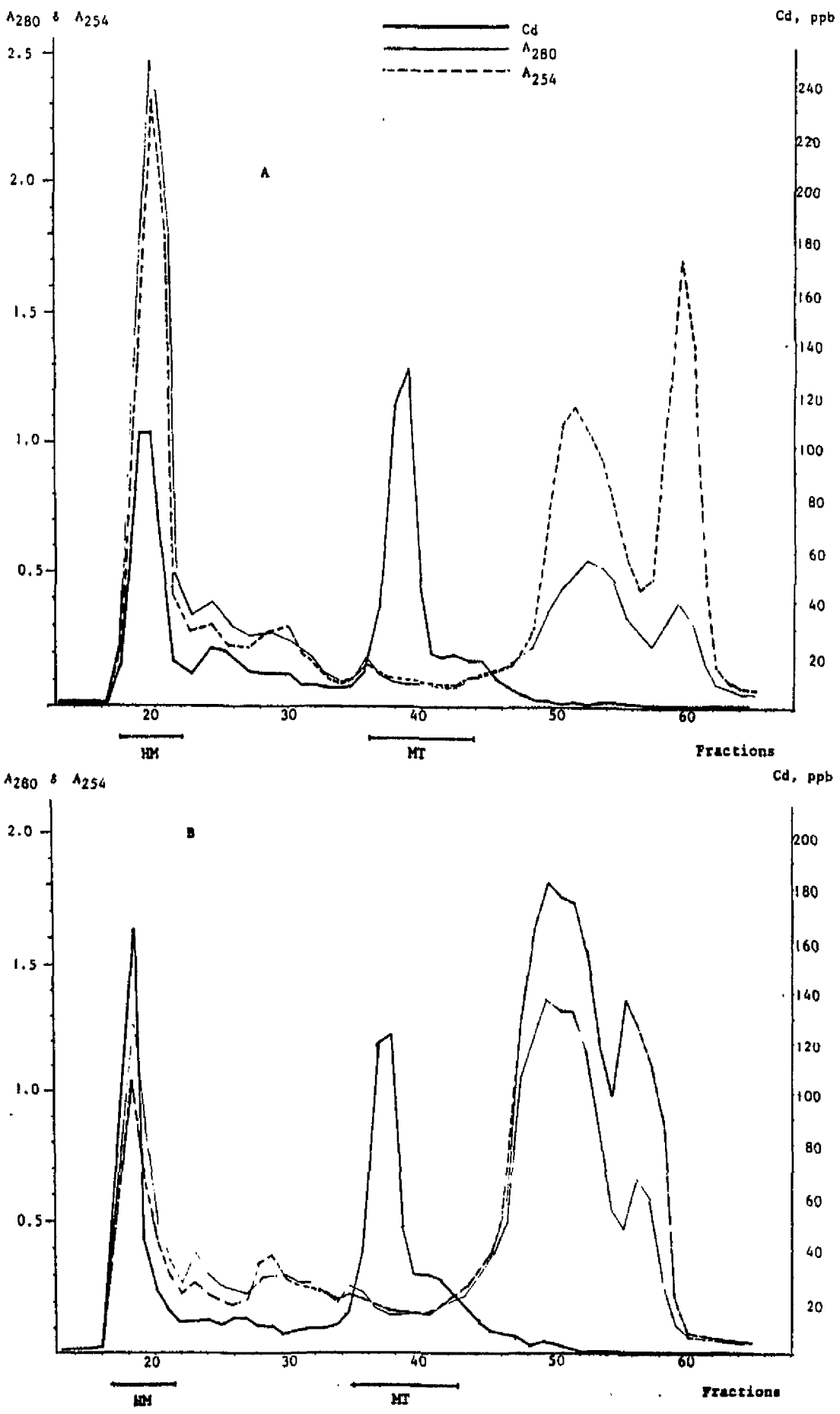

Fig. 4.1. Sephadex G-75 elution profiles of soluble fraction of pyloric caeca from sea stars exposed to 50 ppb Cd for 4 (A) and 8 (B) weeks. Molecular weight decreases with increaseing fraction number.

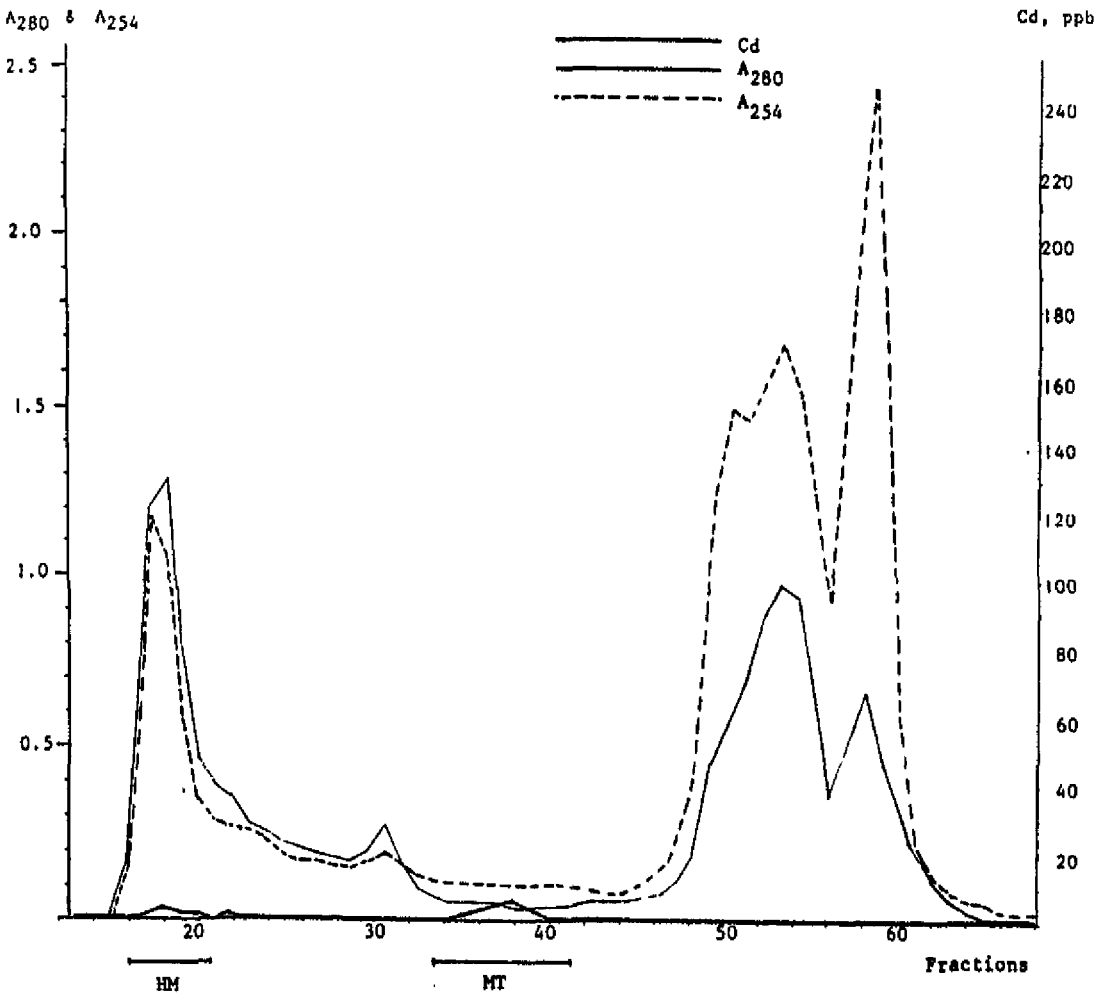

Fig. 4.2. Sephadex G-75 elution profile of soluble fraction of pyloric caeca from control sea star.

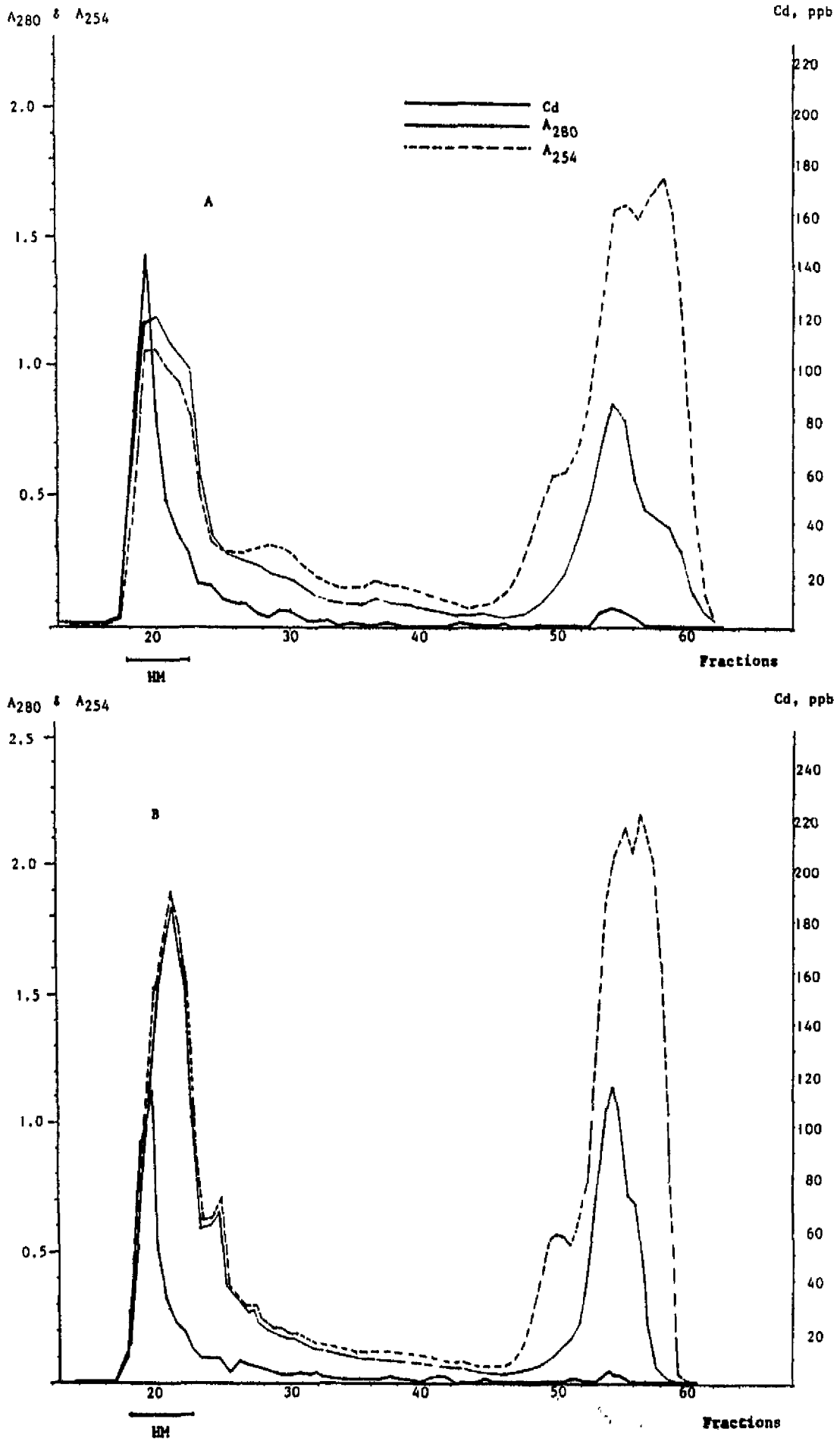

Fig. 4.3. Sephadex G-75 elution profiles of soluble fraction of ovaries (A) and oocytes (B) of sea stars, exposed to 100 ppb CD for 3 weeks.

and Cd became mainly bound to MT. Biochemical analysis of the fraction of Cd bound to MT may provide a sensitive toxicological index.

In this regard the ovaries and oocytes may be very sensitive to cadmium. Although metal-concentrations in gonads were relatively low after Cd-exposure (see chapter 2), oocytes contain Cd which is mainly bound to high molecular weight proteins. Function of these proteins may be disturbed, which might explain the aberrations occurring during early development of the offspring of Cd-exposed sea stars (see chapter 3).

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Chapter 5. CELLPHYSIOLOGICAL EFFECTS OF CADMIUM

5.1 Introduction

In our pilot experiments, it has been demonstrated that cadmium is toxic to *Mytilus edulis*, *i.e.*, the anoxic survival time was significantly shortened after two weeks of exposure to 50 ppb Cd (see Chapter 2). This finding is in contradiction with the supposition that *M. edulis* is less suitable as test organism for metal toxicity because of its high capacity to store cadmium and other heavy metals without noticeable toxic effects. Moreover, recent data have indicated that capacity of *M. edulis* for the production of metal-binding proteins is limited, thereby restricting the organism ability to tolerate the exposure to further increase in metal concentrations (Harrison *et al.*, 1987). Therefore, it is of importance to investigate the toxic effects of cadmium into more details.

Cd may be expected to exert its effects at the biochemical and physiological level either by complexing directly at vital centers or indirectly by interfering with the metabolism of essential metals. In the former case the Cd ion binds to the charged groups, including sulphydryl that occur in proteins, carbohydrates and nucleic acids. Moreover, the metal may affect the active site or even the whole secondary structure of an enzyme producing simulatory effects at low concentration, but inhibitory effects at higher metal concentration (Simkiss *et al.*, 1982). In the latter case Cd has been shown to substitute the functional metal of a metalloenzyme, thus affecting the enzyme activity. That is profoundly studied in mammals and characterized by the kidney damage, gonadal atrophy, bone demineralization, muscle and connective tissue damage (Webb, 1979).

In the cellular pathways and detoxification of heavy metals, metallothioneins (MT) play a crucial role. So far the dynamics of MT's have been studied by conventional biochemical methods such as gel filtration and/or AAS. However, it would be much more interesting to investigate the interaction of heavy metals with a wide range of cellular proteins (in particular, MT) by means of advanced methodology. With these techniques it will be possible to study the interference of cadmium with cellular processes at different levels, *e.g.*, transcriptional (mRNA synthesis), translational (protein synthesis) and post-translational (modifications of proteins). In a variety of organisms and cells in culture, the toxic effects of cadmium have been demonstrated at the molecular level. Cadmium modifies DNA and RNA metabolism. DNA repair and replication processes are inhibited. RNA synthesis may be increased or decreased (Burkart and Ogorek, 1986; Nocentini, 1987). In marine invertebrates, the effects of cadmium at the above-mentioned levels are poorly known. Recently, Baksi and Frazier

(1987) have demonstrated that Cd has an effect on the pattern of protein synthesis in the fish hepatocytes. Furhter, Sanders (1987) has found that both heat shock and Cd can induce heat-shock proteins (HSP) in *M. edulis*, although according to Steinert and Pickwell (1987) Cd appeared to be a poor inducer of these proteins (HSP) in *M. edulis*. In mammals and recently in fish (Chan *et al.*, 1987) a molecular approach has already been chosen to quantify and to make a positive identification of MT, using mRNA isolation and its subsequent translation *in vitro*.

Considering the above-mentioned ideas, the research of Cd effects on the macromolecular metabolism in *M. edulis* was started. The present chapter describes the first approach to study the effects of Cd on protein synthesis. The protein patterns in different organs were analysed by means of one- and two-dimensional electrophoresis with respect to Cd-exposure. The effect of Cd on the incorporation of the radio-labeled amino acid was studied in gill tissue.

5.2 Materials and Methods

Animals

Mussels, *Mytilus edulis*, were collected in the Eastern Scheldt in September 1987. The mean shell length was 6.0±0.5 cm. After transportation to Utrecht, animals were kept in aquaria with recirculating sea water at 12°C. Mussels received no food.

Exposure system

Mussels were exposed to approximately 50 or 250 ppb Cd (for different periods of time) in glass aquaria (volume of 80 l) to which sea water and metal solution were supplied with pomps at rates 1 l/h and 10 ml/h, respectively. The sea water temperature was 12°C. Cadmium was added as $CdCl_2.H_2O$ (Merck No. 2011). Each control or exposed group contained 6, 10 or 12 mussels. After one week of exposure to 50 ppb Cd, the group of six animals was subjected to 1 ppm Cd for 16 h in glass jar (volume of 2.5 l). Prior to further experimental procedures, mussels (from all exposed groups) were kept in "clean" sea water overnight to eliminate the adherent cadmium.

Preparation of protein samples

a) Samples for SDS-polyacrylamide gel electrophoresis:

The excised organs (gills, labial palps, PAM and digestive glands) were pooled from six animals, frozen and lyophilized. The freeze-dried tissues were homogenized with Ultra-Turax in buffer: 20 mM TRIS.HCl, pH 7.4, 100 mM KAc., 0.1 mM EDTA, 10% glycerol, 1 mM DTT. Afterwards, the samples were centrifuged at 15,000g at 4°C for 30 min. The protein concentrations of SN were determined by the method of Bradford (1976). Aliquots of SN containing 50 µg of protein, were mixed 2:1 with loading buffer: 0.125 M TRIS.HCl, pH 6.8, 5% SDS, 25% glycerol, 25% ß-mercapto-ethanol and 20 µM bromophenol blue, incubated at 95°C for 10 min and subjected to SDS-PAGE analysis.

b) Samples for isoelectric focusing:

The above-mentioned lyophilized tissues were broken mechanically into smaller pieces. Tissue fragments where then homogenized with Potter in 20 mM TRIS.HCl, pH 7.1, 2 mM $CaCl_2$ and 1 mM DTT. During homogenization care was taken to minimize heating and foaming which could cause the protein modifications. The homogenates were processed by two different methods:

1. "Lysis". Homogenates were mixed with 0.12 volume of 3% SDS and 10% ß-mercapto-ethanol. After addition of 0.12% volume of DNAse/RNAse mixture (500 µg/ml RNAse A, 1 mg/ml DNAse I, 0.5 M TRIS, pH 7.0 and 50 mM $MgCl_2$), homogenates were incubated at room temperature for 5 min. Equal parts of the pretreated homogenates were frozen and lyophilized. One part was dissolved in 9.5 M ureum and used for protein determination by the modified method of Bradford (Ramagli and Rodriguez, 1985). The second part was dissolved in the equal volume of lysis buffer: 9.95 M ureum, 4% Nonidet P-40, 2% LKB ampholines (1.3% pH range 5.0 - 7.0 and 0.7% pH range 3.5 - 10), 100 mM DTT and 0.3% SDS. The dissolved samples were centrifuged at 16,000 g for 2 min at room temperature to remove unsoluble material and stored at -20°C.

2."Dialysis". The homogenates were centrifuged at 15,000 g for 30 min at 4°C. The protein concentrations of SN were determined by the method of Bradford (1976). The aliquots of SN were pretreated with DNAse/RNAse mixture as described above and then dialysed against 9.5 M ureum, 4% Nonidet P-40 and 0.3% SDS for 3 h at room temperature. To dialysates, DTT and ampholines were added to make the final concentrations of 100 mM and 2%, respectively. Samples were stored at -20°C.

SDS-polyacrylamide gel electrophoresis of protein samples

The analysis of protein samples from Cd-exposed and control animals was performed on 15% SDS-polyacrylamide slab gels by the method of Laemmli (1970) at 40 mA/gel for 4 h. Phosphorylase B (Mr=92 kD), senumalbumin (Mr=67 kD), ovalbumin (Mr=45 kD), catalase (4 subunits, Mr=40 kD) and cytochrome (Mr= 12.4 kD) were used as standard markers. Gels were stained in 40% methanol and 10% acetic acid with 0.25% Coomassie Brillant blue R.

Isoelectric focusing in polyacrylamide gels

The two-dimensional gel electrophoresis was employed (as described by O'Farrel, 1975, O'Farrel *et al.*, 1977 and Garrels, 1979) to resolve the protein samples. Samples, containing 50 µg proteins, were brought from the kathodic end of the gel for isoelectric focusing. The first dimension gels contained 8.5 M ureum, 2% Nonidet P-40, 3.5% acrylamide/bisacrylamide (ratio 28.32:1.58%), and 2% ampholines (1.3% pH range 5.0-7.0, and 0.7%, pH range 3.5-10). These gels were polymerized with 2 µl of 10% ammonium presulphate and 1.4 µl of TEMED per 1 ml of gel mixture. The gels were loaded in the glass tubes (12 mm x 2.5 mm inside diameter) and allowed to set for 2 h. They were prerun for ½ h at 200 V, ½ h at 400 V and 1 h at 600 V in the IEF apparatus. The lower reservoir was filled with 25 mM H_3PO_4 (anode electrode solution) and the upper reservoir was filled with 50 mM NaOH (kathode electrode solution). After the samples were loaded and the reservoirs were refilled, the gels were run for 20 h at 800 V. The gels were then equilibrated 2 x 15 min with gentle shaking in 125 mM TRIS.HCl, pH 6.8, 3% SDS, 50 mM DTT and 0.001% bromphenol blue and loaded on the second demension SDS-PAGE. SDS-PAGE was performed as described above (Laemmli, 1970). Staining of the gels was conducted in 40% methanol and 10% acetic acid with 0.25% Coomassie brilliant blue R. Destaining was carried out in 40% methanol and 10% acetic acid. Afterwards, silver staining was applied.

Silver staining.

Silver staining of 2D gels was performed by method of Morrissey (1981). Gels were fixed in 10% glutaraldehyde for 30 min, rinsed and left in a large volume of distilled water overnight. After reduction with DTT (5 µg/ml), gels were placed in 0.1% silver nitrate for 30 min and developed with developer mixture: 50 µl 37% formaldehyde in 100 ml 3% sodium carbonate until the desired

level of staining was attained. Staining was stopped by adding 5 ml of 2.5M citric acid directly to the developer and agitating for 10 min. Wet gels were photographed.

Incorporation of [^{35}S]-methioneine by isolated gills

Gills were isolated from the mussels exposed to cadmium for different periods of time. The middel parts of the outer gill lamella were incubated individually in 0.5 ml of standard medium (0.45 µ filtered sea water, 32 mM imidazol, pH 7.6, 25 µg/ml chloramphenicol and 1 µM each of 19 unlabeled amino acids) with 12.5 (or 25) µ Ci/ml [^{35}S]-methioneine (specific activity 1000 Ci/mmol, purchased form Amersham International). Incubations were carried out in the wells of multidishes (24 wells, Nucleon) for 4 h at room temperature, using rotation shaker plate (Brouwer Scientific). Incubations were stopped by placing on ice for 5 min. Gill tissues were removed from the incubation medium, rinsed and washed twice with distilled water. After centriguation for 2 min at 15,000 g at room temperature the supernatants were discarded. Pellets were weighed and resuspended 1:5 (w/v) in buffer: 20 mM TRIS.HCl, pH 7.1, 2 mM and 1 mM DTT. Pellets were disrupted by sonification 2x30 sec and recentrifuged at 16,000 g for 40 min at 4°C. A 10 µl aliquot of SN from each sample was used for protein determinations. The SN aliquots of 5 µl were transferred to Whatman 3MM filters to measure [^{35}S]-methioneine incorporation by hot 10% trichloroacetic acid precipitation. Radioactivity was determined in a Beckman LS 7500 liquid scintillation counter.

5.3 Results and Discussion

5.3.1 EFFECTS OF EXPOSURE TO 50 PPB CD ON THE PROTEIN PATTERNS IN DIFFERENT ORGANS OF *M. EDULIS*

One- and two-dimensional electrophoresis was employed to study the protein patterns with respect to Cd-exposure. SDS-PAGE separates the polypeptides strictly according to size, providing information about the molecular weight and the sub-unit composition of any protein complex. However, closely spaced protein bands tend to overlap, so that any one-dimensional separation can resolve only a relatively small number of proteins (generally fewer than 50). The most promising technique to study the interference of Cd with cellular proteins is two-dimensional gel electrophoresis. In two-dimensional electrophoresis, proteins are separated according to the isoelectric point by iso-

electrofocusing in the first dimension, and according to the molecular weight by SDS-PAGE in the second dimension. Due to the high resolution and sensitivity of this method, a protein which constitutes 10^{-4}-10^{-5}% of the total protein content in any biological system can be detected and quantified by fluorography (O'Farrel, 1975). In a recent review on metallothioneins, Hamer (1986) has pointed to the fact that a sensitive, rapid and high resolution method (e.g., two-dimentsional electrophoresis) is lacking to detect the metalloproteins (metalloenzymes) and that the development of such a methodology would revolutionize the field.

Initially, protein samples from different organs were analyzed by SDS-PAGE. After two weeks of Cd exposure protein patterns were identical in the gill, labial palp and PAM tissues (Fig. 5.1). The digestive gland sample from control group contained more protein bands with low molecular weight whereas the protein bands with higher molecular weight were more pronounced in the exposed group (Fig. 5.1). The observed shift of the protein bands in the digestive gland samples was presumably a result of proteolitic acitivity of the released digestive enzymes as no protease inhibitors were added to the samples. Moreover, no similar changes were found in the digestive gland protein patterns after 4 weeks of Cd-exposure (Fig. 5.2). Protein patterns of either gill or labial palp tissue were not affected by Cd-exposure for 4 weeks (Fig. 5.2). Additional band was observed at high molecular weight position (> 100 kD) in the sample of PAM proteins from exposed mussels whereas control samples contained an additional band at approximately 15 kD (Fig. 5.2).

The protein patterns were also studied by means of two-dimensional gel electrophoresis. The preparation of protein samples appeared to be one of the most important steps in 2D analysis. Protein samples from different organs were prepared by two methods (as described in Materials and Methods). Lysis of the total homogenate (method 1) resulted in poor resolution in both dimentsions. Lysates were obviously contaminated with membrane residues and high molecular weight molecules other than proteins. Large amount of sample remained on the origin of IEF gel and the majority of the protein spots were jammed, as shown for PAM tissue in Fig.6.3AB. That is why no comparison can be made between the protein patterns of muscle tissue from the exposed and control animals (Fig.5.3AB). Two-dimensional protein patterns of the gill and labial palp lysates showed similar poor resolution (results not shown).

Sample preparation by dialysis (method 2) conspicuously improved the 2D resolution (Figs.5.4AB - 5.7AB). The best resolution was achieved for PAM tissue proteins, probably due to the homogeneous structure of the organ (Fig.5.5AB). It was found that each organ has its own recognizable protein

pattern. The spot patterns were consistent from run to run, but not necessarily identical in every respect. Further optimalization and standardization of 2D gel patterns are in progress.

2D gel analysis of protein samples from PAM tissue revealed the differences in protein patterns after 2 and 4 weeks of Cd-exposure. As a rule, protein patterns from muscle tissue (as well as from other organs) of the exposed group contained more spots at various positions of the gel. The presence of the similar additional spots in the acidic, low molecular weight quadrant of the gel was observed in the muscle tissue after 2 weeks as well as after 4 weeks of Cd exposure (Figs.5.4AB - 5.5AB). After 2 weeks of Cd-exposure, several spots were more apparent in the exposed gill sample than the protein spots at the same position in the control gill sample. Gill protein sample from control group contained a protein at low molecular weight position which was not present in the pattern of the exposed group (Fig.5.6AB). After 2 weeks of Cd-exposure, labial palp protein sample from the exposed animals contained several proteins with molecular weight lower than 45 kD which were not detected in the protein pattern of the control group, whereas several proteins with higher molecular weight were more discernable in the control sample (Fig.5.7AB).

Alterations of protein patterns in response to short-term Cd-exposure imply that cadmium affects the cellular proteins. Two-dimensional gel analysis, followed by siver staining, is an appropriate method to reveal the total, drastic changes in protein patterns (*e.g.*, after long-term Cd-exposure), and to detect the presence or absence of proteins, but it does not highlight the changes in protein synthesis rate. Radioactive labeling of proteins, followed by gel analysis and fluorography, is required to assess the effect(s) of Cd on actual, *de novo* synthesis of proteins.

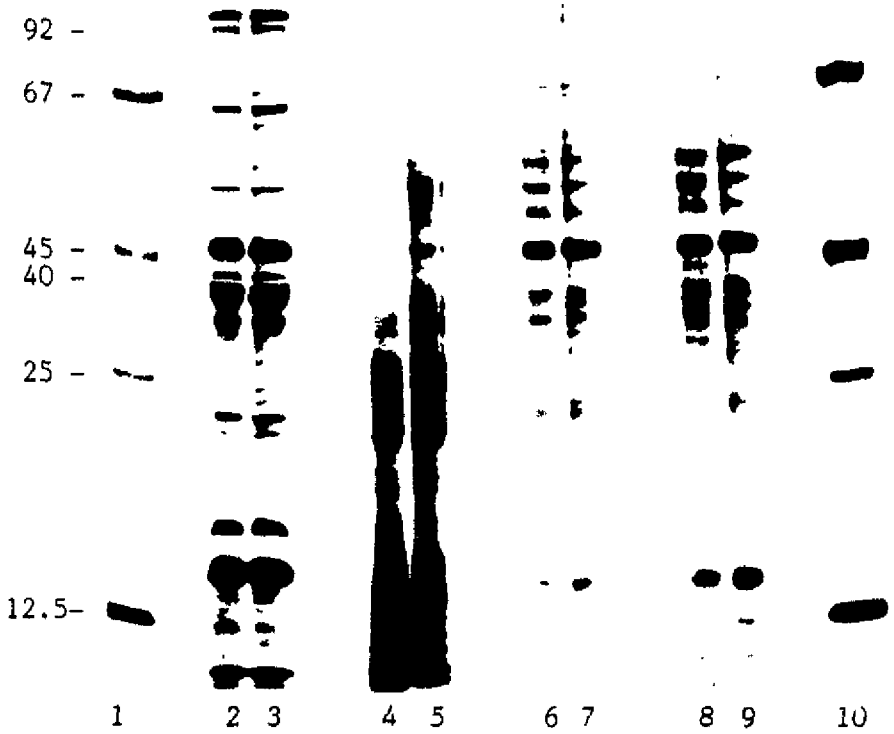

Fig.5.1. SDS-PAGE analysis of proteins from different organs of mussels exposed to 50 ppb Cd for 2 weeks. Lanes: 1 and 10 - molecular weight markers, Mr of markers are given in $\cdot 10^{-3}$; 2 - PAM from control group; 3 - PAM from exposed group; 4 - digestive glands from control group; 5 - digestive glands from exposed group; 6 - labial palps from control group; 7 - labial palps from exposed group; 8 - gills from control group; 9 - gills from exposed group.

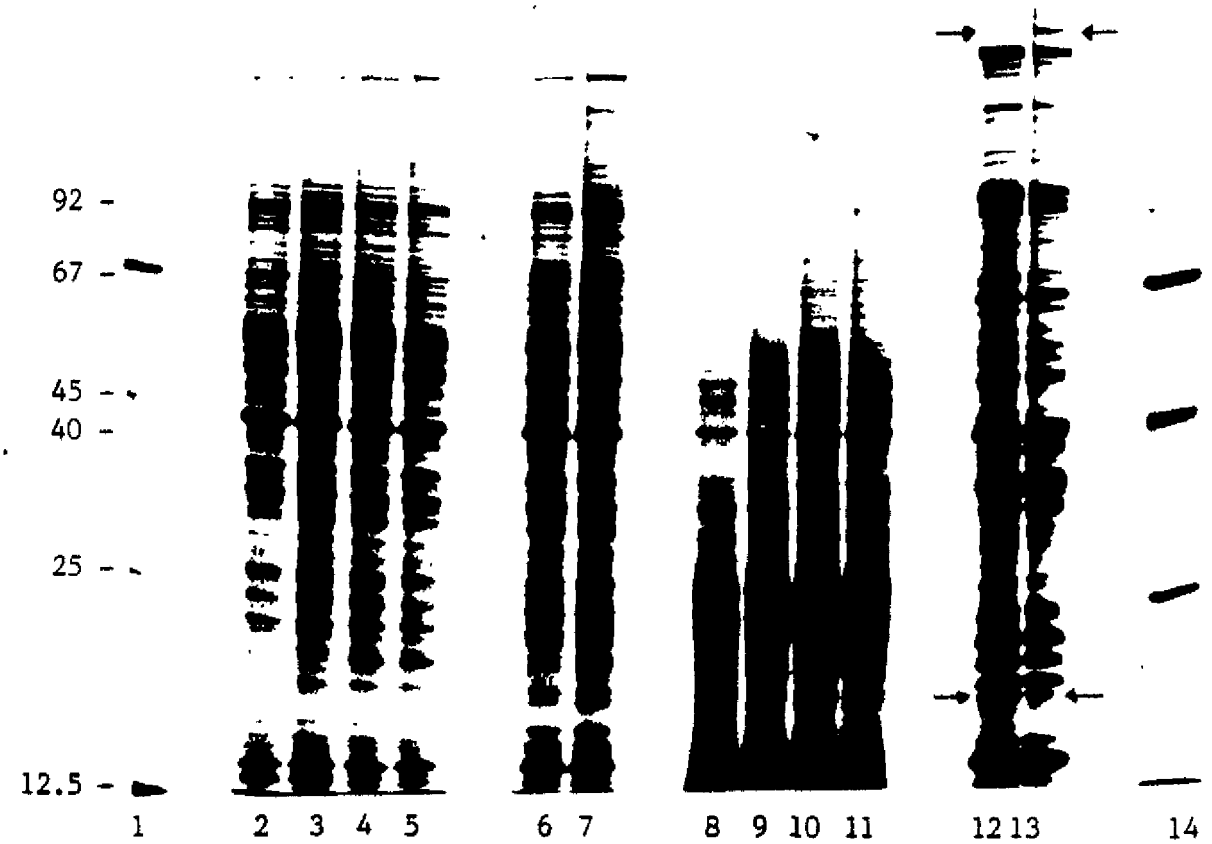

Fig.5.2. SDS-PAGE analysis of proteins from different organs of mussels exposed to to 50 ppb Cd for 4 weeks. Lanes: 1 and 14 - molecular weight markers, Mr of markers are given in $\times 10^{-3}$; 2 and 3 - gills from control group; 4 and 5 - gills from exposed group; 6 - labial palps from control group; 7 - labial palps from exposed group; 8 and 9 - digestive glands from control group; 10 and 11 - digestive glands from exposed group; 12 - PAM from control group; 13 - PAM from exposed group. The arrows indicate the differences between proteins from control and exposed groups.

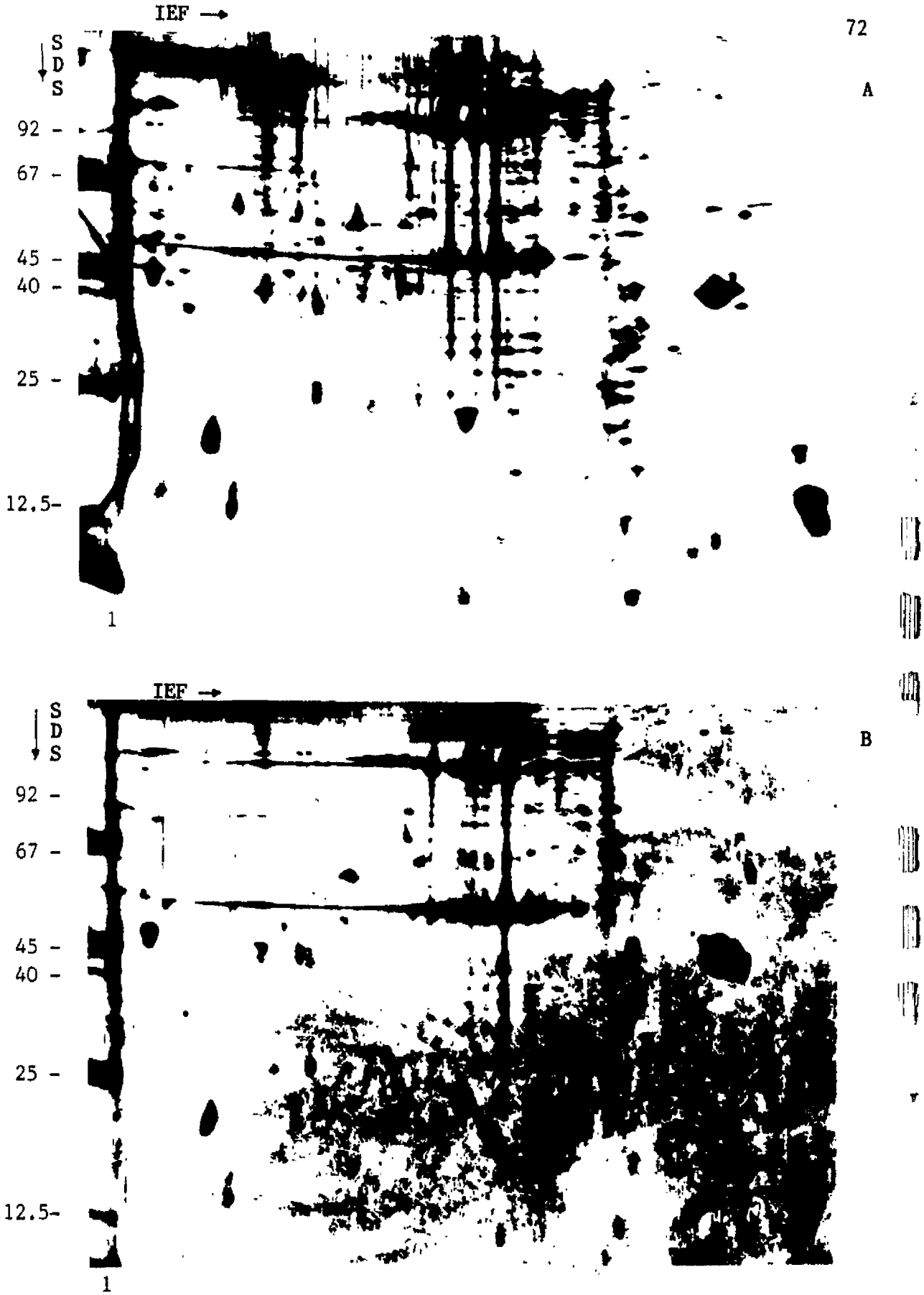

Fig.5.3. Two-dimensional IEF/SDS-PAGE patterns of PAM proteins from mussels exposed to 50 ppb Cd for 2 weeks. A - exposed group; B - control group. Lane 1 - molecular weight markers, Mr of markers are given in $\times 10^{-3}$. Protein samples were prepared by method 1 (lysis).

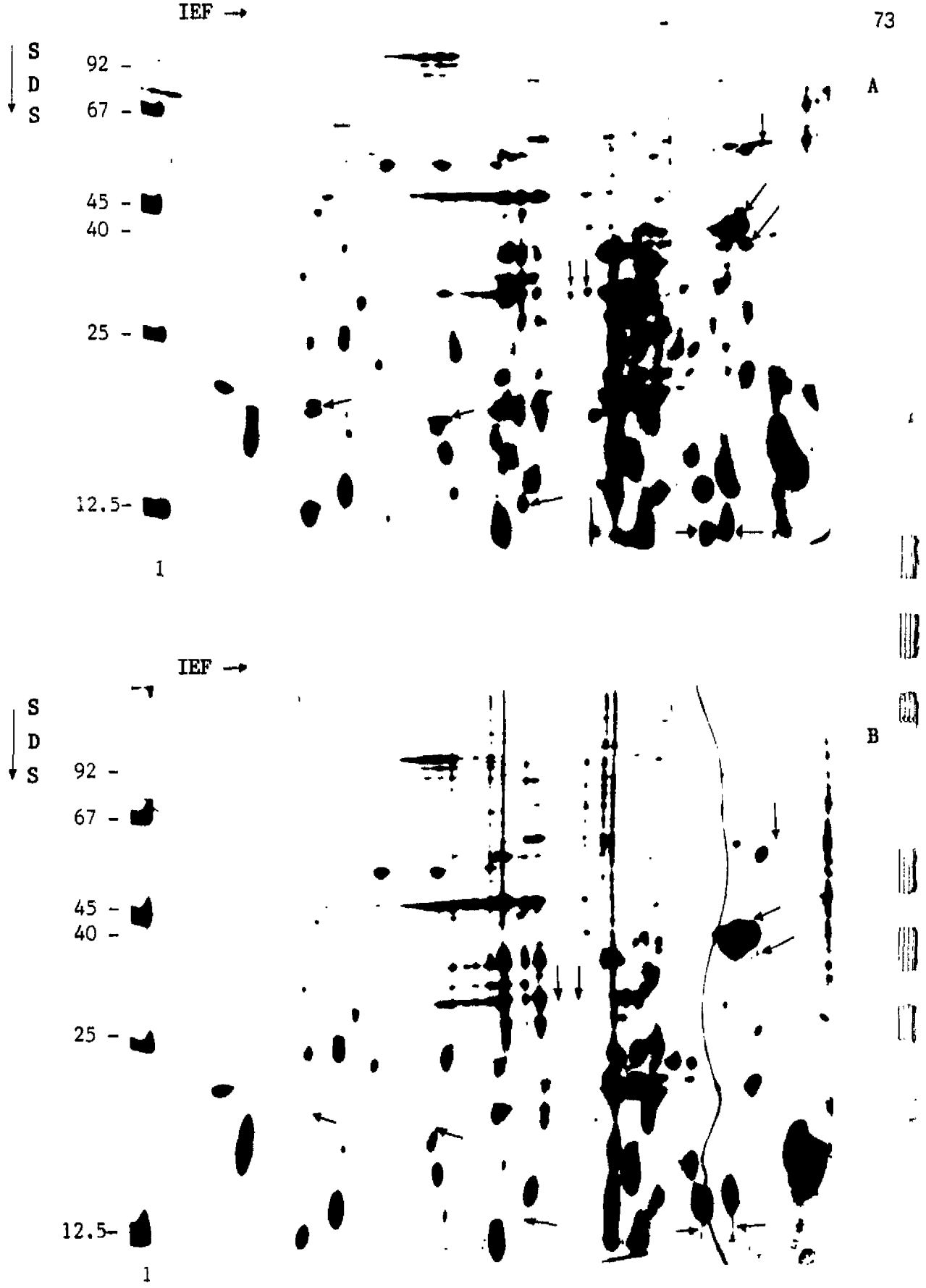

Fig.5.4. Two-dimensional IEF/SDS-PAGE patterns of PAM proteins from mussels exposed to 50 ppb Cd for 2 weeks. A - exposed group; B - control group. Lane 1 - molecular weight markers, Mr of markers are given in $* 10^{-3}$. Protein samples were prepared by method 2 (dialysis). Arrows indicate the differences in protein patterns between the control and exposed groups.

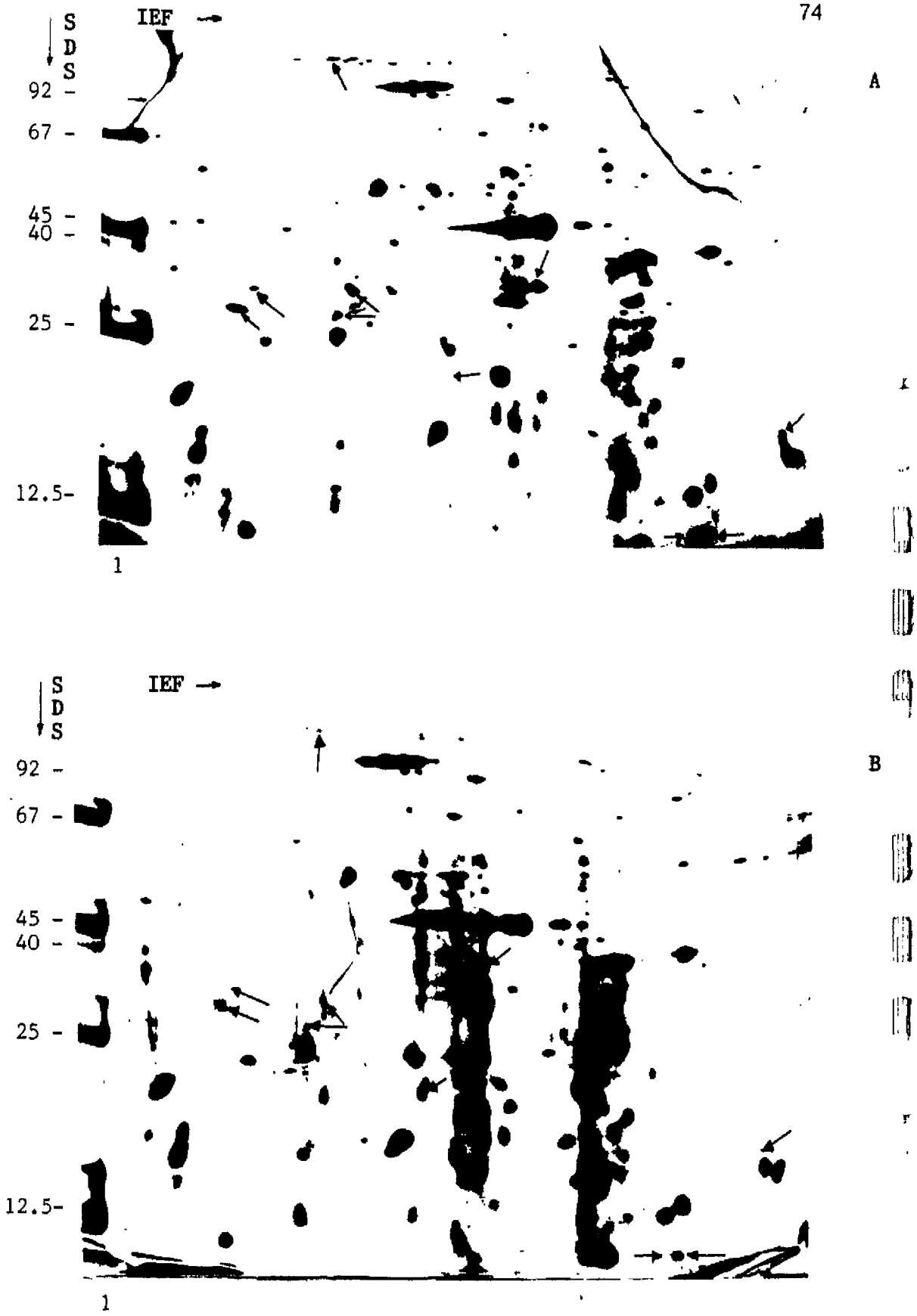

Fig.5.5. Two-dimensional IEF/SDS-PAGE patterns of PAM proteins from mussels exposed to 50 ppb Cd for 4 weeks. A - exposed group; B - control group.Lane 1 - molecular weight markers, Mr of markers are given in $\times 10^{-3}$. Protein samples were prepared by method 2 (dialysis). Arrows indicate the differences in protein patterns between the control and exposed groups.

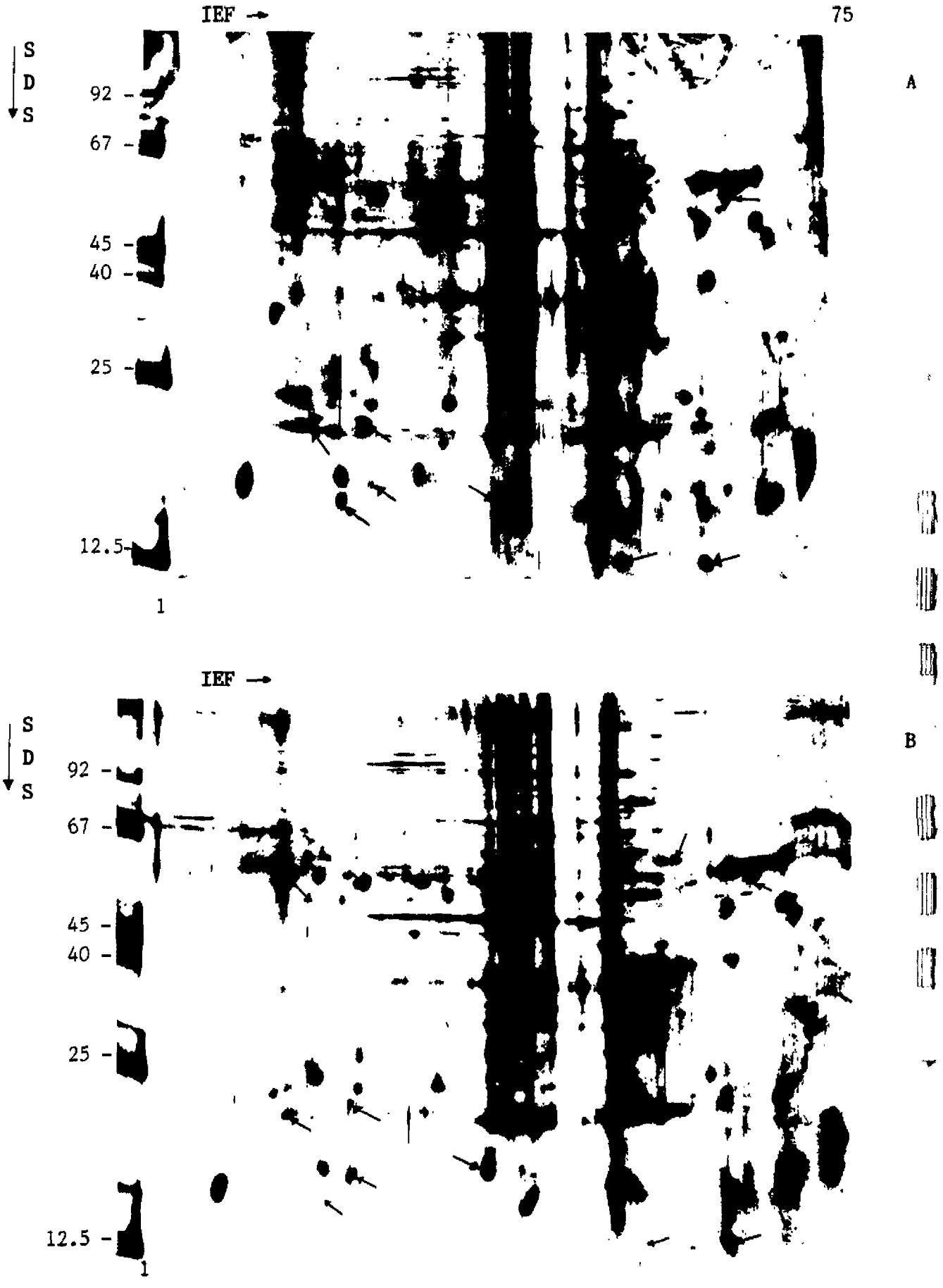

Fig.5.6. Two-dimensional IEF/SDS-PAGE patterns of gill proteins from mussels exposed to 50 ppb Cd for 2 weeks. A – exposed group; B – control group. Lane 1 – molecular weight markers, Mr of markers are given in x 10^{-3}. Protein samples were prepared by method 2 (dialysis). Arrows indicate the differences in protein patterns between the control and exposed groups.

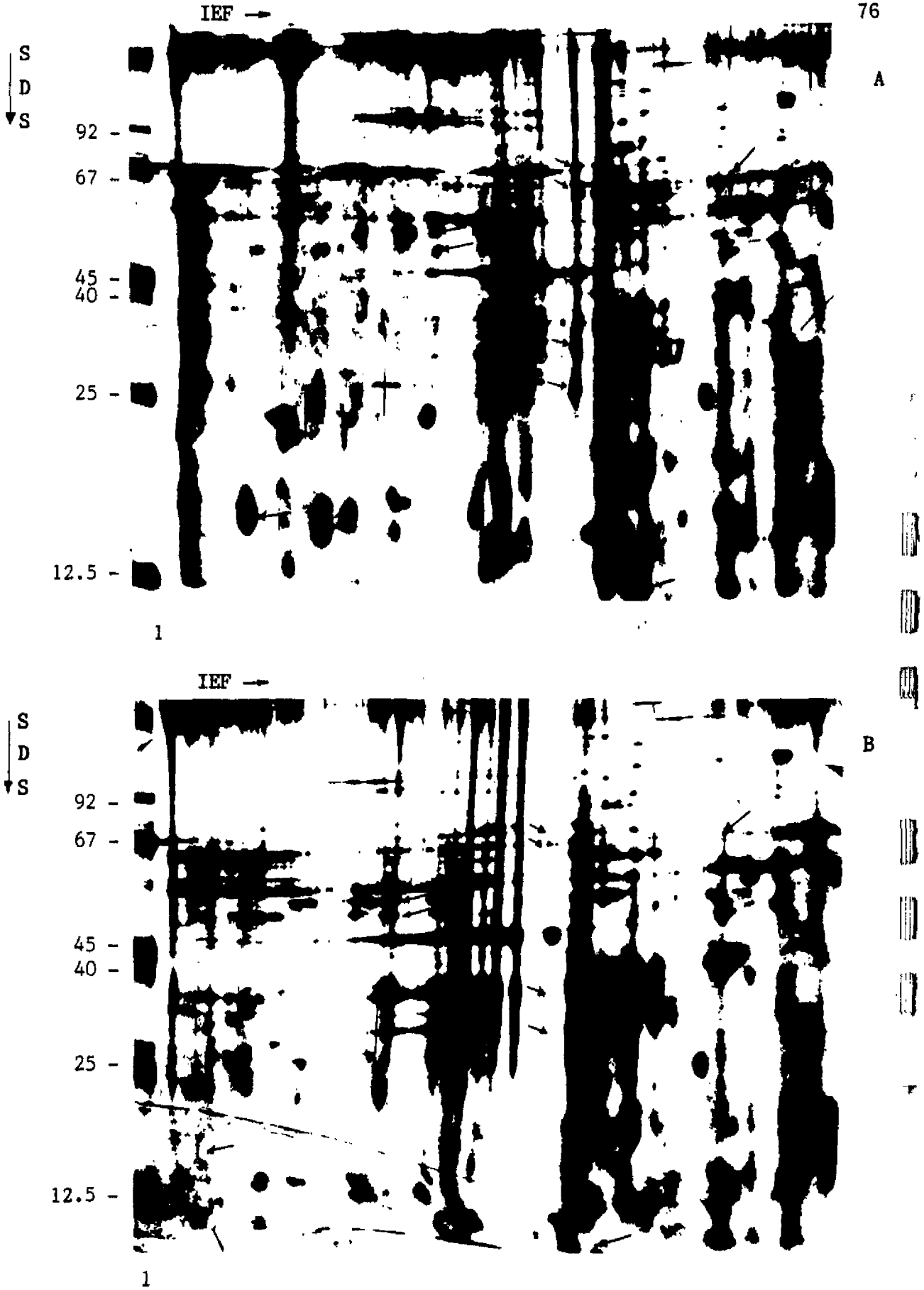

Fig.5.7. Two-dimensional IEF/SDS-PAGE patterns of labial palp proteins from mussels exposed to 50 ppb Cd for 2 weeks. A - exposed group; B - control group. Lane 1 - molecular weight markers. Mr of markers are given in $\times 10^{-3}$. Protein samples were prepared by method 2 (dialysis). Arrows indicate the differences in protein patterns between the control and exposed groups.

5.3.2 EFFECTS OF CD ON [^{35}S]-METHIONEINE INCORPORATION BY THE ISOLATED GILLS OF *M. EDULIS*

To assess the possible effects of Cd on the protein synthesis, the experiments were carried out with isolated gills from mussels exposed to Cd for short periods of time. The gills were chosen for these experiments since they are ideally constructed for an absorbtive role, having a high surface area, a cell monolayer, rich vascularization, and are known to accumulate cadmium (George and Coombs 1977; Carpené and George 1981). Moreover, morphological Cd-induced changes have been found in molluscan gill tissue (Engel and Fowler 1979; Sunila 1981; Sunila and Lindström 1985) which are apparently linked to physiological and biochemical alterations.

In preliminary experiments, the optimal conditions for radioactive label incorporation by the isolated gills were investigated. Fig.5.8AB shows that isolated gills incorporated [^{35}S]-methioneine during 24 h. In the same figure can be seen that variations between the individual mussels can be more than 50%, as each line presents the incorporation values of gills derived from one animal. Fig.5.9AB depicts the temperature effect, indicating the decrease (over 30%) in label incorporation as temperature was lowered from 22°C to 12°C. Additions of chloramphenicol (inhibitor of bacterial protein synthesis) and 19 unlabeled amino acids to the incubation medium had a positive effect on the label incorporation (results not shown). The incorporation rate was dependant on the label concentration added to medium (Fig.5.10AB). Maximum of label incorporation was achieved after 4 h of incubation in standard medium at room temperature (Fig.5.11AB).

Considering the results of preliminary experiments, the incubations of gills from Cd-exposed and control animals were carried out in standard medium with 12.5 (or 25) µCi/ml [^{35}S]-methioneine at room temperature for 4 h. Fig.5.12AB summarizes the incorporation rate values in gill tissue after exposure to 50 ppb Cd for different periods of time. Three and 22 days of Cd exposure caused a slight (not significant) increase in the incorporation rate. After 7 days of Cd-exposure, no differences in the label incorporation were observed. The incorporation rate in the gills from exposed group was significantly ($p < 0.05$) increased after 15 days of Cd-exposure. After 1 week of exposure to 50 ppb Cd, a group of mussels was subjected to an additional metal stress, while kept in 1 ppm Cd for 16 h. Fig.5.13AB shows that this group had lower incorporation rate values, but the difference was significant ($p < 0.05$) only when compared to the values of the control group. The radio-labeled proteins derived from the latter experiment were analysed by SDS-PAGE and fluorography (re-

sults not yet ready).

The exposure to higher Cd concentration (250 ppb) for 7 days caused a significant ($p < 0.05$) decrease (29.7%) in the incorporation rate in the gills from the Cd-exposed animals (Fig.5.14). As exposure to 250 ppb Cd was extended to 15 days, the incorporation rate in the gills from the exposed group continued to decline, attaining a signficant ($P < 0.001$) decrease of 37.3% (Fig.5.14). The radio-labeled proteins obtained from these experiments will be subjected to one- and two-dimensional analysis.

As Cd-exposure has significantly altered the incorporation rate, it can be concluded that cadmium affects the protein synthesis in the gills of *M. edulis*. The enhancement of protein synthesis at relatively low Cd concentration (50 ppb), which corresponds with the presence of the additional spots in protein pattern of the gills from Cd-exposed mussels (Fig.5.6A), and inhibition of protein synthesis at higher Cd concentrations (250 ppb, 1 ppm) fit the concept that at low concentrations cadmium exerts beneficial effects, but at higher concentrations these effects become inhibitory and eventually toxic (Simkiss *et al.*, 1982). No more conclusions can be drawn since the experiments are not completed. The observed effects of Cd on protein synthesis have given rise to a number of questions: Does cadmium interfere with protein synthesis *sec* or does it exert its effects at the level of transcription? The second question to be answered is, does Cd influence the overall protein synthesis or does it affect the synthesis of particular proteins? Does the affected protein synthesis underlie the metabolic processes which led to diminished anoxia survival of Cd-exposed mussels (Chapter 2)? Therefore, further investigation of cellphysiological effects of Cd is required.

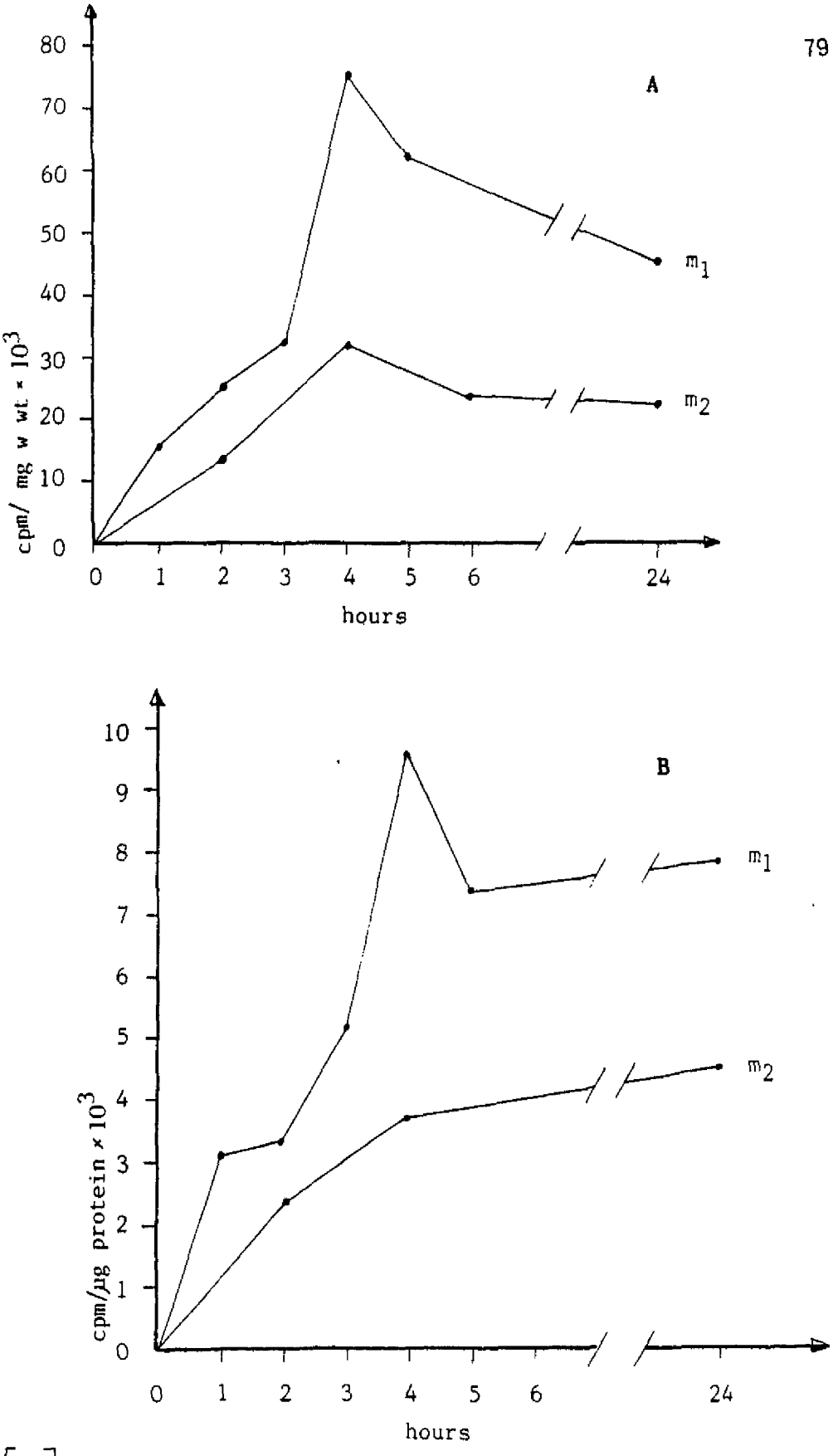

Fig.5.8. $[^{35}S]$-methionine incorporation by the isolated gills. Gills were isolated from two mussels (m_1 and m_2) and incubated with 25 µCi $[^{35}S]$ -methionine at 25°C. The incorporated radioactivity is expressed as cpm/mg wet weight (A) and cpm/µg supernatant protein(B).

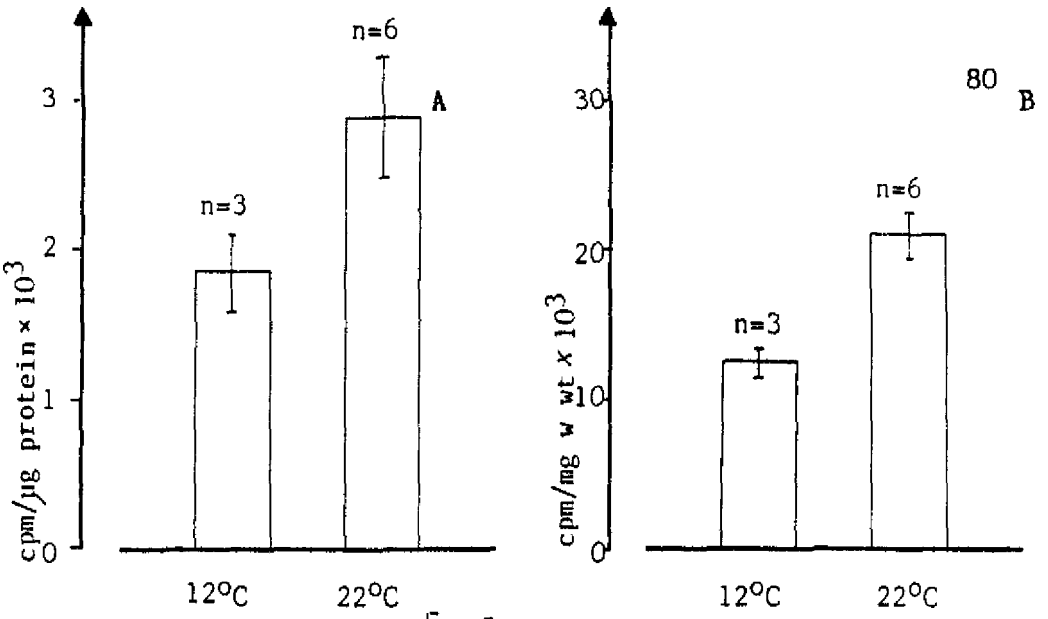

Fig.5.9. Temperature effect on [^{35}S]-methionine incorporation. Isolated gills were incubated with 12.5 µCi [^{35}S]-methionine for 16 h. The incorporated radioactivity is expressed as cpm/µg supernatant protein (A) and cpm/mg wet weight (B). Mean of n animals ± SEM.

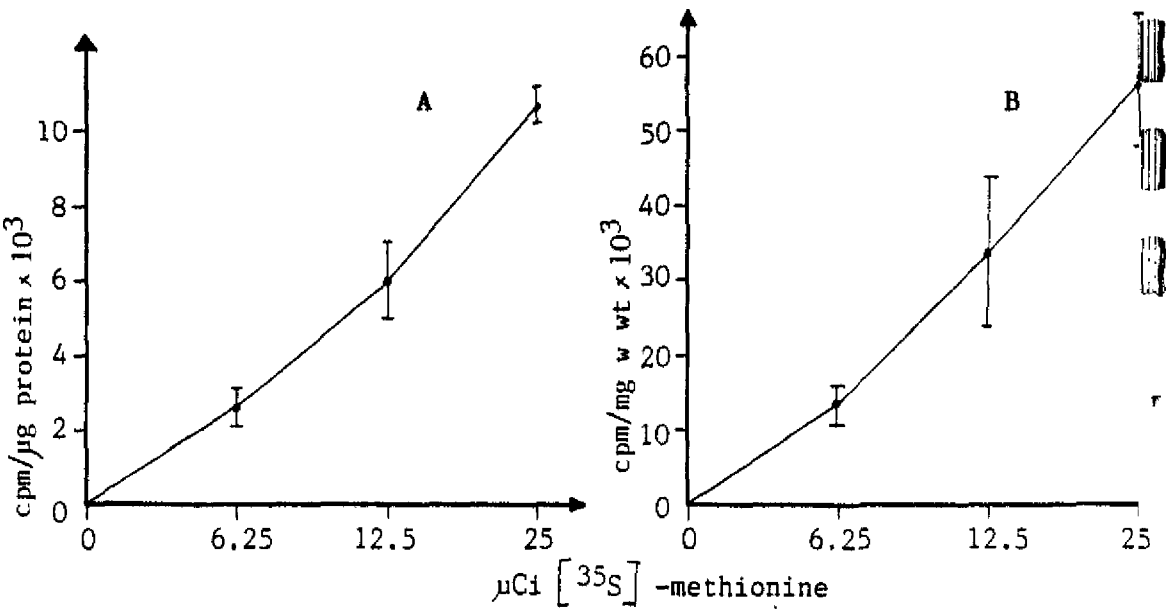

Fig.5.10. Incorporation rate vs label concentration in the incubation medium. Isolated gills were incubated for 21 h at 20°C. The incorporated radioactivity is expressed as cpm/µg supernatant protein (A) and cpm/mg wet weight (B). Mean of 3 animals ± SEM.

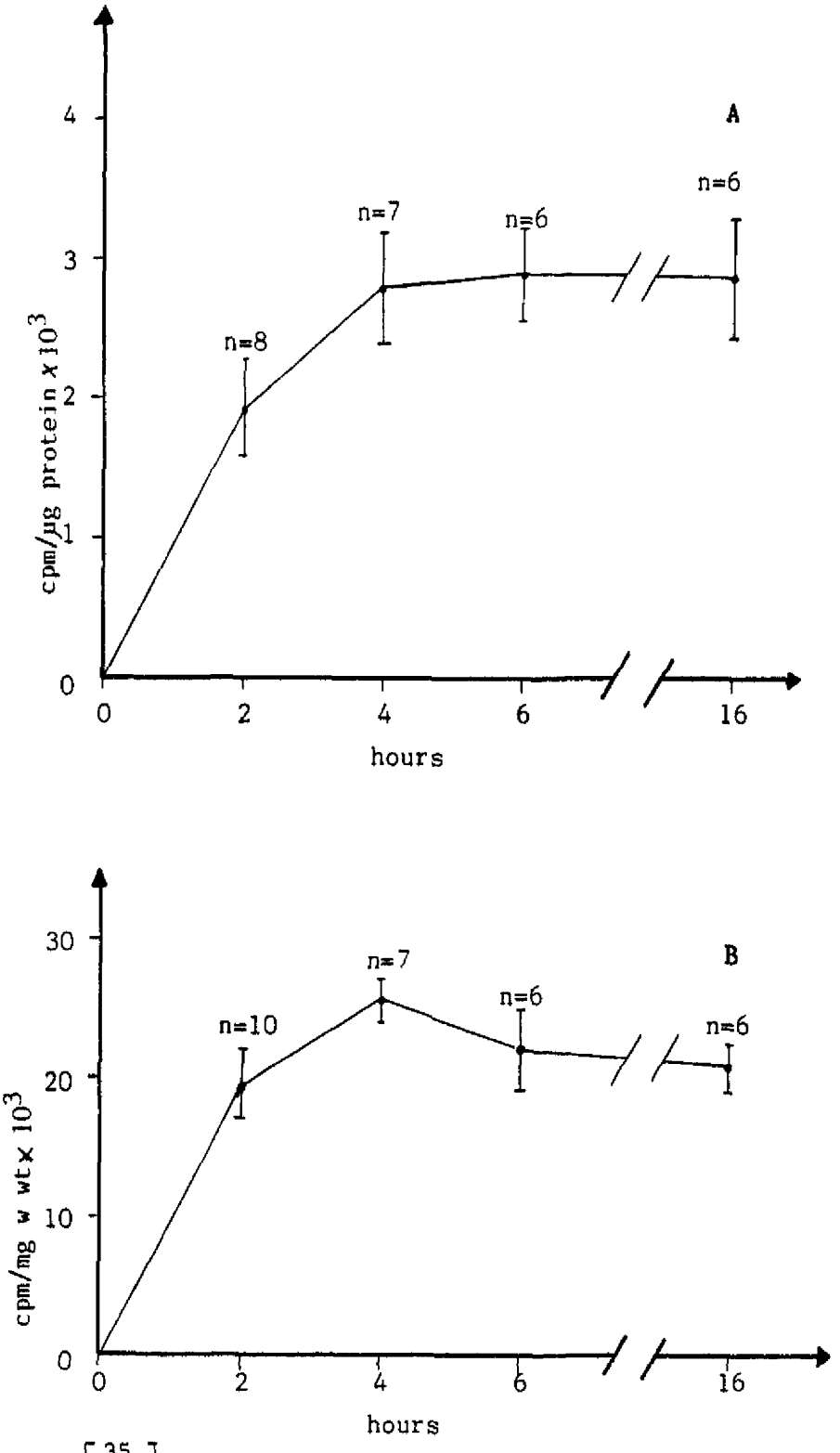

Fig.5.11. [^{35}S]-methionine incorporation **vs** incubation time. Isolated gills were incubated with 12.5 µCi [^{35}S] -methionine at 22.5°C. The incorporated radioactivity is expressed as cpm/µg supernateant protein (A) and cpm/mg wet weight (B). Mean of n mussels ± SEM.

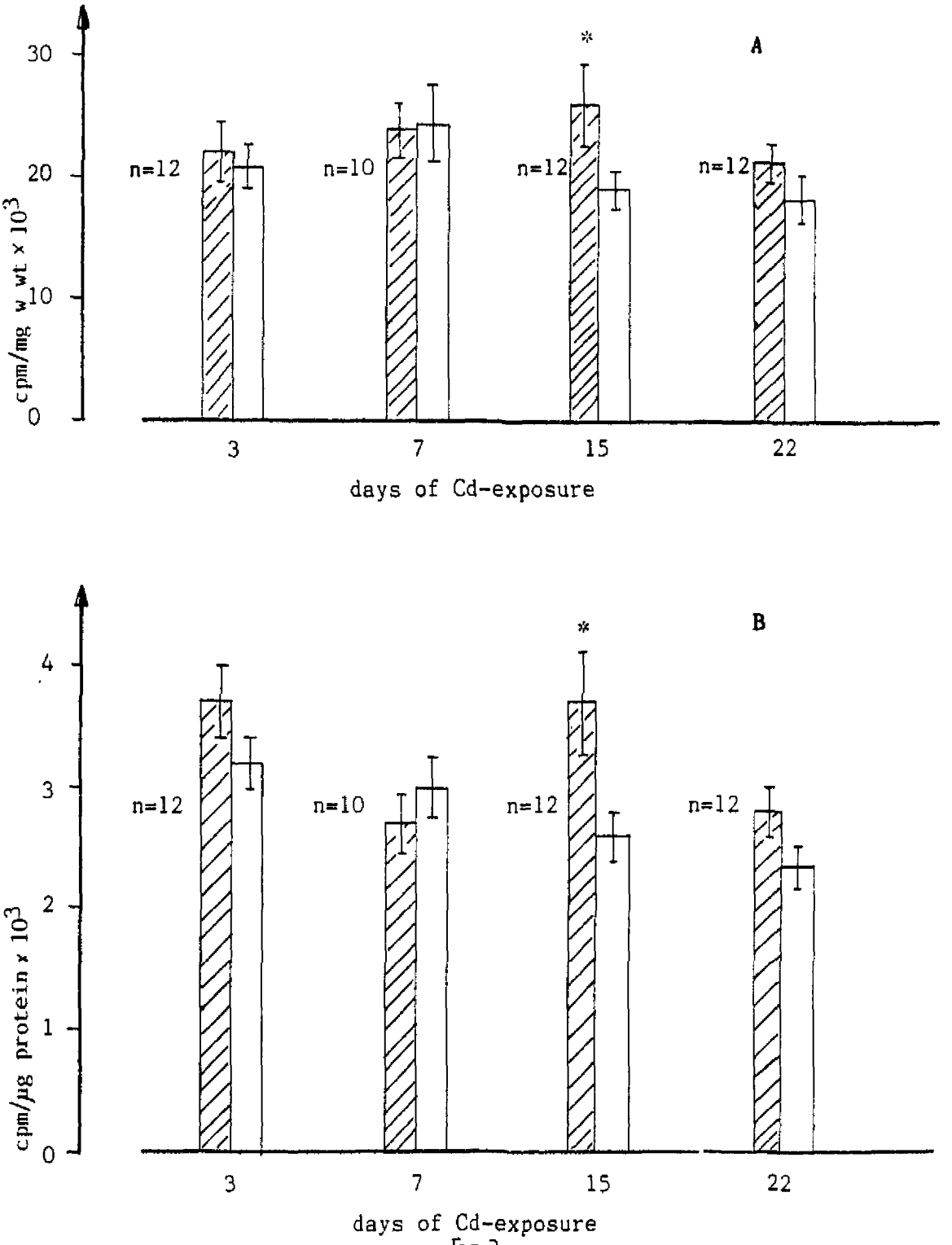

Fig.5.12. Effect of cadmium on the $[^{35}S]$ -methionine incorporation rate. Isolated gills were incubated with 12.5 µCi $[^{35}S]$ -methionine for 4 h at 21 - 22°C. ▨ - gills from mussels exposed to 50 ppb Cd. □ - gills from control animals. The incorporated radioactivity is expressed as cpm/mg wet weight (A) and cpm/µg supernatant protein (B). Mean of n animals ± SEM.

*Statistically significant difference when compared with the values of control mussels ($p < 0.05$).

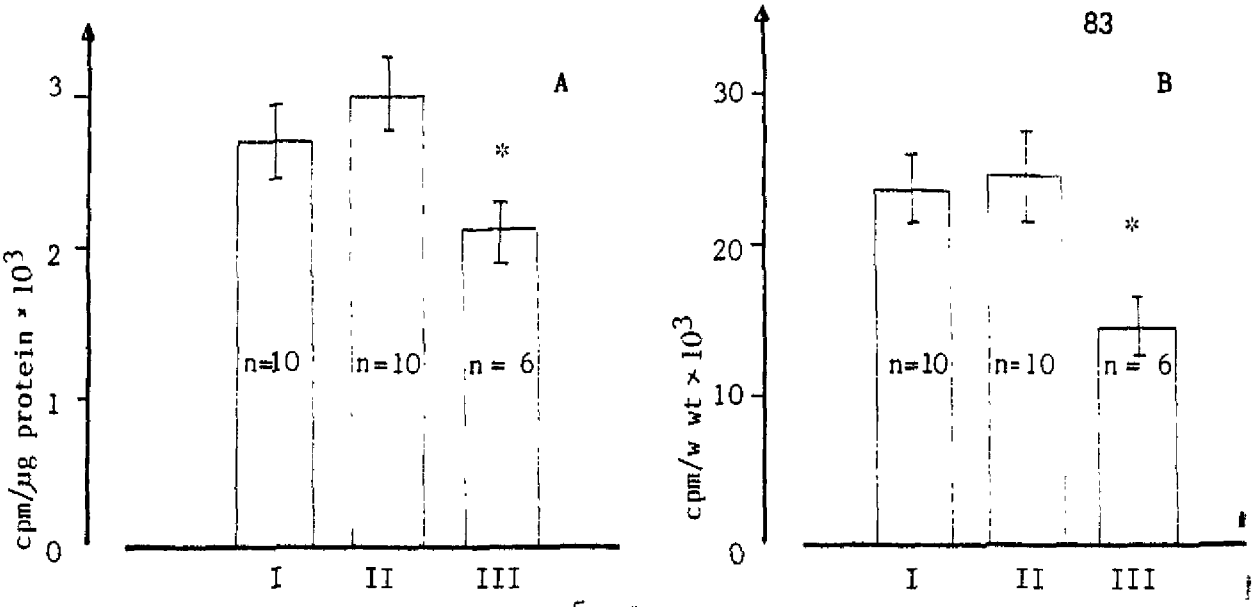

Fig.5.13. Effect of cadmium on the $[^{35}S]$ -methionine incorporation rate. Isolated gills were incubated with 12.5 µCi $[^{35}S]$ -methionine for 4 h at 22°C. I - gills from mussels exposed to 50 ppb Cd for 7 days; II - gills from control animals; III - gills from mussels exposed to 50 ppb Cd for 7 days and afterwards to 1 ppm Cd for 16 h. The incorporated radioactivity is expressed as cpm/µg supernatant protein (A) and cpm/mg wet weight (B). Mean of n animals ± SEM.

Fig.5.14 Effect of Cd on the $[^{35}S]$-methionine incorporation rate. Isolated gills were incubated with 25 µCi $[^{35}S]$ -methionine for 4 h at 20°C. ▨ - gills from mussels exposed to 250 ppb Cd; □ - gills from control mussels. The incorporated radioactivity is expressed as cpm/mg wet weight. Mean of 10 animals ± SE..

*Statistically significant difference when compared with the values of control mussels ($p < 0.05$).

5.4 Abbreviations

cpm	counts per minute
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
IEF	Isoelectrofocusing
kD	kilodalton
M_r	Molecular ratio
mRNA	messenger ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
PAM	posterior adductor muscle
SDS	Sodium dodecyl sulfate
TRIS	Tris(hydroxymethol)-aminomethane

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