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TOXICANTS AND OSMOREGULATION IN FISH

by

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ABSTRACT

Fish are extremely sensitive to many water-borne toxicants, because these affect the gills by increasing the permeability to water and ions of the gill epithelium and by inhibition of the ion exchange activity of the chloride cells. The compensatory responses of the fish will significantly increase the energy required for maintenance of water and ion homeostasis, and this will result in reduced growth and reproduction. The effects of toxicants are to a great extent comparable to those of stressors such as confinement, transport, and handling, not only where the endocrine and metabolic responses are concerned, but also with respect to the osmoregulatory disturbances produced. Stressors may affect osmoregulation indirectly through the action of catecholamines on the gills. Furthermore, stressors induce immunosuppression and this may result in gill damage by infectious agents. Many toxicants evoke a stress response, and thus it is difficult to determine the mechanism of action of toxicants on the gills, because the specific effects of the toxicants are hard to distinguish from the effects of non-specific stress responses on the gills. This further implies that the negative effects of many toxicants and non-toxicant stressors on gill structure and hydromineral balance are additive. This aspect needs more attention in aquaculture.

KEY WORDS: chloride cells, fishes, gills, ion fluxes, osmoregulation, stressors, stress adaptation, toxic metals.

INTRODUCTION

Toxic substances (toxicants) may affect the physiological functions of aquatic organisms in a variety of ways. In fish, for example, many water-borne toxicants commonly disturb water and ion homeostasis, in particular Na^+ , Cl^- and Ca^{2+} , of the blood plasma. These changes are mainly caused by a damaged structure of the gills affecting processes such as water and ion exchange, respiratory gas exchange, acid-base balance, and the excretion of waste products. In addition, gills are an important route for the uptake, biotransformation and excretion of toxicants (EVANS, 1987). In this respect gills combine the function of lungs with some of the functions of kidneys and intestine in terrestrial vertebrates. To this end, the numerous gill lamellae, constituting up to 90% of the total body surface, are covered with a thin multifunctional epithelial layer. This layer contains, besides the respiratory or pave-

ment cells, also specialized cells for ion transport (chloride cells) and mucus secretion. To maintain water and ion homeostasis of the body fluids, the fish produce relatively large volumes of dilute urine. To compensate the excretory and diffusional ion losses—and to eliminate metabolic waste products—fish accumulate ions such as Na^+ Cl^- in exchange for NH_4^+ , H^+ and HCO_3^- . The chloride cells and, possibly, the gill lamellae are implicated in these processes (EVANS, 1987; MAYER-GOSTAN *et al.*, 1987). In spite of their great physiological importance, gills are delicate structures, vulnerable to all kinds of environmental influences: physical changes of the water, microorganisms, and organic pollutants (EDDY, 1981). In particular, gill lamellae and chloride cells are the targets, representing the weak spots in the integument of fish. Any damage to the gills will have immediate effects on ion homeostasis and will evoke compensatory osmoregulatory responses. As a consequence the metabolic costs to freshwater fish of maintaining water and ion homeostasis, which are already much higher in fish than in terrestrial animals (FURSPAN *et al.*, 1984), will increase rapidly. If the disturbance is chronic, this will have negative effects on growth and reproduction.

In this paper we shall briefly analyze the common structural and functional aspects of the interaction of some aquatic toxicants, in particular heavy metals, with the gills of freshwater teleost fish. We make a comparison of the particular effects of these toxicants with the effects of other water pollutants, and stressors such as handling or confinement. Finally, we discuss to what extent the osmoregulatory responses of fish to toxicants can be considered part of a “general adaptation syndrome” of fish to stressors.

EFFECTS OF TOXICANTS ON THE GILLS

In an extensive review of the literature, MALLATT (1985) summarized the main structural damage to the gills by aquatic irritants, including many toxic substances, as follows: uplifting of the lamellar epithelium from the underlying tissue (fig. 1, 2), necrosis of pavement cells and chloride cells, epithelial swelling by increased intercellular spaces, rupture of the epithelium, and lamellar fusion. This damage was often accompanied by hyperplasia and hypertrophy of pavement cells and chloride cells, and by leucocyte infiltration of the branchial epithelium. A short survey of the same and more recent literature concerning the effects of toxic substances on freshwater fish shows that, as far as measured, the above disturbances of gill structure were invariably accompanied by a drop in the electrolyte concentrations of the blood

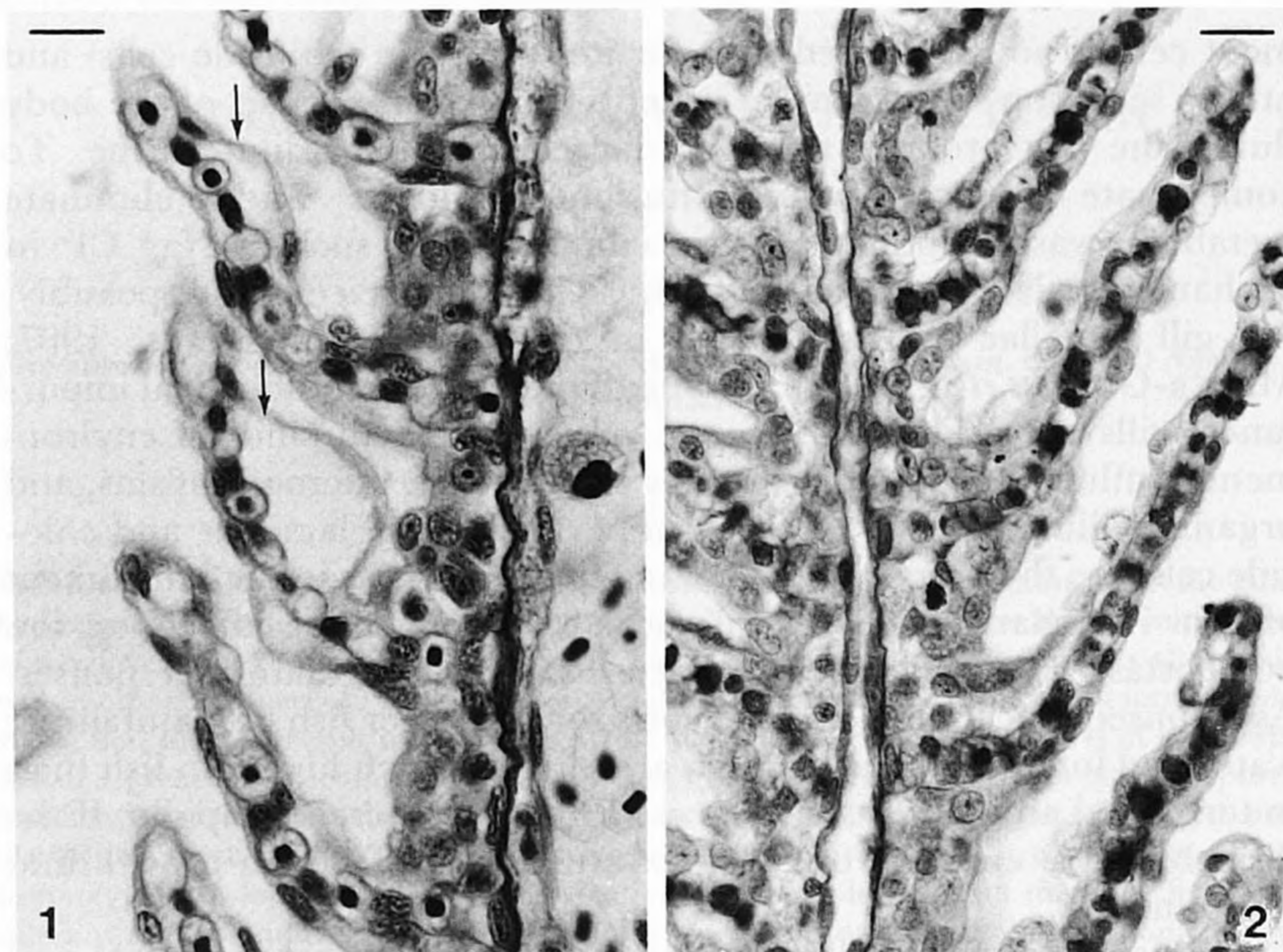


Fig. 1. Branchial filament with lamellae of rainbow trout (*Oncorhynchus mykiss*) exposed for 7 days to mercuric chloride (100 µg/l); the lamellae show epithelial uplifting (arrows); bar represents 10 µm.

Fig. 2. Branchial filament of control rainbow trout; bar represents 10 µm.

plasma, in particular Na^+ and Cl^- . Electrolyte losses have been described for instance after exposure of fish to pesticides, chlorinated hydrocarbons (*e.g.* HAUX & LARSSON, 1979; MALLATT & STINSON, 1990), and heavy metals (LOCK *et al.*, 1981; SPRY & WOOD, 1985; REID & McDONALD, 1988; FU *et al.*, 1989). In fig. 3 a concentration-dependent reduction of plasma electrolytes in the cichlid fish *Oreochromis mossambicus* is shown as obtained in our studies on the effects of cadmium.

There is consensus now that the drop of plasma electrolyte levels has two important causes. First, there is an increased passive efflux of ions across the gills, due to a more or less non-selective rise of the permeability of the gills, especially the gill lamellae, to water and ions. This may lead to hemodilution by enhanced osmotic uptake of water across the gills, and to increased losses of ions associated with the stimulation of urine flow. Second, the inhibition of active ion uptake by the chloride cells of the gills may further contribute to the negative ion balance of the blood.

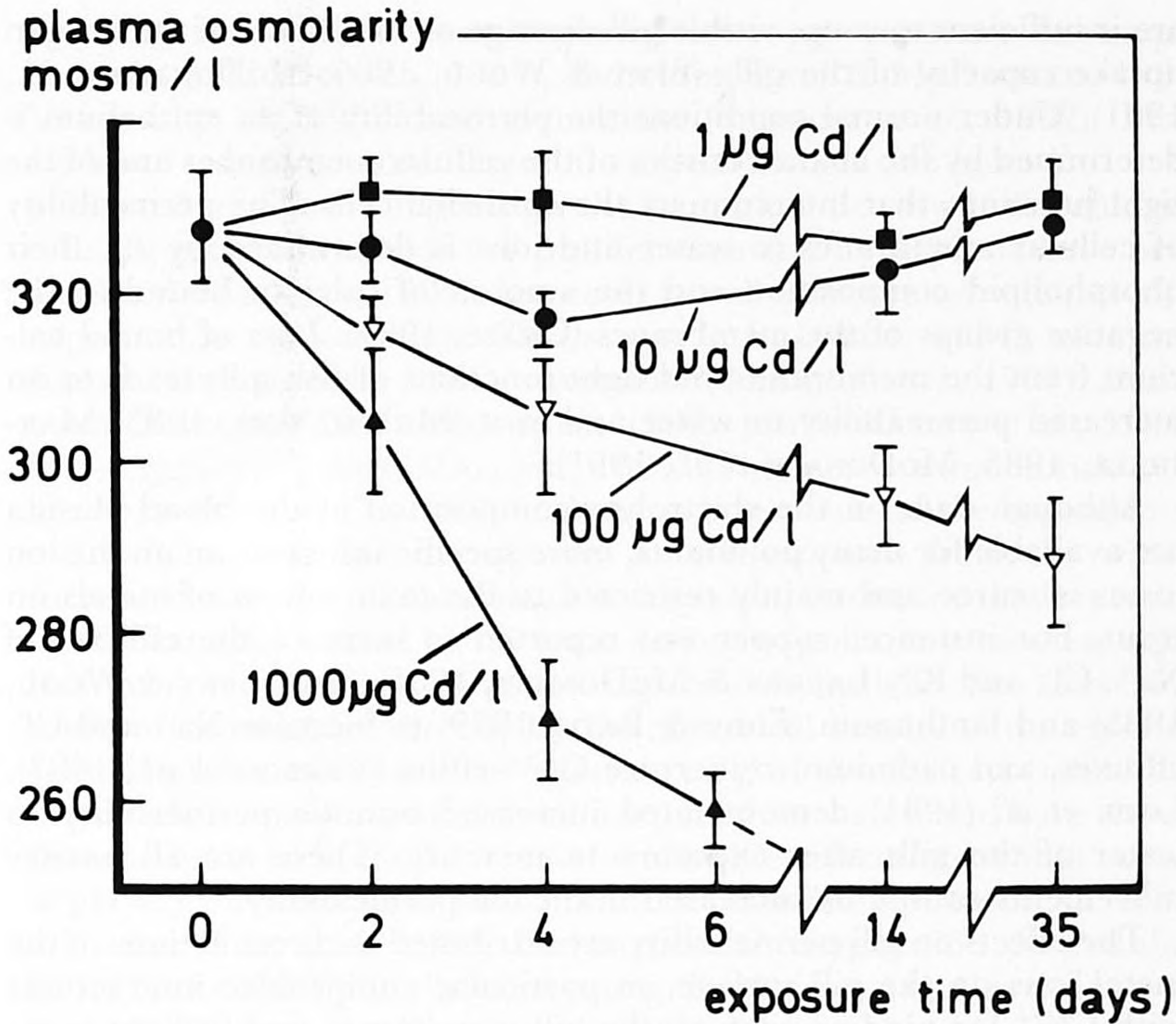


Fig. 3. Plasma osmolarity of freshwater *Oreochromis mossambicus* after exposure to indicated concentrations of water-borne cadmium; means \pm S.E. of 8 fish; at 1000 $\mu\text{g}/\text{l}$ all fish died within 8 days (from: FU *et al.*, 1989).

EFFECTS ON GILL PERMEABILITY

The increase in permeability of the gills to ions is considered the most important factor for the drop in plasma electrolyte levels caused by heavy metals. Structural lesions of the gills certainly will contribute to the increase in permeability. However, when epithelial uplifting and necrosis become visible at the light microscopic level, the fish most probably have already been very seriously affected and suffer from a disturbed osmoregulation and an impaired oxygen uptake capacity. Indeed, in our experiments with cadmium, copper, and aluminium in acid water, we already found losses of plasma electrolytes before any light microscopic lesions became visible (PRATAP & WENDELAAR BONGA, 1992). Other investigators have also reported that loss of plasma electrolytes may occur at concentrations or exposure times that

are insufficient to cause visible gill damage or inhibition of the oxygen uptake capacity of the gills (SPRY & WOOD, 1985; McDONALD *et al.*, 1991). Under normal conditions the permeability of an epithelium is determined by the characteristics of the cellular membranes and of the tight junctions that interconnect the epithelial cells. The permeability of cellular membranes to water and ions is determined by *e.g.* their phospholipid composition and the amount of calcium bound to the negative groups of the membranes (CHASE, 1984). Loss of bound calcium from the membranes and tight junctions of fish gills leads to an increased permeability to water and ions (McWILLIAMS, 1983; MARSHALL, 1985; McDONALD *et al.*, 1991).

Although data on the electrolyte composition of the blood plasma are available for many pollutants, more specific information on the ion losses is scarce and mainly restricted to the toxic effects of metals on trout. For instance, copper was reported to increase the effluxes of Na^+ , Cl^- and K^+ (LAURÉN & McDONALD, 1985), zinc (SPRY & WOOD, 1985) and lanthanum (EDDY & BATH, 1979) to increase Na^+ and Cl^- effluxes, and cadmium to increase Ca^{2+} -efflux (VERBOST *et al.*, 1987). LOCK *et al.* (1981) demonstrated increased osmotic permeability to water of the gills after exposure to mercury. These are all passive movements caused by increased branchial permeability.

The effects on gill permeability are attributed to direct actions of the metal ions on the gill surface, in particular competitive interactions with Ca^{2+} for binding sites on the gill membranes and in tight junctions (NIEBOER & RICHARDSON, 1980; LAURÉN & McDONALD, 1986). This explanation is supported by the well-known protective effect of high water calcium levels on heavy metal toxicity (LAURÉN & McDONALD, 1985; PRATAP *et al.*, 1989). Calcium ions can effectively compete with heavy metals such as cadmium for binding sites on membrane phospholipids (SORENSEN *et al.*, 1985) and regulatory proteins such as calmodulin (FLIK *et al.*, 1987).

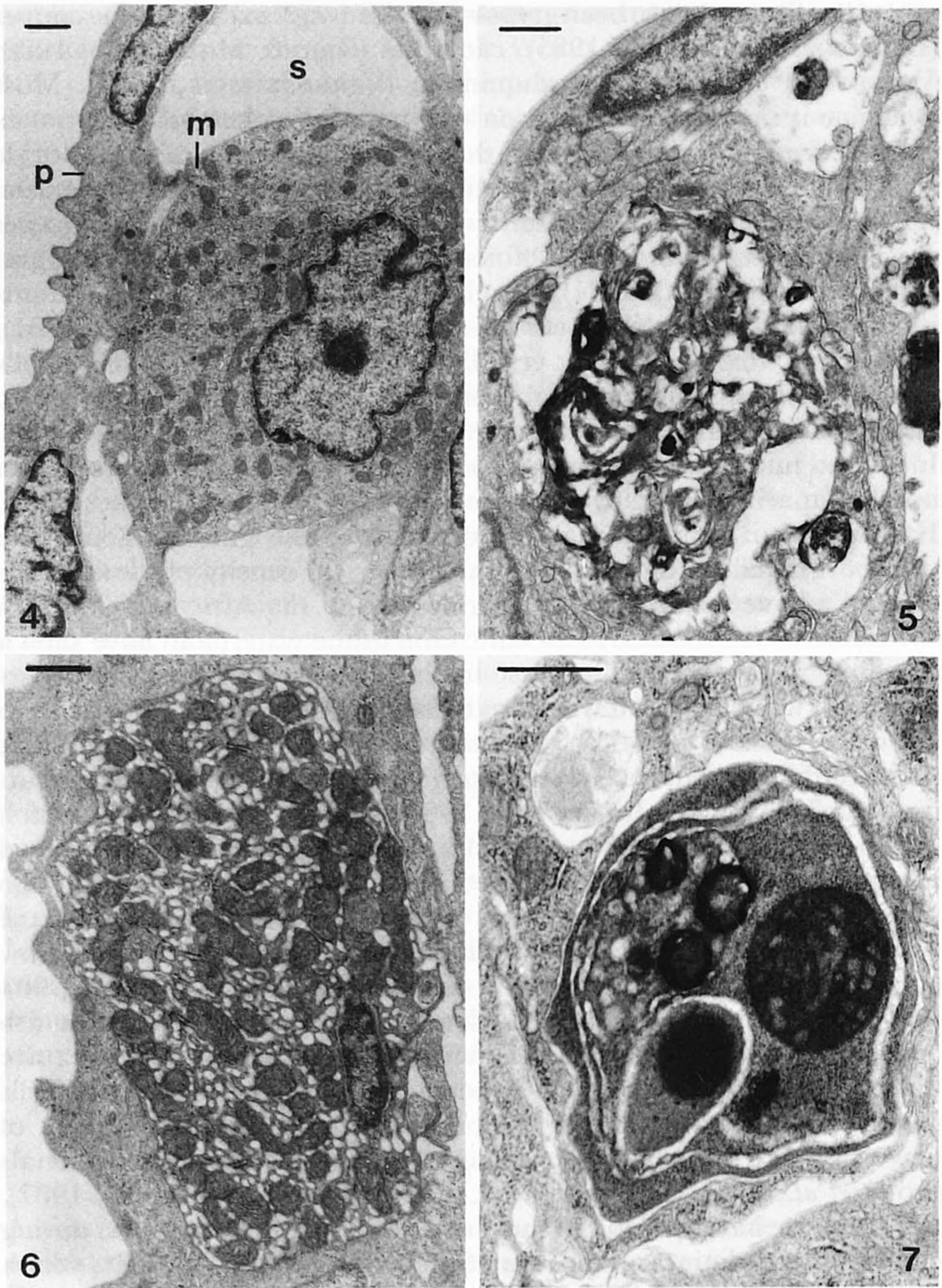
EFFECTS ON ACTIVE ION UPTAKE

Active ion uptake in the gills takes place via the chloride cells (fig. 3), which contain a tubular system with several ion-transporting ATPases (Na^+/K^+ ATPase, a presumptive Na^+/H^+ ATPase, Ca^{2+} -ATPase) and exchangers (FLIK *et al.*, 1985; MAYER-GOSTAN *et al.*, 1987). These highly specialized cells are readily affected by many kinds of pollutants, including toxic substances (MALLATT, 1985; EVANS, 1987). Reports on the effects of toxicants on chloride cells are scarce. Most reported effects on ion uptake concern metals. Inhibition of Na^+ , and/or Ca^{2+}

and Cl^- fluxes have been reported after *e.g.* exposure to copper (LAURÉN & McDONALD, 1985), cadmium (REID & McDONALD, 1988; VERBOST *et al.*, 1987) and aluminium (VERBOST *et al.*, 1992). Most evidence is indirect and based on enzyme studies (see below).

More frequent are reports on the numerical density of the chloride cells. In general, chloride cell proliferation and hyperplasia have been observed (MALLATT, 1985). Increases in cell numbers were found after exposure to *e.g.* cadmium (ORONSAYE & BRÄFIELD, 1984), zinc and copper (CRESPO *et al.*, 1981), chromium (TEMMINK *et al.*, 1983), aluminium (KARLSSON-NORRGREN *et al.*, 1986), nitrite (GAINO *et al.*, 1984), and organic toxicants (MALLATT, 1985). The increase in chloride cells has been explained by most authors as a response of the organism to maintain or increase its capacity to take up ions from the water. Increased numbers of chloride cells may compensate for the loss of ion uptake capacity caused by the toxic actions of pollutants on these cells. However, it is unlikely that the capacity of the gills for active ion transport is reflected directly by the numerical density of the chloride cells. We have examined the chloride cells of the African cichlid fish *Oreochromis mossambicus* after acidification of the water, and have shown that the four- to fivefold increase in chloride cell numbers that occurs at pH 4.5 was associated with a reduced branchial uptake of Na^+ from the water (FLIK *et al.*, 1989). Ultrastructural examination showed that many of the chloride cells were young developing stages or degenerating cells (WENDELAAR BONGA *et al.*, 1990). Mature chloride cells, which may be considered as the functioning cell stages, were slightly reduced in number, explaining the observed reduction in Na^+ uptake through the gills. This interpretation was supported by the finding that gill Na^+/K^+ ATPase activity, the driving force of Na^+ uptake, was below control level in the acid-exposed fish (WENDELAAR BONGA *et al.*, 1990).

How then should the increased numbers of chloride cells in fish exposed to toxic substances be interpreted? Ultrastructural data are scarce, but the few reports have shown that many of the chloride cells are affected. Degeneration of chloride cells and the development of new cells have frequently been reported after exposure to toxic metals (LOCK *et al.*, 1981; TEMMINK *et al.*, 1983; YOUSON & NEVILLE, 1987). Recently, we have quantified the chloride cells in *O. mossambicus* during exposure to cadmium. Exposure of the fish to $10 \mu\text{g.l}^{-1} \text{Cd}^{2+}$, which caused a slight reduction in plasma osmolarity (fig. 3) and enlargement of the intercellular spaces of the branchial epithelium (fig. 4), produced a transient increase in chloride cell density, with a maximum of about 30% above control levels after two weeks (fig. 8). Further analysis of these cells gave the same picture as observed earlier in the gills of fish from acid water: many of the chloride cells were young, immature cells



Figs. 4-7. Epithelium of gill filaments of *Oreochromis mossambicus* exposed to cadmium (10 $\mu\text{g}/\text{l}$); bars represent 1 μm .

Fig. 4. Mature chloride cell (m); the intercellular spaces (s) are enlarged; p, pavement cell; 4 days.

Fig. 5. Necrotic chloride cell; 4 days.

Fig. 6. Apoptotic chloride cell; 35 days.

Fig. 7. Apoptotic body, probably remnant of a chloride cell, in the cytoplasm of a macrophage; 4 days.

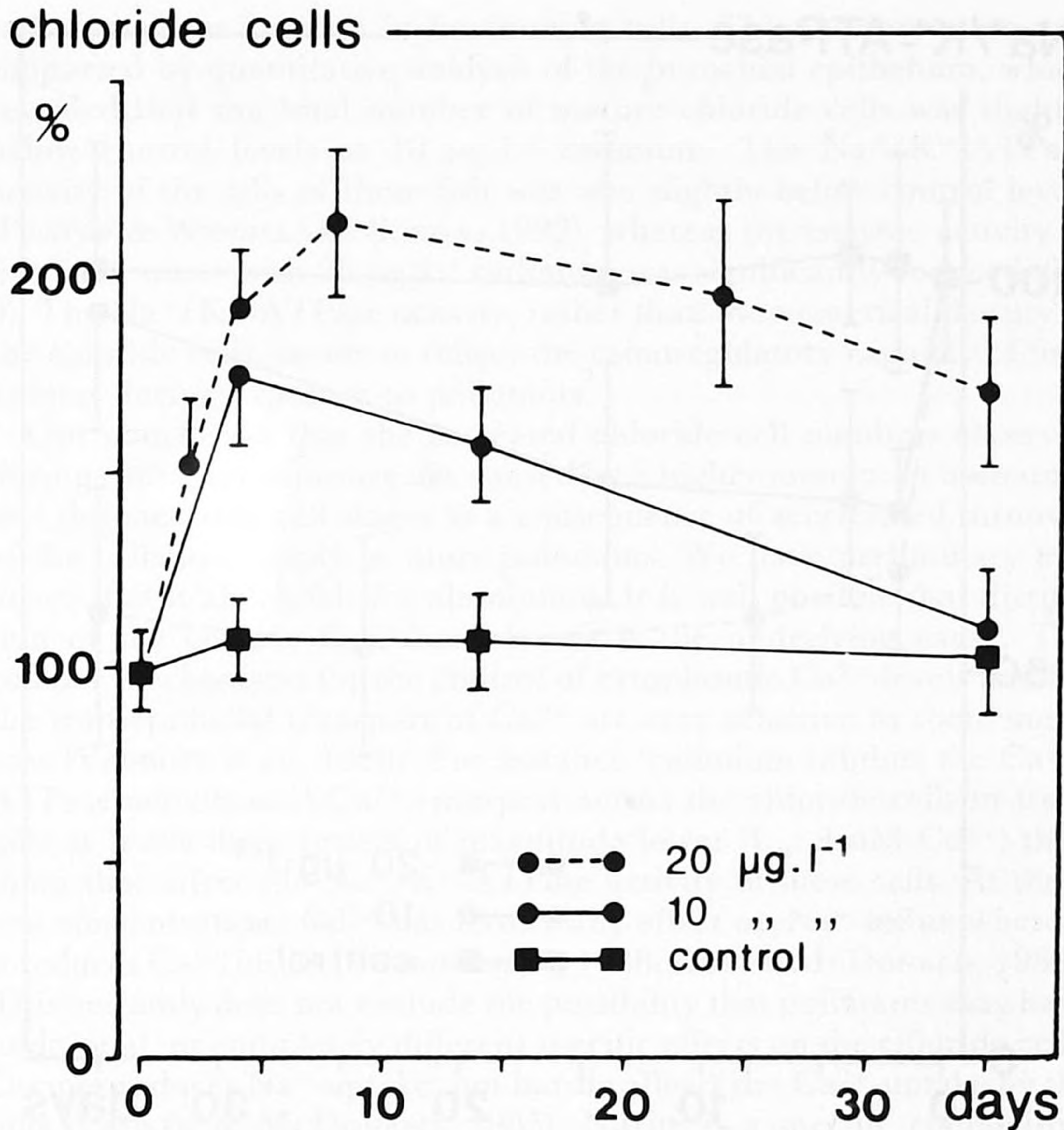


Fig. 8. Effects of different concentrations of water-borne cadmium on the numerical density of chloride cells in the opercular epithelium of *Oreochromis mossambicus*, as determined after DASPEI-staining in a fluorescence microscope (number of cells per surface area as percentage of controls at day 0); means \pm S.D. of 8-10 fish per group (for description of method see WENDELAAR BONGA *et al.*, 1990).

or cells degenerating by necrosis or apoptosis. Necrosis is accidental cell death by lethal injury. Necrotic cells show swelling of the cytoplasm and of cellular organelles, and rupture of cellular membranes (fig. 5). Apoptosis is the normal physiological controlled cell death, and its increase indicates acceleration of cellular turnover (WYLLIE, 1981). Apoptotic cells show shrinkage, resulting in densification of the cytoplasm, mitochondria and nuclei and, in chloride cells, swelling of the tubular system of these cells (fig. 6). The remnants of apoptotic chloride cells are finally removed by macrophages (fig. 7). Significantly

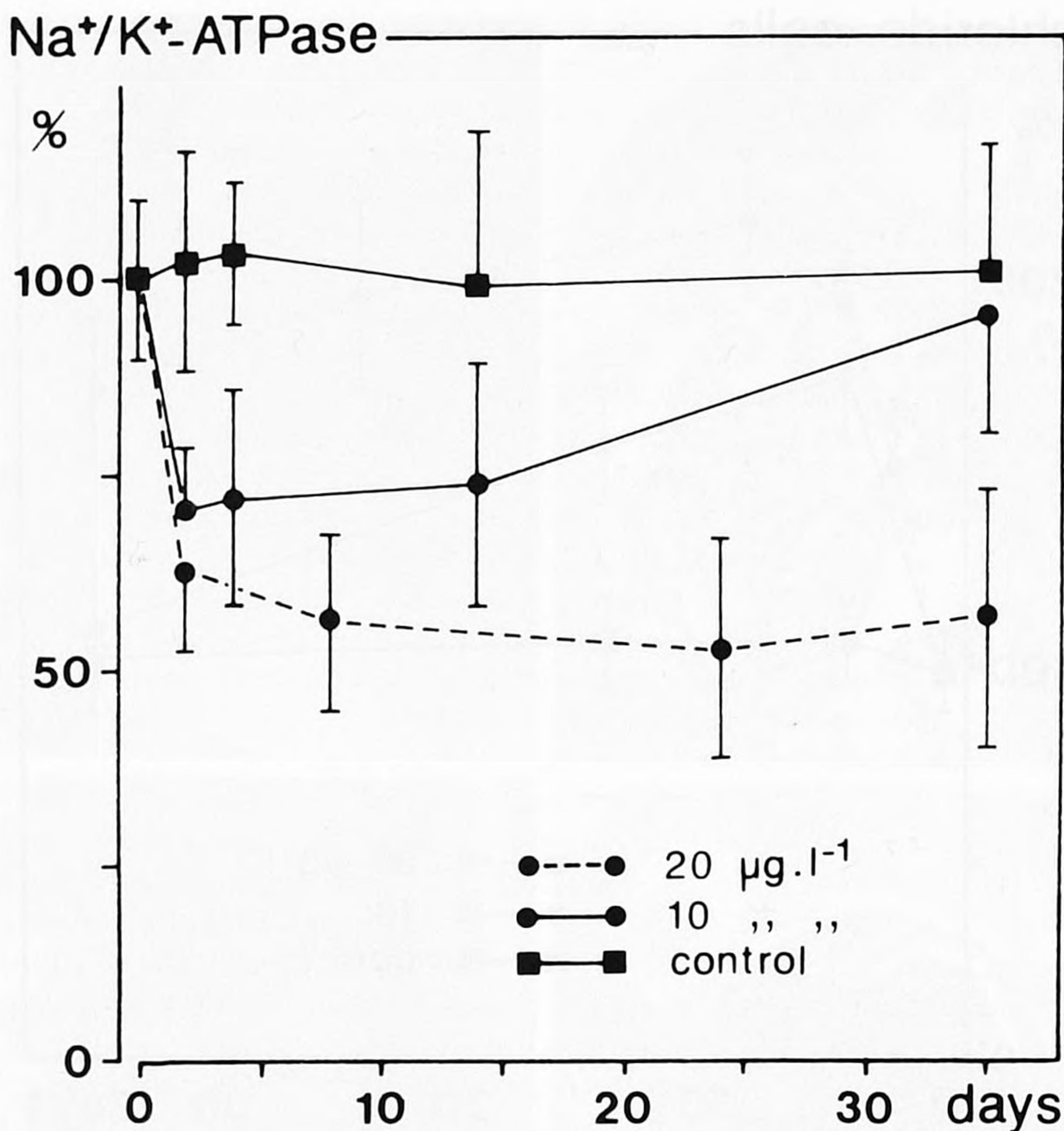


Fig. 9. Na⁺K⁺-ATPase activity (expressed as µg P_i released per mg protein per hour as percentage of controls at day 0) in the gills of *Oreochromis mossambicus* exposed to different cadmium concentrations; means ± S.D. of 8-10 fish per group (for description of method see WENDELAAR BONGA *et al.*, 1990).

increased rates of necrosis and apoptosis were observed throughout the four weeks experimental period of exposure to 10 µg.l⁻¹ Cd²⁺. During exposure to 25 µg Cd.l⁻¹ for the same period, a significant 10% drop in plasma osmolarity occurred; chloride cell density increased with about 50% in the first week and stabilized at a level about two times that of controls (fig. 8). Again, necrotic and apoptotic cell stages were very common, as were young and maturing chloride cells. These data indicate that the increase in chloride cell density is mainly a reflection of increased replacement of the chloride cells under these conditions,

rather than an increase in functioning cells. This interpretation was supported by quantitative analysis of the branchial epithelium, which revealed that the total number of mature chloride cells was slightly below control levels at $10 \mu\text{g.l}^{-1}$ cadmium. The Na^+/K^+ -ATPase activity of the gills of these fish was also slightly below control levels (PRATAP & WENDELAAR BONGA, 1992), whereas the enzyme activity in fish from water with $25 \mu\text{g.l}^{-1}$ cadmium was significantly reduced (fig. 9). The Na^+/K^+ -ATPase activity, rather than the numerical density of the chloride cells, seems to reflect the osmoregulatory capacity of fish, at least during exposure to pollutants.

Our conclusion that the increased chloride cell numbers observed during cadmium exposure are caused by a higher amount of immature and degenerating cell stages as a consequence of accelerated turnover of the cells may apply to more pollutants. We have preliminary evidence that it also holds for aluminium. It is well possible that disruption of the cellular Ca^{2+} -homeostasis is the underlying cause. The cellular mechanisms for the control of cytoplasmic Ca^{2+} -levels and for the transepithelial transport of Ca^{2+} are very sensitive to some metal ions (VERBOST *et al.*, 1988). For instance, cadmium inhibits the Ca^{2+} -ATPase activity and Ca^{2+} -transport across the chloride cells in trout gills at levels three orders of magnitude lower (I_{50} : 3 nM Cd^{2+}) than those that affect the Na^+/K^+ -ATPase activity of these cells. At these low concentrations Cd^{2+} has hardly any effect on Na^+ -influx whereas it reduces Ca^{2+} influx (VERBOST *et al.*, 1988; REID & McDONALD, 1988). This certainly does not exclude the possibility that pollutants may have additional, or completely different specific effects on the chloride cells. Copper reduces Na^+ -uptake, but hardly affects the Ca^{2+} -uptake by the gills (LAURÉN & McDONALD, 1985). Nitrite is a specific competitive inhibitor of active chloride uptake (WILLIAMS & EDDY, 1986), whereas chlorinated hydrocarbons such as DDT may inhibit Na^+/K^+ -ATPase activity (HAUX & LARSSON, 1979).

TOXICANTS AND STRESS

Although loss of blood plasma electrolytes is a common type of osmoregulatory disturbance when fish are in contact with toxicants, it is by no means restricted to exposure to aquatic pollutants. It may also occur under many other conditions, varying from capture, handling or confinement, to social interactions such as lost battles for rank. Under the latter conditions, the decline in electrolytes takes place without direct physical or chemical interference with the gill tissue. Increased tissue necrosis, possibly caused by infectious agents, and proliferation of chloride cells are commonly observed when the stressors have a

more chronic nature (PETERS & HONG, 1984). Thus, the phenomena observable in the gills during stress are similar to those described during exposure to toxicants. The question then presents itself whether osmoregulatory disturbances in fish during toxicant exposure should be partly or fully considered as a more general response to stressors. After reviewing the literature, GRONOW (1973) concluded that the general stress concept—originally developed as the general adaptation syndrome for higher vertebrates—was also applicable to fishes. He noted many similarities between terrestrial and aquatic vertebrates in their responses to stressors and in the effects of severe stress: depletion of energy stores, disturbed acid-base balance, and, during prolonged stress, impaired growth, immunosuppression, and reduced reproduction. His conclusion has been well substantiated by more recent evidence on the effects of water-borne irritants and other stressors, such as capture, handling, confinement, and transport, or some types of social interactions (*e.g.* MAZEAUD *et al.*, 1977; PICKERING, 1981; PETERS & HONG, 1984; MAULE *et al.*, 1988, 1989). The similarities include stimulated glycogenolysis and glyconeogenesis, hyperglycemia and increased plasma levels of amino acids, and effects on the cardiovascular system. In the higher vertebrates these processes are under control of the autonomic nervous system and of the pituitary-interrenal axis, with catecholamines and cortisol as the primary endocrine messengers. These hormones are also important stress hormones in fish, produced in the homologue of the adrenal glands, the interrenal cells (PICKERING, 1981, 1989; MAULE *et al.*, 1988, 1989; XUEMIN *et al.*, 1991). The similarities between higher vertebrates and fishes indicate that the fundamental mechanisms of the general stress response had been developed before the water-to-land transition in vertebrate evolution.

GRONOW (1973) made one exception when summing up the similarities between higher vertebrates and fishes, and this concerned the changes in water and ion balance. In mammals, stress may result in hemoconcentration associated with sodium retention and loss of potassium. In freshwater fish, stress results in hemodilution caused by ion losses and osmotic water uptake across the gills. We therefore conclude that the main difference in the stress effects between terrestrial vertebrates and fishes is connected with their habitat and the possession of gills. This difference may explain why in fish cortisol has effects on both energy balance and hydromineral balance, whereas in terrestrial vertebrates this stress hormone has lost its hydromineral actions.

Many reports in the literature have shown effects of toxicants on typical stress parameters. Elevated levels of plasma glucose (fig. 10a), ammonia, lactate, cortisol (fig. 10b) and catecholamines have been

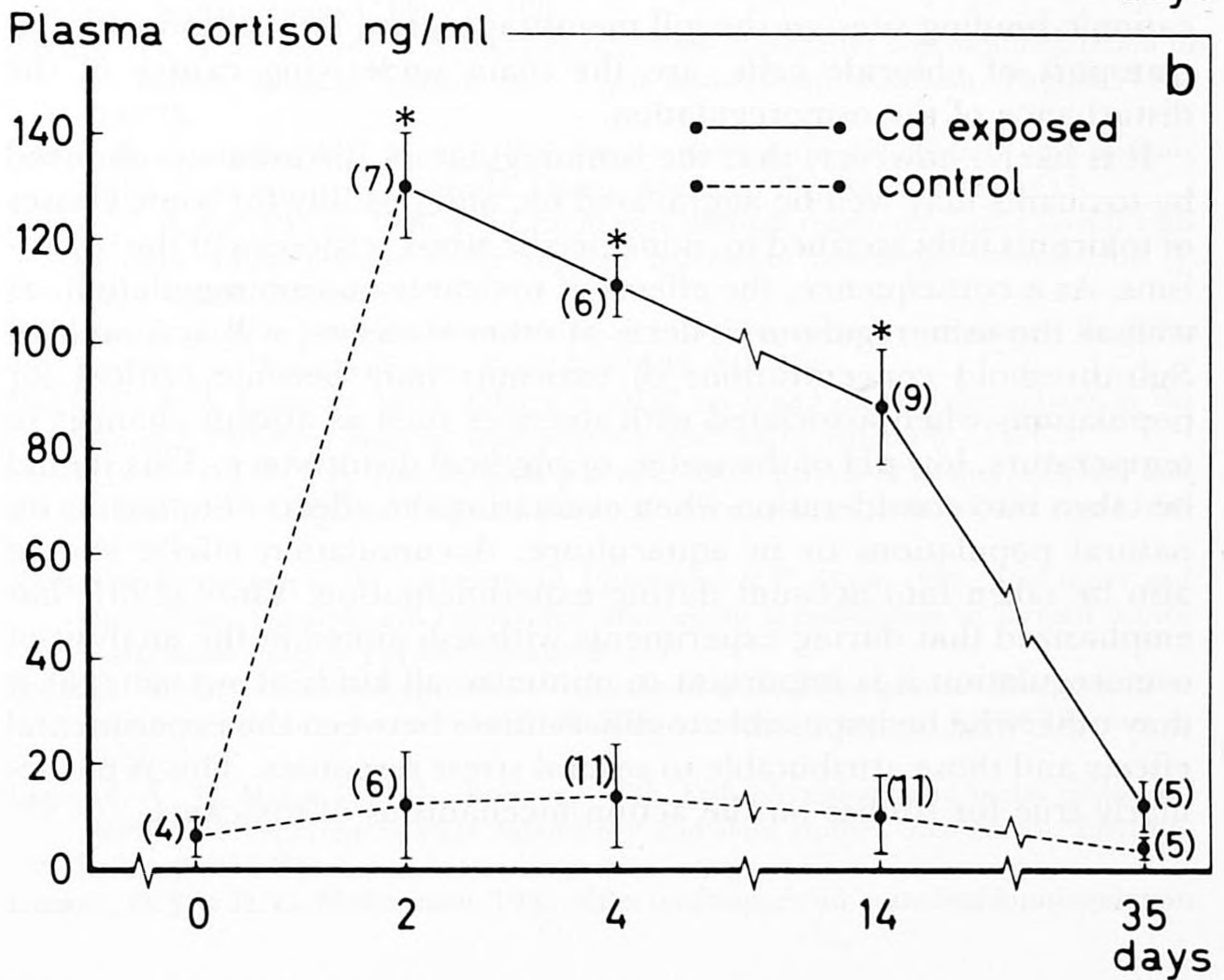
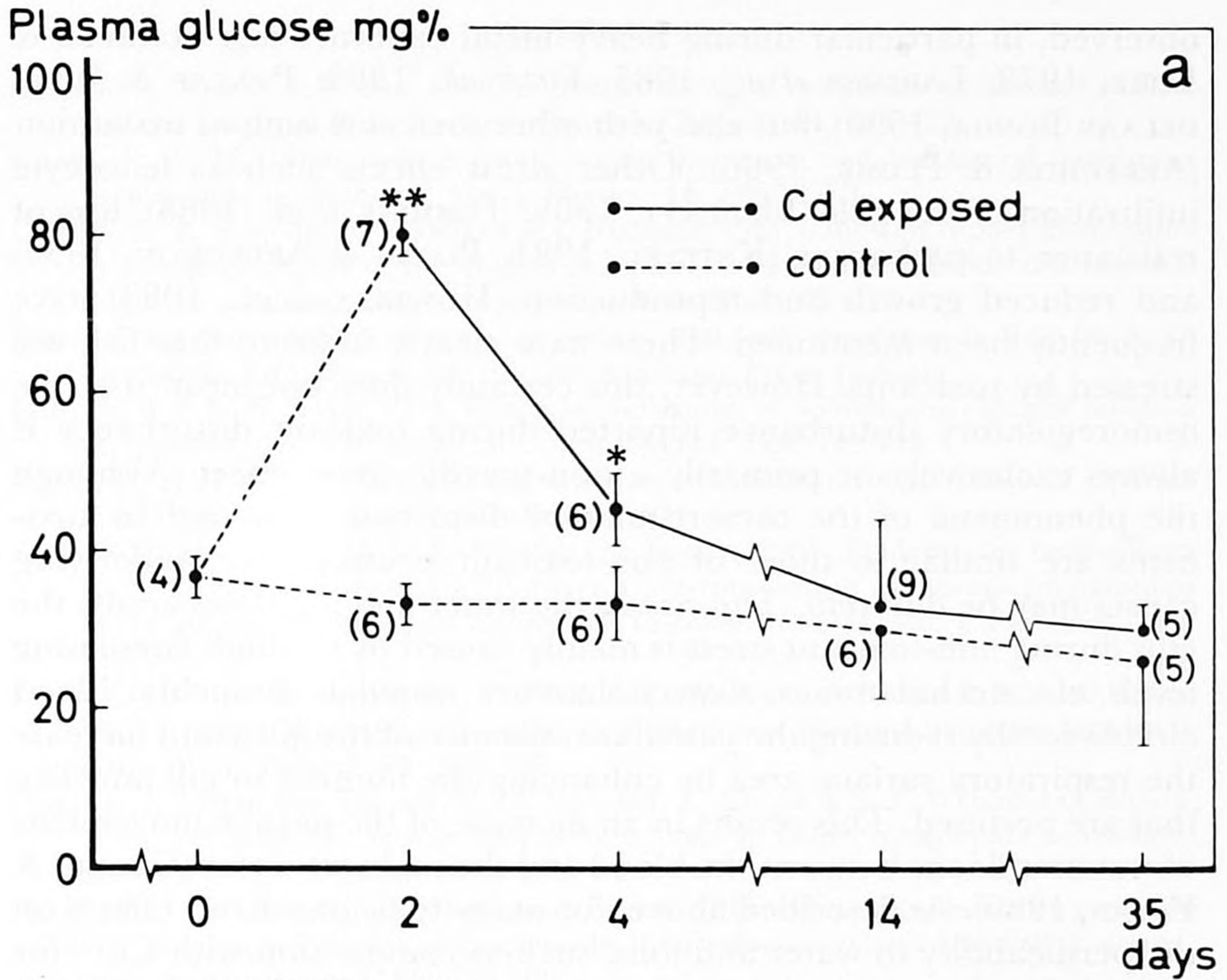


Fig. 10a,b. Effect of exposure to water-borne cadmium (10 $\mu\text{g/l}$) for 2, 4, 14 and 35 days on plasma glucose (fig. 10a) and plasma cortisol (fig. 10b) of *Oreochromis mossambicus*; means \pm S.D.; the number of fish sampled is indicated between brackets (from: PRATAP & WENDELAAR BONGA, 1990).

observed, in particular during heavy metal exposure (*e.g.* SCHRECK & LORZ, 1978; LARSSON *et al.*, 1985; FU *et al.*, 1989; PRATAP & WENDELAAR BONGA, 1990), but also with other toxicants such as malathion (AREECHON & PLUMB, 1990). Other stress effects such as leucocyte infiltration of the gills (MALLATT, 1985; TEMMINK *et al.*, 1983), loss of resistance to pathogens (KNITTEL, 1981; PLUMB & AREECHON, 1990) and reduced growth and reproduction (HOWELLS *et al.*, 1983) have frequently been mentioned. These data clearly indicate that fish are stressed by toxicants. However, this certainly does not mean that the osmoregulatory disturbance reported during toxicant disturbance is always exclusively or primarily a non-specific stress effect. Although the phenomena of the osmoregulatory disturbances caused by toxicants are similar to those of non-toxicant stressors, the underlying causes may be different. The rise in the water and ion flows across the gills during non-toxicant stress is mainly caused by the high circulating levels of catecholamines. Catecholamines stimulate branchial blood circulation by reducing the vascular resistance of the gills, and increase the respiratory surface area by enhancing the number of gill lamellae that are perfused. This results in an increase of the passive movements of water and ions between the blood and the ambient water (GIRARD & PAYAN, 1980). As described above, for many toxicants direct effects on the permeability to water and ions, such as competition with Ca^{2+} for cationic binding sites on the gill membranes, and interaction with ion transport of chloride cells, are the main underlying causes of the disturbance of the osmoregulation.

It is likely, however, that the osmoregulatory disturbances induced by toxicants may well be aggravated by, and possibly for some classes of toxicants fully ascribed to, non-specific stress responses of the organisms. As a consequence, the effects of toxicants on osmoregulation, as well as the osmoregulatory effects of other stressors, will accumulate. Sub-threshold concentrations of toxicants may become critical for populations when associated with stressors such as abrupt changes in temperature, low pH of the water, or physical disturbance. This should be taken into consideration when evaluating the effects of toxicants on natural populations or in aquaculture. Accumulation effects should also be taken into account during experimentation. EDDY (1981) has emphasized that during experiments with fish aimed at the analysis of osmoregulation it is important to minimize all kinds of stressors, as it may otherwise be impossible to differentiate between the experimental effects and those attributable to several stress responses. This is particularly true for studies on the action mechanisms of toxicants.

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