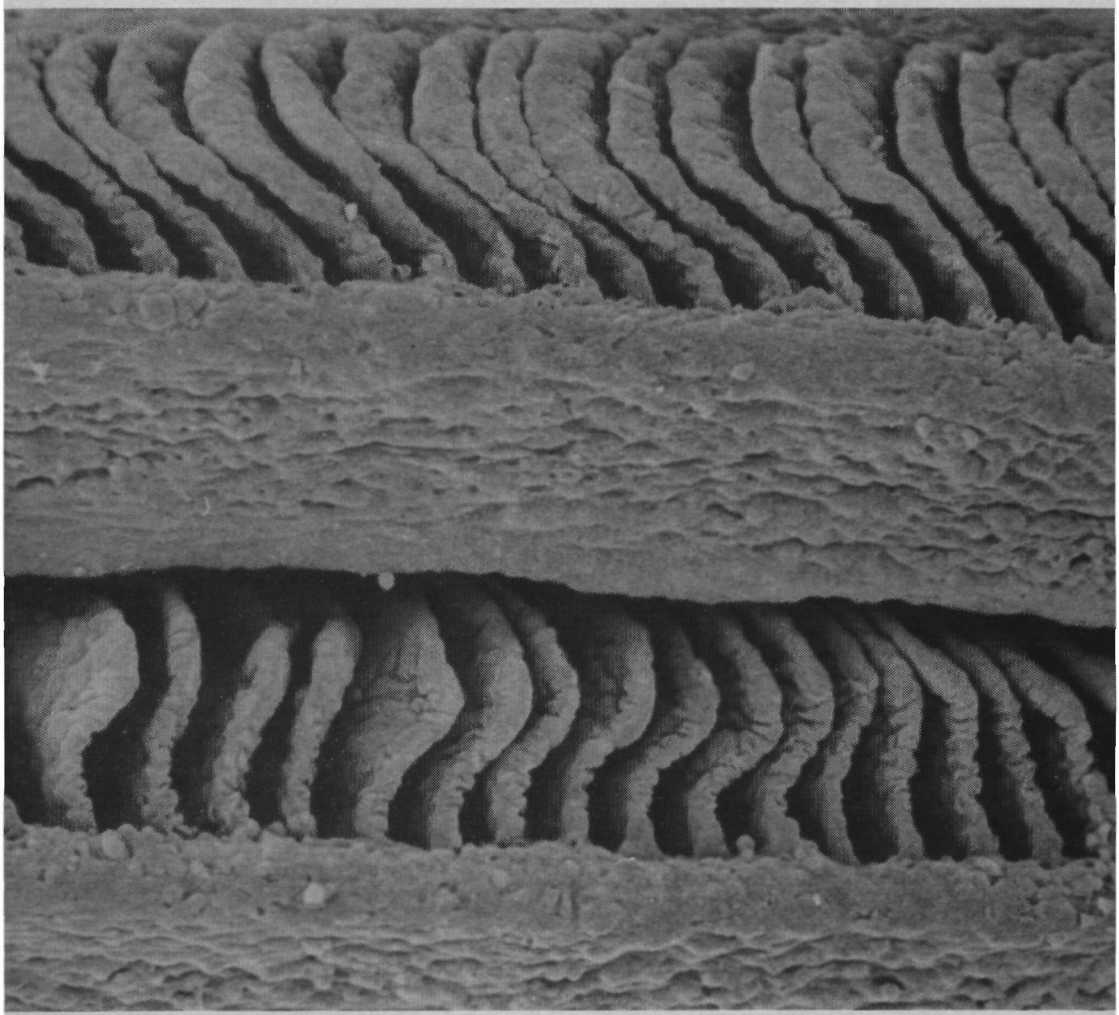
# EFFECTS OF MERCURIC CHLORIDE AND METHYLMERCURIC CHLORIDE ON MUCUS PRODUCTION AND OSMOREGULATORY FUNCTION OF THE GILLS IN RAINBOW TROUT, *SALMO GAIRDNERI* RICHARDSON



R.A.C. Lock

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Promotor:
Prof. Dr. A.P. van Overbeeke

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
OP GEZAG VAN DE RECTOR MAGNIFICUS
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**ROBERT ARIE CORNELIS LOCK**
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All the vital mechanisms, however
varied they may be, have only
one object, that of preserving
the conditions of life in the
internal environment

Claude Bernard

Aan Joke, Nicolien en Erik-Jan

Hartelijk dank aan iedereen die op enigerlei wijze aan de totstandkoming van dit proefschrift heeft bijgedragen!
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ABBREVIATIONS

| | |
|---|---|
| PPB | Parts per billion |
| PPM | Parts per million |
| MC | Mercuric chloride |
| MMC | Methylmercuric chloride |
| MS 222 | Tricaine methanosulphonate |
| NANA | N-acetylneuraminic acid |
| $Na^+$-$K^+$-ATPase | Sodium plus potassium stimulated (ouabain sensitive) adenosine triphosphatase |
| $Mg^{2+}$ ATPase | Magnesium stimulated (ouabain insensitive) rest adenosine triphosphatase |
| $P_i$ | Inorganic phosphate |
| TCA | Trichloroacetic acid |

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# CHAPTER 1

## GENERAL ASPECTS ON THE OCCURRENCE OF MERCURY IN THE AQUATIC ENVIRONMENT

Mercury is a relatively rare element, comprising approximately $5.10^{-6}$% of the earth's crust (Haissinsky and Adloff, 1965). The element has always been present in the biosphere, although mostly in minute concentrations, probably as a consequence of the evaporation of mercury from the earth's crust and the subsequent distribution through aerial circulation and precipitation (Stock and Cucuel, 1934). In the natural state, mercury occurs mainly in the form of various sulphides, especially red sulphide (cinnabar) and as metallic mercury. Both forms have been known by man already for millennia. Arab and Greek physicians used mercury compounds as early as the 6th century B.C. for treating various skin diseases, infections and disorders (Goldwater, 1971, 1974).

Because of their toxic properties, mercury compounds have also long been known as agents for suicide or murder. Ivan the Terrible, Charles II of England and Napoleon are all suspected of having died of mercury poisoning (Goldwater, 1971). The toxicity of mercury is generally believed to be related to the strong affinity between mercury compounds and sulphydryl groups in biological materials, in particular enzymes (Hughes, 1957; Webb, 1966; Jocelyn, 1972). Chemical binding of mercury compounds to proteins or phospholipids of a cell membrane may alter the distribution of ions, change electric potentials and thus interfere with the movement of fluids across the membrane (Passow *et al.*, 1961; Rothstein, 1959, 1970; Magnaval *et al.*, 1973).

It was not until the beginning of this century that industrial communities locally induced an appreciable increase in the flow of mercury through the biosphere.

Since that time, world production of mercury steadily increased up to 10,000 metric tons per year (Hammond, 1971; Nelson *et al.*, 1971). A large part of this eventually escapes into waterways, air and soil. Stock and Cucuel (1934) already reported mercury in freshwater and seawater fish from waters near industrial areas in concentrations that were surprisingly higher than those found in fish from uncontaminated waters. Isolated observations have not attracted much attention until the time of the disaster in Minamata Bay, Japan, in the early fifties. In these years more than a hundred people died or became seriously ill after consuming fish or shellfish from the nearby bay. This food contained high levels of methylmercury (27 to 120 ppm Hg; Irukayama, 1969). The neurotoxicological syndrome characterized by sensory disorders, ataxia and constriction of the visual field has since been known as the Minamata disease. Similar poisoning was reported in the early sixties in Niigata, Japan, where several people died after consuming mercury-contaminated fish. In both cases, mercury-containing effluents had been discharged into the water by industrial plants (Kitamura, 1968). Since this time mercury pollution received much attention. For reviews on the subject see Hartung and Dinman, 1972; Saha, 1972; D'Itri, 1972; Friberg and Vostal, 1972; Krenkel, 1974 and Chang, 1977.

It appeared that most discharges of mercury into the water, including that of Minamata Bay, occurred as inorganic mercury and practically none as methylmercury. It was therefore already postulated in 1960 by Kurland (cited by Saha, 1972), that the high methylmercury concentration in the fish from Minamata Bay could have been the result of biological methylation. Also in Sweden, Westöö (1966, 1968) found that practically all the mercury in fish is present as methylmercury, even in waters lacking industrial sources of this compound. The experimental support for biological methylation of mercury was furnished in

a study by Wood *et al.*, (1968) using methanogenic bacterial cell extracts and by Jensen and Jernelöv (1969) who demonstrated that microbe-mediated methylation takes place in bottom sediments. The capacity for methylation might be universal among bacteria, although quantitative differences between different species under varying conditions are large (Landner, 1971; Vonk and Kaars Sijpestein, 1973; Hamdy and Noyes, 1975; Olson and Cooper, 1975, 1976; Jacobs and Keeney, 1974). In addition, Spangler *et al.*, (1973a,b) demonstrated methylmercury synthesis as well as methylmercury degradation by bacteria isolated from lake sediments. Furthermore, methylation has also been shown to occur non-biologically when inorganic mercury reacts with methylcobalamin, a vitamin $B_{12}$-derivative and a known methyl donor in biological systems (Imura *et al.*, 1971; Bertilsson and Neujahr, 1971; Wood, 1971, 1976). As most experiments on rates of methylation have been performed under conditions whereby the two processes, methylation and demethylation, could not be distinguished from one another, results should not be interpreted as a measure of gross methylation rate but rather as a net methylation rate (Fagerström and Jernelöv, 1974). Despite repeated attempts, it could not be demonstrated that fish itself may methylate mercury. Experiments with inorganic mercury injected into muscle and liver of fish failed to result in a measurable increase in methylmercury concentration (Fagerström and Jernelöv, 1974). The discharged mercury, irrespective of its chemical form, is likely to end up in the sediment, either as a result of precipitation with organic and inorganic colloids or as a result of sedimentation of the discharge (de Groot *et al.*, 1971; Gavis and Ferguson, 1972; Förstner and Müller, 1973; Clifton and Vivian, 1975; Ramamoorthy and Kushner, 1975; Andrew and Harriss, 1975; Cowgill, 1975; Thomas and Jaquet, 1976). Microbial methylation of mercury in the bottom sediments appears therefore the most important pathway through which mercury contributes towards environmental pollution. A scheme of the biological cycle of mercury is presented in fig. 1.1.

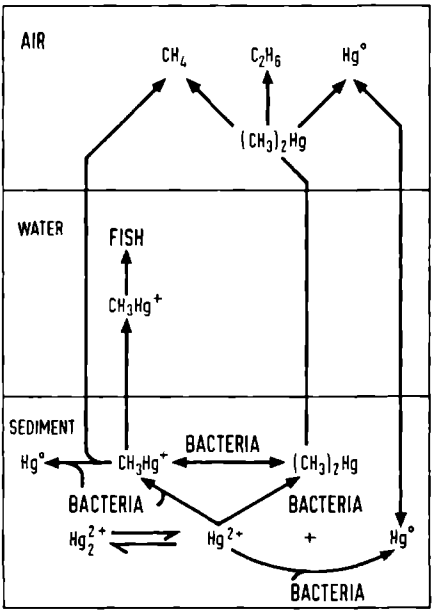



Fig 1 1 The biological cycle of mercury (from Wood, 1974)

Methylmercury is highly lipophilic. After its formation in the sediments it is quickly released into the water and rapidly taken up by organisms. This phenomenon explains the failure to measure methylmercury in the water (Fagerstrom and Jernelov, 1974). Direct uptake of methylmercury by fish from bottom sediments to which inorganic mercury had been added, has been shown to occur under laboratory conditions (Gillespie and Scott, 1971, Gillespie, 1972) It was suggested that in this process the gills function as the major route of uptake for the organic compound. Experimentally, this was proved by Olson *et al.*, (1973), who compared the mercury accumulation in the rainbow trout, *Salmo gairdneri* with or without ligation of the esophagus. Indeed, when mercury compounds are administered with the food (Hannerz, 1968) or directly by way of injection (Giblin and Massaro, 1973), the

mercury concentration in the gills, relative to that in other tissues and organs, is low.

It is important to realize that in the natural environment uptake via the gills is not the only manner in which fish accumulate mercury. There is evidence, that at least in eutrophic waters, the main route of methylmercury accumulation by fish is with the food This notion has prompted numerous studies, in particular in view of the "bio-magnification" which characterizes this route of uptake (Jernelov and Lann, 1971, Knauer and Martin, 1972, Fagerstrom and Asell, 1973, Lock, 1975, Prabhu and Hamdy, 1977). Nevertheless, gills play an important role in the concentration of mercury compounds in fish. In particular, in many toxicological investigations involving fish, mercury is dissolved in the water. Under these conditions uptake via the gills was found to be very rapid and effective (Hannerz, 1968; McKone *et al.*, 1971, Burrows and Krenkel, 1973, Burkett, 1974, Olson *et al.*, 1973, 1978, Lock, 1975, McKim *et al.*, 1976).

In preliminary experiments in our laboratory, uptake of both mercuric chloride (MC) and methylmercuric chloride (MMC) from the water by rainbow trout was studied. The results* are shown in fig. 1.2. It appears that under these conditions, the mercury concentration in the gills by far exceeds those of other tissues and organs. MMC in particular is accumulated in the gills at a very high rate and also its concentration factor (ratio between mercury concentration in the tissue and that in the water) has the highest value. That the rapid accumulation of mercury in the gills reflects their role as the main route of uptake and does not point to the existence of a special affinity for these compounds, is indicated by the results of an elimination experiment. In this experiment, exposure to mercury lasted 24 hours, after which fish were kept in mercury-free water for 1 week. The results are shown in fig. 1.3

*Unpublished results belonging to a study reported elsewhere (Lock, 1974).*

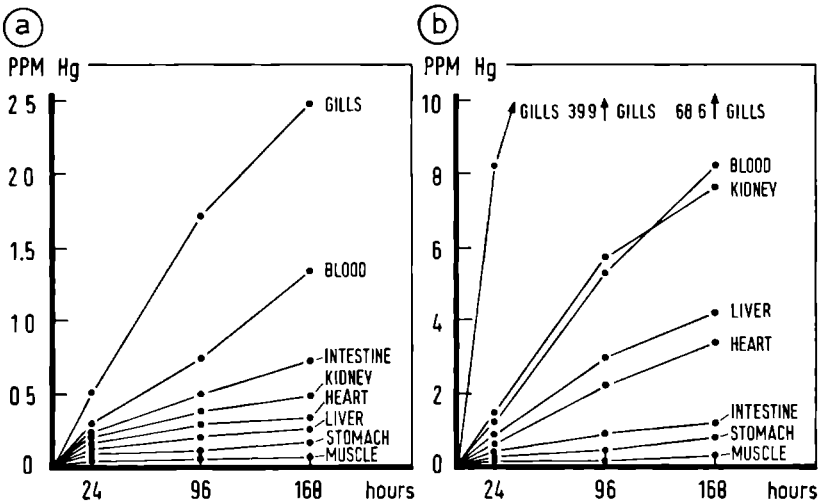



Fig. 1.2. Concentration of mercury in the various organs and tissues of rainbow trout after continuous exposure to 5 ppb Hg as either (a), mercuric chloride (MC) or (b), methylmercuric chloride (MMC) for different periods of time. Data are the mean values of eight fish.

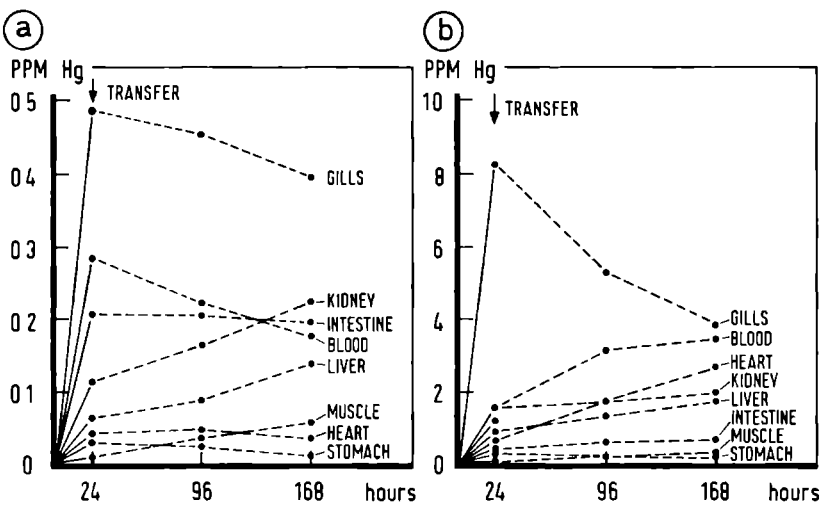



Fig. 1 3 Elimination of mercury by the various organs and tissues of rainbow trout after a 24-hour exposure to 5 ppb Hg as either (a), mercuric chloride (MC) or (b), methylmercuric chloride (MMC) and subsequent transfer to mercury-free water. Data are the mean values of eight fish

Mercury content in the gills rapidly decreased following this transfer, particularly in the case of MMC, while that in most other organs continued to increase. This rapid loss of mercury from the gills is likely caused by two processes, (1) sloughing of mercury containing mucus as already hypothetized earlier (McKone *et al.*, 1971; Olson and Fromm, 1973; Lock, 1975) and (2) a rapid permeation of this compound through the gill epithelium into the blood. The latter possibility is supported by the observation that during the first week of elimination, the rate of mercury decrease from the gills is almost proportional to the rate of mercury increase in the blood. The fact that the rates concerning MMC are higher than those for MC probably reflects the relatively high lipid solubility of MMC as compared to that of MC. This last difference probably accounts also for the fact that MMC is more toxic to organisms than MC as illustrated by the different 96-hour $LD_{50}$ values (amount of poison, lethal to 50% of the animals after a 96-hour exposure period). For rainbow trout, these values are 275 µg Hg/l and 24 µg Hg/l for MC and MMC respectively (Lock, 1974).

The initial purpose of our study was to compare the effects of MC and MMC on the structure and osmoregulatory function of the gills in rainbow trout. Chapter 2 reports the general morphological effects on the gills as observed by the light microscope. In addition, the chloride cells of the gill epithelium were analysed electron microscopically.

Conspicuous mucification at the body surfaces of the trout, following exposure to mercury was noted. Such effect of mercury as well as of other heavy metals has been reported in other investigations but to our knowledge, no quantitative study concerning this phenomenon has been published. It was furthermore considered probable that enhanced mucus production affects the gills' osmoregulatory function and the rate of mercury uptake (Lock, 1975). Therefore, it was decided to undertake a quanti-

tative analysis of the influence of MC and MMC on mucus, involving mucous cells, mucus content of several tissues, and mucus release into the water. The results are presented in chapter 3.

In chapter 4, first the effects of MC and MMC on osmolality and electrolyte concentrations of the bloodplasma are reported. We then tried to answer the question whether the observed decrease in the osmotic value of the plasma following mercury treatment was the consequence of a disturbed functioning of the gills' $Na^+$-$K^+$-ATPase or whether it was related to a change in osmotic water uptake via the gills.

Chapter 5 is a general discussion of the various results and an attempt to relate the data to one another.

# CHAPTER 2

## MORPHOLOGICAL EFFECTS OF MERCURIC CHLORIDE AND METHYLMERCURIC CHLORIDE ON THE GILLS OF RAINBOW TROUT

### INTRODUCTION

When fish are exposed to heavy metals in their environment, the gill tissue rapidly accumulates these compounds in such a manner that their concentration, at least initially, reaches values exceeding by far those in other tissues. This has been shown to be the case with copper (Sellers *et al.*, 1975), zinc (Matthiesen and Brafield, 1973, 1977) and cadmium (Sangalang and Freeman, 1979). Similarly, as was demonstrated in chapter 1, rainbow trout accumulate both mercuric chloride (MC) and methylmercuric chloride (MMC) to levels not found in the other organs and tissues. This conspicuous mercury accumulation in the gills is associated with the fact that, at least when toxic compounds are dissolved in the water, the gills function as the main route of uptake (Hannerz, 1968, Rucker and Amend, 1969, Olson and Fromm, 1973; Olson *et al.*, 1973, 1978; Bouquegneau and Gilles, 1979).

In addition to their respiratory function, teleost gills are involved in the maintenance of the ionic composition and water content of the body fluids. Already the classic study by Keys in 1931 (see Conte, 1969) and by Krogh (1939) identified the gill-buccal region as a probably site for extrarenal salt secretion in euryhaline teleosts. Since that time major advances have been made in our understanding of the significance of active transport of salts across the gill epithelium. For reviews on this subject see Maetz (1971) and Maetz and Bornancin (1975). Particularly, a number of specialized epithelial cells, the so-called chloride cells (Keys and Willmer, 1932) are responsible for ion transport across the epithelium. Both the gills' osmoregulatory role and their respiratory function require large

amounts of water to flow over their surface. It is this process that enables polluting substances such as mercury to enter the fish's body and to concentrate in the gill tissue up to thousands of time the level found in the water. It is to be expected, therefore, that the structure and the function of the gills will be affected by exposure of the fish to mercury.

The aim of the study reported hereafter was to investigate the effects of mercury on the structure of the gills in rainbow trout, after exposure of the fish to different concentrations of MC or MMC during various lengths of time. The general morphological effect was studied with the light microscope, while the influence on the chloride cells was analysed electron microscopically.

## MATERIALS AND METHODS

Rainbow trout, *Salmo gairdneri* Richardson, were obtained as young brood from the Nederlandse Forellen Mij., Apeldoorn, The Netherlands. Fish were maintained in 1000-l round, polyethylene tanks, continuously supplied with Nijmegen tap water of 12 ± 1 C. They were fed daily with commercial trout pellets according to the manufacturer's scheme (Trouvit, Putten, The Netherlands).

Concentrations of the main electrolytes (mg/l) in the water were as follows: $Na^+$, 9.8; $K^+$, 1.1; $Ca^{2+}$, 19.8; $Mg^{2+}$, 10.0; $Fe^{2+}$, 0.01; $NH_4^+$, 0.03; $NO_3^-$, 3.1 and $NO_2^-$, 0.03.

*General experimental procedure*. At least 4 days prior to commencing an experiment, the groups of fish to be used were placed in polyethylene experimental tanks of 75 l. Waterflow through the tanks was continuous at a rate of 1 l/min. Oxygen concentrations were kept above the 90% air saturation point by bubbling compressed air through the water with air diffuser stones. Temperature of the water was kept at 12 + 1 C. The desired mercury concentrations in the tanks were maintained by continually mixing the inflowing tap water with the appropriate amounts of stock

solutions of MC or MMC using a 12-channel dosing pump (Cenco, Breda, The Netherlands). In this system, considerable adsorption of the mercury compounds to the surface of the tanks took place. A period of approximately three hours of flow was therefore necessary before mercury levels had reached the desired (nominal) concentrations. As a consequence of this "equilibrium period", exposure time to mercury was considered to commence 3 hours after introducing the mercury stock solution. As, in general, the actual concentrations deviated less than 5% from the nominal ones, the latter values are used in presenting the results.

*Effect of mercury on the gill structure.* To determine the effect of MC and MMC on the gill structure, a group of at least eight fish was placed in each of a series of tanks. Fish were exposed to MC concentrations of 50, 100, 500, 1000 and 2000 µg Hg/l for 4 hours; 50, 100, 250, 500 and 1000 µg Hg/l for 24 hours; and 10, 25, 50, 100 and 200 µg Hg/l for 1 week. MMC concentrations were 5, 10, 50, 100 and 200 µg Hg/l for 4 hours; 1, 5, 10, 25 and 50 µg Hg/l for 24 hours; and 1, 2.5, 5, 10 and 20 µg Hg/l for 1 week. Periodically, water samples were taken for measuring the mercury concentration in the water by flameless atomic absorption at 254 nm on a LDC Mercury Monitor, Model 1205, using the method described by Olson *et al.* (1975). Samples of at least four fish were removed at the time periods indicated above for histological examination of the gills. To study the effect of mercury elimination, the flow of mercury in the tanks was stopped immediately after taking the last samples, and new gill samples of two fish each were collected 24 hours and 1 week later.

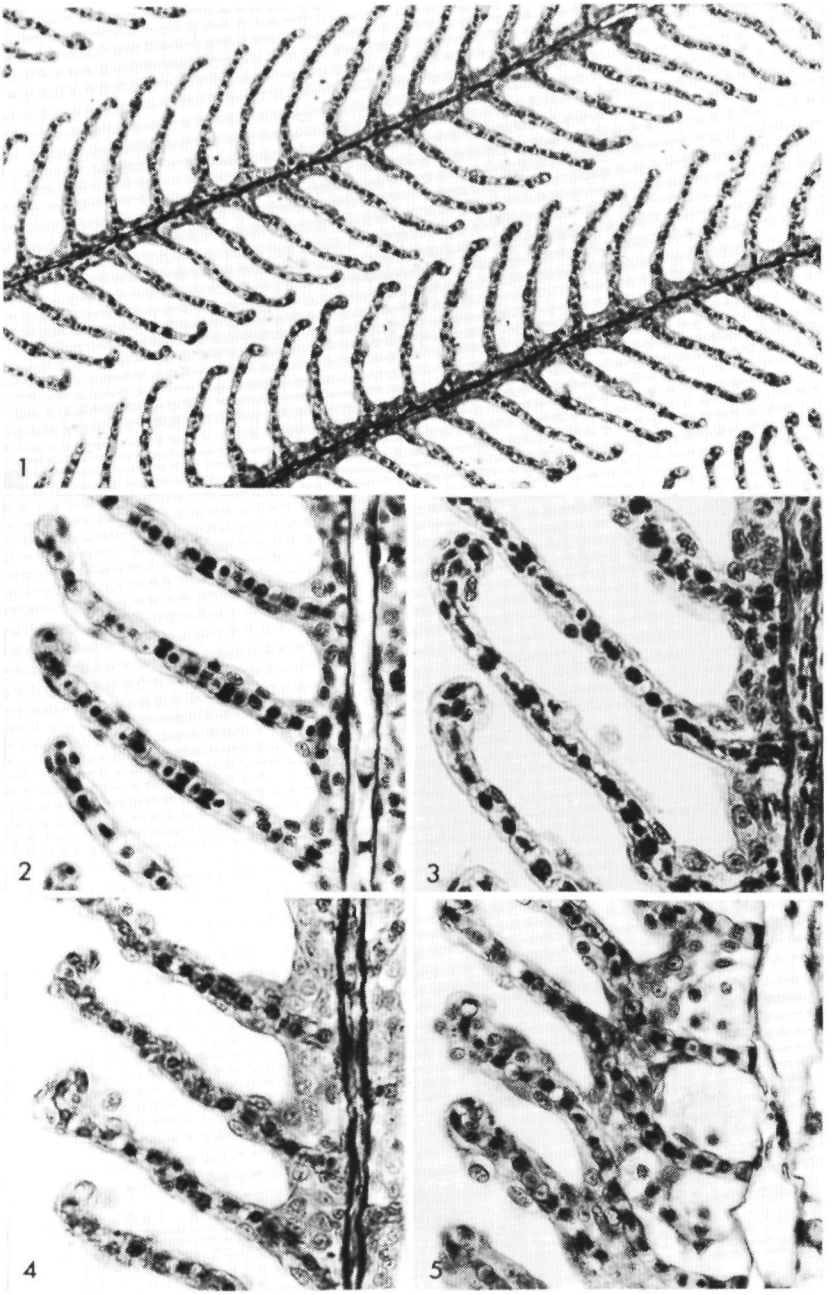
*Histological techniques.* For light microscopical examination, gills were fixed for 24 hours in Bouin's fluid, dehydrated, and embedded in paraplast. Transverse sections of 7 µ thickness were stained with 1% Alcian Blue in 3% acetic acid, followed by Azan according to Heidenhain (Romeis, 1948). Gill tissue selected for

electron microscopic examination was prefixed in 0.1 M cacodylate buffered (pH 7.2) 3% glutaraldehyde for 10 minutes at room temperature. Fixation was done in a similarly buffered solution of 2% osmium tetroxide, 3% glutaraldehyde and 5% potassium dichromate for 1 hour at 0 C. Tissues were postfixe in 1% uranyl acetate in distilled water, dehydrated in mixtures of ethanol and 1% uranyl acetate and embedded in Spurr's resin. Ultrathin sections were examined with a Philips 201 electron microscope. Cytoplasmic and mitochondrial areas in the chloride cells were determined by morphometrical analysis of electron micrographs. For each experimental group six chloride cells per fish were analysed at a final magnification of 12,000 x, using a Kontron Digiplan Analyser. For statistical evaluation of the data Student's t test was used. All tests were two-sided at the 5% significance level.

## RESULTS

In all experiments exposure to MC and MCC in concentrations exceeding the 96-hour $LD_{50}$ values induced abnormal behaviour, namely erratic swimming followed by surfacing and progressive loss of blance, turning over, and loss of swimming ability. These phenomena were accompanied by gradual darkening of the skin and, in advanced stages of intoxication, visible mucus accumulation on skin and gill operculum as well as frequent coughing.

*Normal gill structure.* A light micrograph of gill filaments with secondary lamellae of a control fish is shown in fig. 1. Characteristically, the lamellae (leaflets) alternatingly arise at $45^{o}$-$60^{o}$ angles from both sides of the gill filament. The multi-layered epithelium of the filaments is continuous with the single-layered epithelium covering the leaflets. Mucous cells and chloride cells are mainly located on the edges of the epithelial layer of the filaments. The epithelium of the fila-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ment rests on a collagenous basal membrane which in turn lines connective tissue, containing blood and lymph vessels. This membrane is continuous with the supporting columns of the pillar cells of the secondary lamellae The flanges of the pillar cells form the enclosure of the lamellae where the marginal channels are partly lined by separate endothelial cells (fig. 2).

*Changes induced by MC.* In general, the higher the concentration of MC and the longer the exposure time were, the stronger were the morphological effects For instance, a 4-hour exposure to 1000 µg Hg/l brought about the same degree of damage as a concentration of 100 µg Hg/l for a period of 1 week. The weakest effect concerned a slight disarray as well as some curling of the secondary lamellae (fig. 3). This was seen at concentrations of 100 µg Hg/l in the 4-hour experiment, 50 µg Hg/l in the 24-hour experiment and 10 µg Hg/l in the 1-week experiment. At higher concentrations, the epithelium became slightly swollen and contained small intercellular lymphoid spaces (fig. 4). This was apparent at concentrations of 500 µg Hg/l in the 4-hour experiment, 100 µg Hg/l in the 24-hour experiment and 25 - 50 µg Hg/l in the 1-week experiment. At the highest concentrations used, the epithelial cells became detached from the basal membrane, commencing at the proximal regions and progressing towards the distal regions of the lamellae. This resulted in the development of large sub-epithelial spaces (fig. 5). Generally, the final stages of cellular disintegration in the

Plate I Structural effects of mercury on the gills of rainbow trout
Fig 1 Transverse section of two adjacent gill filaments with alternating secondary lamellae of control fish, x 400
Figs 2-5 Structure of gill filaments and secondary lamellae of fish exposed to various concentrations of mercuric chloride (MC) for 24 hours Compare condition of control fish (fig 2) with the hypertrophic effect of dosages ranging from 50 µg Hg/l (fig 3), 100 µg Hg/l (fig 4) and 250 µg Hg/l (fig 5), x 730

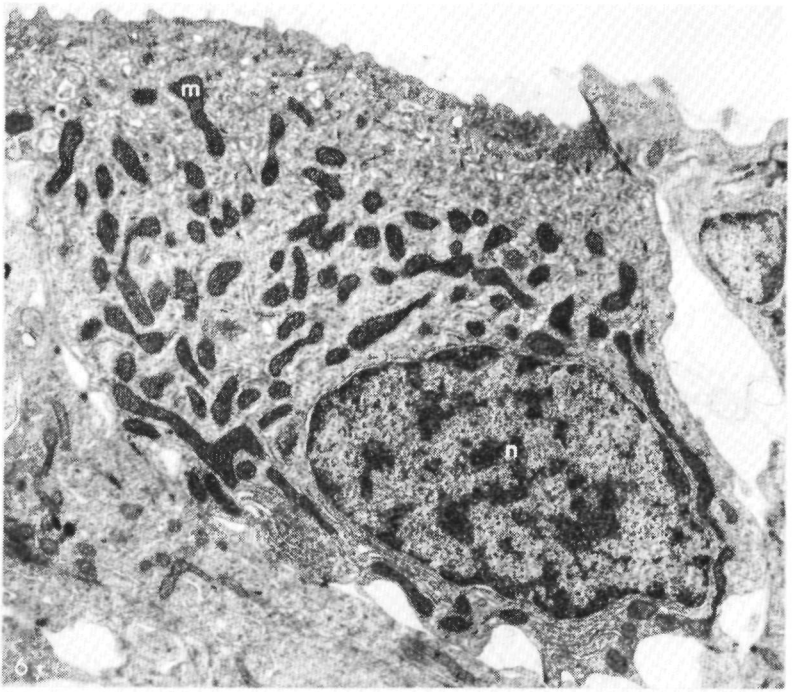
case of acute lethal concentrations showed cellular debris between the lamellae, an apparent result from the sloughing of dead epithelial cells.

*Changes induced by MMC.* The progressive structural deterioration in the gills of trout exposed to various MMC concentrations for different time periods was similar to that observed with MC (figs. 3, 4 and 5). However, MMC induced these changes at lower concentrations, namely approximately one twentieth of those of MC. Mitotic figures in the epithelium were more abundant in MMC and MC treated fish than in control fish.

*Removal of mercury.* In those cases where mercury treatment had induced moderate effects, e.g. hypertrophy with some vacuolization of the gill epithelium, recovery was usually complete 24 hours to 1 week after transfer of the fish to mercury-free water. Usually, recovery following MC treatment took 2 - 3 times longer than after MMC exposure at equitoxic concentrations. In cases, where the epithelium had undergone considerable desquamation, the damage proved to be irreversible and the fish died soon after mercury was withdrawn.

*Chloride cells.* The most conspicuous features of the chloride cell are the large number of mitochondria, which are variable in size and shape, and a closely-packed tubular membrane system between the mitochondria (fig. 6). The tubular system is continuous with the plasma membrane. The tubules are about 50 - 100 nm in diameter and are of low electron density, while the intertubular spaces may contain electron-dense matter including free ribosomes (fig. 7). Chloride cells were mainly located around the bases of

Plate II. Fig. 6. Chloride cell of gill epithelium of untreated control rainbow trout; x 9,000. Fig. 7. Detail of chloride cell of control fish, showing structure of mitochondria and tubular membrane system; x 23,000. Fig. 8. Detail of chloride cell of fish exposed to 100 µg Hg/l as mercuric chloride (MC) for 1 week; x 23,000. Note the swollen appearance of the tubular membrane system. n = nucleus; m = mitochondria; t = tubular membrane system; rer = rough endoplasmic reticulum.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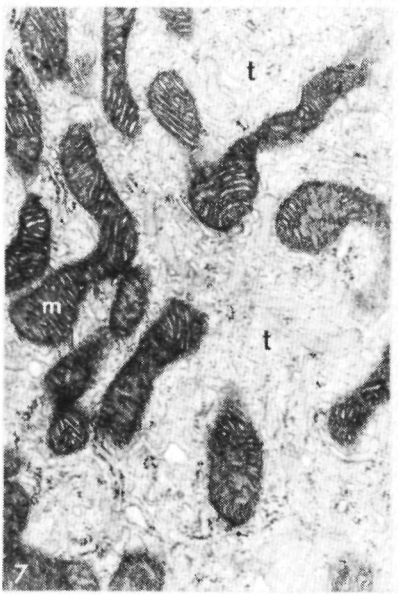
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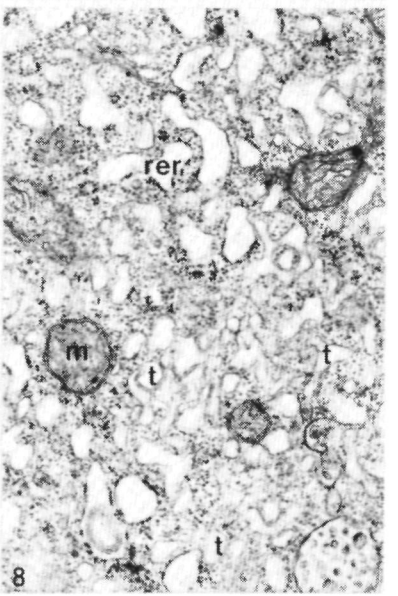
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were the effects. The quantified results, obtained by morphometrical analysis of the cytoplasmic and mitochondrial areas are presented in table 2.1. After exposure of trout to MC concentrations of 100 µg Hg/l or lower for 4 hours, 50 µg Hg/l for 24 hours and 10 µg Hg/l for 1 week, there were no noticeable differences with the control chloride cells. The majority of the mitochondria had retained their normal appearance. The endoplasmic reticulum and the tubular membrane system on the whole appeared normal. Exposure to MC concentrations of 100 - 500 µg Hg/l for 4 hours, 100 - 250 µg Hg/l for 24 hours and 25 - 50 µg Hg/l for 1 week, resulted in a rather varied response. Adjacent to normal looking chloride cells other were found with diffuse cytoplasm and swollen mitochondria. Often within the same cell, swollen mitochondria appeared randomly distributed among normal looking ones. Also, localized swelling of the tubular membrane system was apparent. Exposure to the highest MC concentrations, namely 1000 µg Hg/l or higher for 4 hours, 500 µg Hg/l or higher for 24 hours and 100 µg Hg/l for 1 week, resulted in prominent changes: increased vacuolization of the cytoplasm and an irregular, damaged,and highly swollen tubular membrane system (fig. 8). Mitochondria often appeared swollen and some were disintegrated.

*Changes induced by MMC.* In general, the morphological changes observed in the chloride cells of trout exposed to MMC did not differ from those induced by MC. However, MMC induced such changes at concentrations that were only approximately 5 - 10% of those of MC. The results are presented in table 2.1.
the secondary lamellae in the multi-layered epithelium of the gill filaments. Occasionally, chloride cells were found in the epithelium of the secondary lamellae as well.

*Changes induced by MC.* Just as with the light microscope observations, the analysis revealed that the higher the mercury concentrations were and the longer the exposure time, the stronger

Table 2 1 Effect of mercuric chloride (MC) and methylmercuric chloride (MMC) on the cytoplasmic area and average mitochondrial area of chloride cells in the gill epithelium of rainbow trout after continuous exposure to various concentrations of mercury for 4 hours (A), 24 hours (B) or 1 week (C) Values are expressed as mean ± S E M , n = 6, *p < 0 05

| | MC ; g Hg/l | Cytoplasmic area ($\mu^2$)** | Average mitochondrial area ($\mu^2$)*** | MMC g Hg/l | Cytoplasmic area ($\mu^2$)** | Average mitochondrial area ($\mu^2$)*** |
|---|---|---|---|---|---|---|
| A | 0 | 63 7 7 2 | 0 23 0 04 | 0 | 67 3 5 8 | 0 27+0 05 |
| | 50 | 58 3+6 1 | 0 27 0 06 | 5 | 69 1±6 4 | 0 31+0 06 |
| | 100 | 71 4+9 3 | 0 21 0 03 | 10 | 63 7+5 1 | 0 28 0 03 |
| | 500 | 69 5+3 3 | 0 30 0 07 | 50 | 72 8+4 7 | 0 30 0 04 |
| | 1000 | 73 7±8 2 | 0 24 0 04 | 100 | 79 1±3 2* | 0 34 0 06 |
| | 2000 | 74 1 6 9* | 0 26+0 05 | 200 | 76 2_6 1 | 0 32 0 04 |
| B | 0 | 59 8±5 7 | 0 25 0 03 | 0 | 65 3 4 1 | 0 24 0 03 |
| | 50 | 61 1+4 3 | 0 26 0 04 | 1 | 63 2+3 8 | 0 27 0 04 |
| | 100 | 58 6+5 1 | 0 29 0 04 | 5 | 68 1+5 5 | 0 26+0 06 |
| | 250 | 64 3 3 3 | 0 27+0 05 | 10 | 68 9±3 1* | 0 27+0 04 |
| | 500 | 66 8_5 1 | 0 32+0 03 | 25 | 71 8 2 9* | 0 28+0 04 |
| | 1000 | 73 4+4 7* | 0 33 0 02* | 50 | 74 3 4 3* | 0 34+0 03* |
| C | 0 | 64 1+4 9 | 0 22+0 02 | 0 | 62 4 3 7 | 0 26 0 03 |
| | 10 | 66 3+5 7 | 0 21+0 03 | 1 | 67 6±5 8 | 0 22 0 04 |
| | 25 | 68 2±2 3 | 0 24+0 03 | 2 5 | 65 9 9 3 | 0 29+0 04 |
| | 50 | 71 4 6 1 | 0 28 0 05 | 5 | 66 3 3 3 | 0 30+0 05 |
| | 100 | 75 6 4 2* | 0 31 0 03* | 10 | 71 4 5 1* | 0 33±0 04* |
| | 200 | 79 3+3 4* | 0 39+0 04* | 20 | 75 3±4 3* | 0 34+0 03* |

** otal cell area minus nuclear area

*** otal mitochondrial area per cel civ ded by the number of mitocrondr a

## DISCUSSION

The gills of fishes are organs especially adapted for gas exchange and ion transport. The morphological adaptation for these functions is the great increase in surface area as illustrated by the numerous secondary lamellae. Our observations concerning the gill structure in the rainbow trout are in agreement with data provided by others concerning the same species (Morgan and Tovell, 1973) as well as with reports on other teleosts (Newstead, 1967; Hughes and Morgan, 1973).

When fish are exposed to mercury or other heavy metals dissolved in the water, the gills function as the major route of uptake for these compounds. Experimental evidence for this thesis was provided by Olson *et al.* (1973) who compared mercury uptake in rainbow trout with and without ligation of the esophagus. Indeed, when mercury compounds were administered with the

food (Hannerz, 1968) or directly by way of injection (Giblin and Massaro, 1973), the concentration of mercury in the gills, relative to those in other tissues and organs, remained low.

It is obvious from our results that the transport function of the gills for mercury is accompanied by a considerable deleterious effect on the gill structure and, therefore, possibly on the gill functions. Qualitatively, the effects on the gill structure caused by the two mercury compounds employed are similar, but when the degree of the effects is considered, MMC is by far the more toxic of the two: roughly 10 - 20 times more MC was required to induce histopathological changes comparable to those evoked by MMC. This ratio is about the same as that concerning the 96-hour $LD_{50}$ values for the two mercury compounds (see chapter 1). As the more lipophilic MMC accumulates much faster than MC and, consequently, reaches higher concentrations in the tissues, we must conclude that the degree of the damaging effect on the gill structure is a function of the mercury concentration in the tissue itself rather than being related to the compound's chemical structure.

The observed effect of mercury on the gills is not specific for this heavy metal (see also Lindahl and Hell, 1971; Wobeser, 1975), but applies to other ones as well e.g. copper (Baker, 1969), cadmium (Voyer *et al.*, 1975), zinc (Skidmore and Tovell, 1972) and chromium (Strik *et al.*, 1975). Our observation of a mercury-induced increase in mitotic activity of the gill epithelium suggests proliferation of one or more cell types, including chloride cells, as a compensation for epithelial cell damage and decreased osmoregulatory capacity of the gills. This is reminescent of an investigation by Wobeser (1975) who, using the same species, reported a mercury-induced hyperplasia of the gill epithelium.

Another suggested toxic effect of heavy metals in fish is the development of hypoxia, not only caused by damage of the

gill tissue but also due to coagulation and precipitation of mucus (Westfall, 1945; Ultsch and Gros, 1979). Although our study did not involve an analysis of gas exchange, the observed effect on the gill structure of both mercury compounds used, leaves little doubt that the respiratory function was adversely affected. Already the weakest effect observed, namely a disarray of the gill secondary lamellae, probably caused increased friction of the water flow over the gill surface which, at least theoretically, would result in reduced efficiency of the gas exchange mechanism. In addition, hypertrophy of the epithelial cells means an increase in the diffusion distance. More important is the swelling and detachment of the interlamellar epithelium. In many cases the proximal part, i.e. roughly one third of the pillar cell system of the leaflets can probably no longer participate effectively in the process of gas exchange. Morgan and Tovell (1973) however, suggested that lifting of the epithelium may constitute a protective effect, as it would hinder uptake of pollutants by increasing the diffusion distance. Clearly, this last interpretation seems incomplete as such a response will take place at the expense of the respiratory efficiency. Ultimately, the respiratory handicap imposed by epithelial lifting must outweigh any beneficial effect with regard to mercury uptake.

Through the chloride cells the gills of fish are directly involved in the process of osmoregulation. In freshwater adapted teleosts, these cells actively take up monovalent ions and thus assist in maintaining the osmotic and ionic composition of the body fluids (Maetz, 1971, 1974; Maetz and Bornancin, 1975). For the uptake of at least one of these ions, namely $Na^+$, the tubular membrane system is of crucial importance as this is the morphological structure with which the $Na^+$-$K^+$-ATPase, an enzyme directly involved in the transport of $Na^+$, is associated (Karnaky *et al.*, 1976). Also involved in this $Na^+$ pump are the mitochondria which generate the enzyme's

substrate. Thus, the fact that mercury compounds in affecting the chloride cells caused damage to both the tubular membrane system and the mitochondria makes it likely that the osmoregulatory function of the chloride cells in the mercury treated trout is affected.

Another interesting observation concerns the general swelling of the chloride cells following mercury treatment. Disturbance of cell volume regulation easily occurs when $Na^+$-$K^+$-ATPase is inhibited. This was demonstrated for a variety of cell types by Oschman *et al.* (1974). In freshwater adapted fish, such as the rainbow trout under the conditions of our experiments, a dysfunctioning of the $Na^+$-pump in the chloride cells would certainly cause increased osmotic water uptake. However, the same response is to be expected when the structural integrity of the cell membrane is affected. In this connection it seems worthwile to point out that several investigators reported that mercury affects the cell by altering the structure of the cell membrane and,consequently, its permeability (Rothstein, 1959, 1970; see also Webb, 1966). Whether in trout the osmoregulatory capacity of the gills is indeed affected by exposure to mercury, and whether such effect is to be attributed to a disturbed $Na^+$-$K^+$-ATPase activity of the gills or to a change in cell permeability is the subject of an investigation reported in chapter 4.

# CHAPTER 3

## EFFECTS OF MERCURIC CHLORIDE AND METHYLMERCURIC CHLORIDE ON THE ACTIVITY OF MUCOUS CELLS IN RAINBOW TROUT

### INTRODUCTION

Mucus or slime is a product that results from an interaction between mucin, a glycoprotein containing secretion, and water. In teleosts fishes mucin is secreted by mucous cells, which are found throughout the epidermis, the linings of the buccal cavity, the pharynx and the gills, as well as in epithelia of a variety of internal organs.

The layer of mucus covering the external surface of the fish may serve several purposes. Continuous production and release of mucus could prevent the settling of pathogenic organisms (Jakowska, 1963). Indeed, the presence of bacteriolytic enzymes, antibodies, and lysozyme activity in surface mucus (Fletcher and Grant, 1969) indicates a protective function. Furthermore, a layer of mucus prevents or reduces skin damage caused by abrasia (Yamazaki, 1972). A related function is the capacity of mucus to coagulate and precipitate particles in suspension, thus providing protection to delicate tissues such as the gill filaments. The general lubricating effect of fish slime has also been recognized. An investigation by Rosen and Cornford (1971) supports the notion that mucus provides assistance in the fish's locomotion by strongly reducing the friction with water. In addition, mucus is used by a number of teleosts for specialized functions related to their reproduction, e.g. nestbuilding and feeding of the young.

It has long been discussed whether mucus affects the osmoregulatory capacity of teleosts (Van Oosten, 1957; Hughes and Wright, 1970). This idea itself is not surprising as mucous cells abound at the sites where ionic interactions between the fish and its environment take place: the gills, the skin, the intestinal tract and, in some cases, the kidney. A pertinent observation was made by Burden (1956) who noticed that in the euryhaline

species *Fundulus heteroclitus* the number of mucous cells in the gills was higher in freshwater adapted fish than in seawater adapted fish. Similar observations on several different species have been published by others (Potts and Evans, 1966, Mattheij and Sprangers, 1969; Blanc-Livni and Abraham, 1970).

Effects of heavy metals on mucus production in teleost fishes have been reported by several investigators. Increased mucus accumulation on the gills and skin of fish has been observed following exposure of goldfish to lead nitrate (Westfall, 1945); of catfish to copper- and zinc sulphate (Lewis and Lewis, 1971), of goldfish to mercuric chloride (McKone *et al.*, 1973; Lock, 1975; Varanasi *et al.*, 1975), and of rainbow trout to methylmercuric chloride (Olson *et al.*, 1973; Lock, 1975).

To our knowledge, no systematic, quantitative study on the effect of mercury compounds on mucus production in fish has been published. The aim of our investigation was to analyze quantitatively the effects of mercuric chloride (MC) and methylmercuric chloride (MMC) on mucous cell activity in the rainbow trout, *Salmo gairdneri*. These mercury compounds were compared in view of their differences in uptake ratio and toxicity. Three different regions were considered the skin of the gill operculum, the skin of the tail peduncle, and the gill epithelium.

## MATERIALS AND METHODS

*Fish.* For general information on source and maintenance of the rainbow trout, see chapter 2. Length of the fish used for the experiments reported here ranged from 135 - 175 mm, their weight varied from 34 - 48 grams. Three different experiments were done:

*1. Effect of MC and MMC on tissue mucus content and mucous cell density.* Groups of 15 fish were exposed to MC in concentrations ranging from 50 - 2000 µg Hg/l, or to MMC in concentrations varying from 5 - 100 µg Hg/l, for periods of 4 hours, 24 hours or 1 week. At the end of these periods, fish were killed in a

neutralized MS 222 solution. Samples of the gill operculum, peduncular skin and gills were collected in preweighed test tubes for mucus determination. After removing the opercula, their insides were carefully cleaned with cotton swabs so as to exclude mucus from the buccal cavity and the gills. The mucus determination, therefore, included only mucus derived from the outside of the opercular skin. For each assay 6 - 9 pieces of tissue were used to assess whether a correlation existed between the weight of the sample and its mucus content. For mucus determination, see below. At the same time, samples of the three tissues were fixed in Bouin's fluid, dehydrated and embedded in paraplast for histological examination. For histological techniques, see below.

2. *Comparison between mucus content of the gills and opercular movements.* Groups of 6 trout were exposed to MC or MMC in concentrations of the same range as employed in the previous experiment. The frequency of the opercular movements was determined during the last hour of the exposure period. For mucus determination, see below.

3. *Effect of MC and MMC on mucus release into the water.* Groups of eight fish were continually exposed to MC or MMC in concentrations ranging from 0 - 50 µg Hg/l for periods of 4 hours, 24 hours or 1 week. Immediately thereafter, the flow of the water was stopped to allow accumulation of mucus for a period of 4 hours. For mucus determination, see below.

*Mucus determination.* For the determination of mucus the thiobarbituric acid assay of Warren (1959) was used. In short, the method is based on coupling liberated and oxidized sialic acids to thiobarbituric acid. The resulting chromophore was quantitatively determined by spectrophotometry at 549 nm after extraction in cyclohexanone. In a preliminary experiment the sialic acid composition of the skin mucus of control fish was compared with that of mercury-exposed fish. After carefully collecting

the mucus in test tubes, particulate matter (e.g. scales) was removed by centrifugation at 1,000g for 20 minutes and the supernatant freeze-dried. To free the terminal sialic acids from the side chain of the glycoproteins, the freeze-dried sample was hydrolyzed in 1 ml of 0.1 N $H_2SO_4$ at 80 C for 1 hour. Partial purification of the hydrolyzed sample was achieved by ion exchange column chromatography according to Svennerholm (1958). Sialic acids were further separated by two-dimensional paper chromatography according to Svennerholm and Svennerholm (1958). Identification and quantification of sialic acids was done according to Pickering (1976). Two sialic acids were found. One, comprising approximately 80% of the total, was identified as N-acetyl-neuraminic acid (NANA). The other one, comprising about the remaining 20% was not identified, but may have been an O-acetylated NANA (Buscher *et al.*, 1974). No difference in the sialic acid composition and the calculated sialic acid percentage (0.9% on a dry weight basis) was found between control and mercury-treated fish. As the Warren technique was applied with NANA as a standard sialic acid, all values were expressed as NANA equivalents.

To determine the mucus content of the water in which fish had been maintained, three 2-liter samples of water were filtered through Whatman No. 1 paper. The filter with the collected mucus was cut into small pieces, added to 25 ml of 0.1 N $H_2SO_4$ and hydrolyzed at 80 C for 1 hour. Unused filters served as blanks. After removal of the paper fibres by centrifugation, the supernatant was further purified by column ion exchange chromatography according to Svennerholm (1958). The eluate was freeze-dried and the sialic acids redissolved in 0.3 ml of distilled water and quantified by the thiobarbituric acid assay of Warren (1959). The rate of mucus production was expressed as µmole NANA equivalents per gram fish per hour.

*Histological techniques.* To show the mucous cells, 7-µm thick

sections were stained with a 1% Alcian Blue solution in 3% acetic acid, followed by Heidenhain's Azan technique (Romeis, 1948). Mucous cell density was determined as follows. *Gill operculum:* since during the dissection procedure removal of the mucus from the inside of the operculum often damaged the inside epidermis, for the analysis only the skin at the operculum's outside was used. With the help of an ocular micrometer, from a series of adjacent vertical sections the number of mucous cells in the epidermis was counted. Per fish, approximately 10 - 12 sections, totalling at least 100 mm length, were examined and the average number of mucous cells per mm length of the epidermis was calculated. *Peduncular skin:* transversal sections of the tail peduncle were used, whereby the lateral line system served as a reference point. Of a total of approximately 12 adjacent sections all mucocytes present in that part of the epidermis which extended 2 mm above and below the lateral line, on both sides, were counted and the average number per mm length of the epidermis calculated. *Gill epithelium:* cross sections of one gill, involving the two hemibranchs and showing a series of cross-cut filaments were used. Mucocytes were counted in comparable sections, all at the same distance distal from the insertion of the adductor muscles. Per fish, a total of approximately 40 such filament sections were counted. The density of the mucous cells was expressed as the average number per section of the filament.

Mercury concentrations in the water were determined by flameless atomic absorption spectrophotometry (see chapter 2). For statistical evaluation of the data Student's t test was used. All tests were two-sided at the 5% significance level.

## RESULTS

*Effect of MC and MMC on mucus content and mucous cell density.* Under all three different experimental conditions, MC caused a significant increase in mucus content of the operculum, pedun-

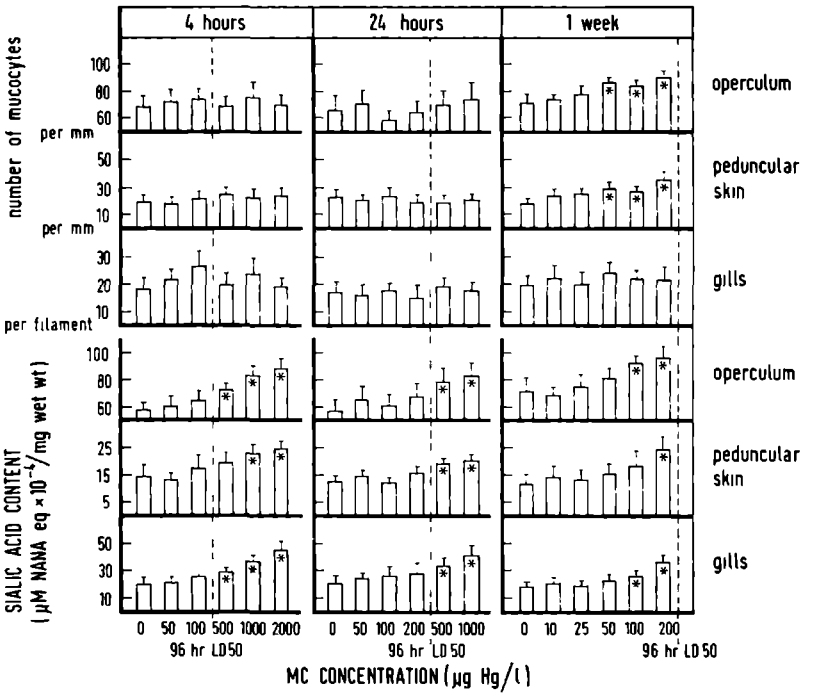



Fig. 3.1. Effect of mercuric chloride (MC) on the number of mucous cells and the mucus content of the operculum, peduncular skin and gill filaments of rainbow trout after continuous exposure to various concentrations in the water for 4 hours, 24 hours or 1 week. Mean values ± S.D.; n = 22; *p < 0.05.

cular skin and gills, but only at the two or three highest concentrations employed (fig. 3.1). MMC also exerted such effect except that in the 1-week experiment the response of the operculum and the peduncular skin, even at the highest concentration used, was not statistically significant (fig. 3.2). Both MC and MMC induced an increase in the mucous cell density of the opercular and peduncular skin but only in the 1-week experiment and, in this case too, the effect was noted at the higher concentrations only. No effect could be found on the mucous cell density in the gills (figs. 3.1 and 3.2).

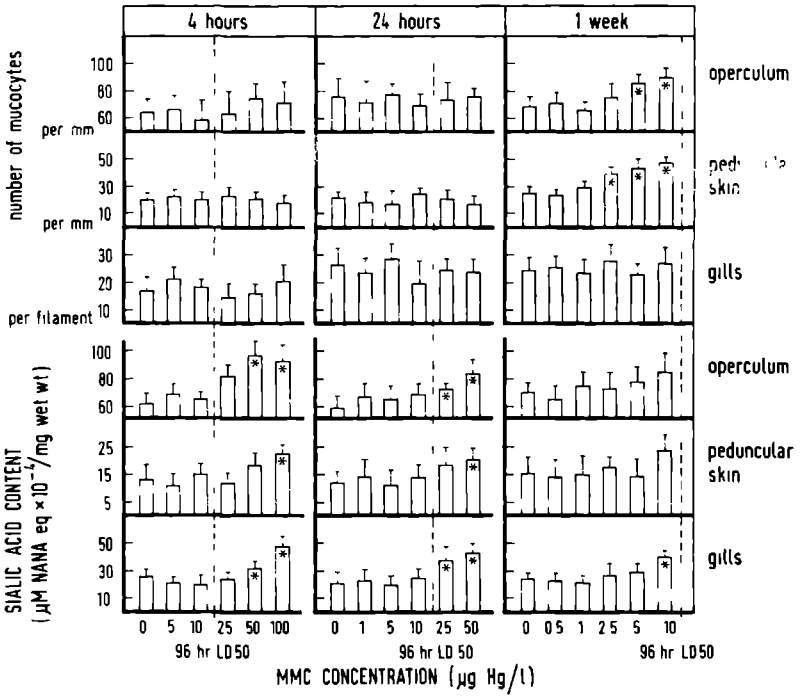



Fig. 3.2. Effect of methylmercuric chloride (MMC) on the number of mucous cells and the mucus content of the operculum, peduncular skin and gill filaments of rainbow trout after continuous exposure to various concentrations in the water for 4 hours, 24 hours of 1 week. Mean values ± S.D.; n = 18; *p < 0.05.

*Comparison between mucus content of the gills and opercular movements*. Both MC and MMC, when administered for 4 hours, 24 hours or 1 week, caused significant increases of the opercular movements (figs. 3.3 and 3.4). In general, the frequency of the movements increased with increasing concentrations of mercury and reached levels significantly different from the control levels at the higher concentrations. The results, however, clearly show a tendency for a reduced effect at the highest concentrations. In the same fish the mucus content of the gills was enhanced at the higher concentrations of MC and MMC (fig. 3.3 and 3.4).

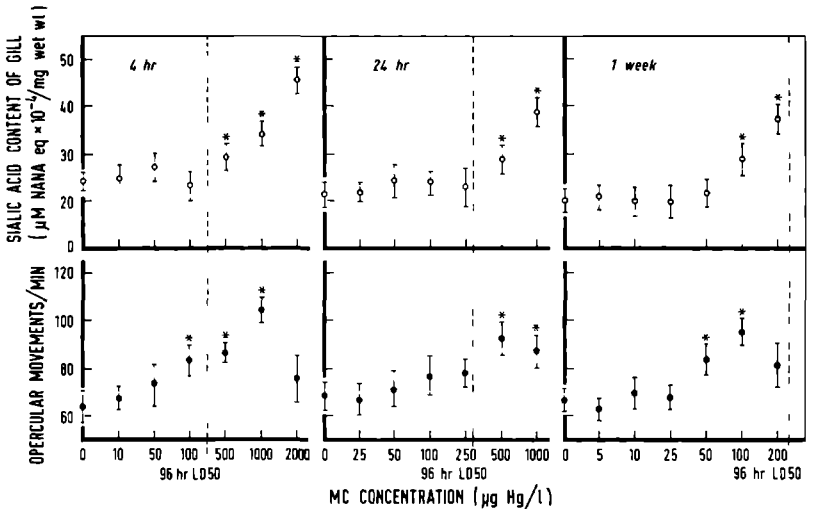



Fig. 3.3. Mucus content of the gills and rate of opercular movements of rainbow trout after continuous exposure to various concentrations of mercuric chloride (MC) in the water for 4 hours, 24 hours or 1 week. Mean values ± S.D.; n = 8; *p < 0.05.

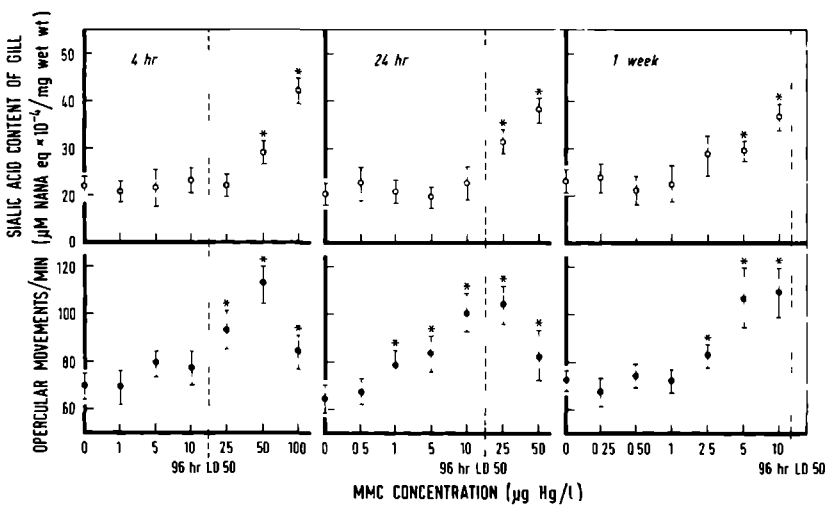



Fig. 3.4. Mucus content of the gills and rate of opercular movements of rainbow trout after continuous exposure to various concentrations of methylmercuric chloride (MMC) in the water for 4 hours, 24 hours or 1 week. Mean values ± S.D., n = 8; *p < 0.05.

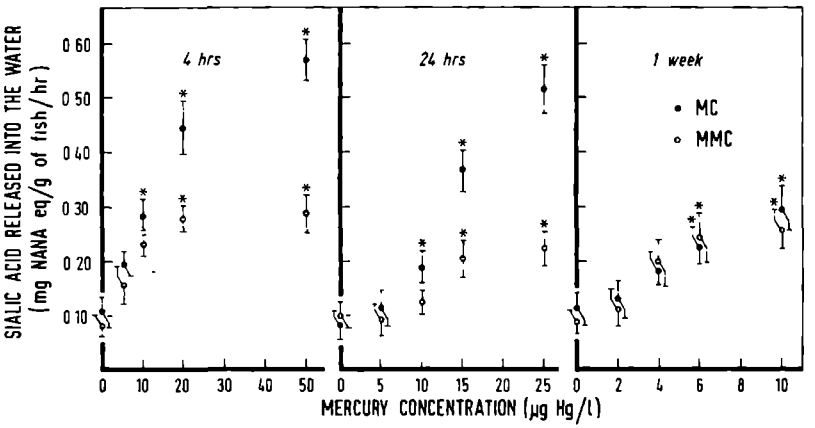



Fig. 3.5. Release of mucus into the water by rainbow trout after continuous exposure to various concentrations of mercuric chloride (MC) or methylmercuric chloride (MMC) for 4 hours, 24 hours or 1 week. Water samples were taken 4 hours after the flow of water had been stopped. Data represent mean values ± S.E.M. of three experiments. *p < 0.05.

It should further be noted that in most cases minimal significant enhancement of the opercular movements was observed at mercury concentrations lower than those causing minimal significant increases in mucus content of the gills.

*Effect of MC and MMC on mucus release into the water.* In all three experimental situations MC as well as MMC caused a significant increase in the amount of mucus released, except at the lowest levels of the different ranges of concentrations (fig. 3.5). For each of the three periods of exposure there was a clear dose-response relationship. In the 4-hour and 24-hour treatments, MC at concentrations of 10 µg Hg/l or higher, exerted a much stronger effect than MMC. No such difference could be seen following an exposure period of 1 week.

DISCUSSION

In all three experiments treatment of rainbow trout with MC or MMC strongly induced mucus secretion. Of the three parameters used to measure the effect on mucous cell activity, mucus release into the water appeared to be the most sensitive. For instance, MC in the 4-hour exposure period already significantly enhanced the release of mucus at a concentration of 10 µg Hg/l (exp. 3), a concentration of 500 µg Hg/l was required to induce an increase in the mucus content of the tissues in the same period, while a dosage as high as 2000 µg Hg/l did not yet affect the number of mucocytes (exp 1) The results concerning the 24-hour treatment clearly show the same trend. When one also considers the results of the 1-week treatments it is, however, clear that to elicit a response, not only the mercury concentration but also the duration of exposure is of importance, this is particularly obvious from the data concerning the effect on mucocyte density With regard to the mercury exposure for 1 week in experiment 1, the results at first seem somewhat confusing Mucous cell densities in both areas of the skin were significantly enhanced by MC in a concentration of 50 µg Hg/l Yet, mucus content of these tissues increased only at concentrations of 100 - 200 µg Hg/l This 'discrepancy" may be explained by the observation in experiment 3 that MC in a dosage as low as 6 µg Hg/l already induced an increase in release of mucus. The same conclusions may be drawn for the effect of MMC.

In contrast to the two epidermal tissues, the gills did not respond to treatment for 1 week with either mercury compound by increasing their mucous cell density Yet, a clear increase in mucus content of the gills was noted It is unlikely that this inconsistency may be explained by assuming that the exposure time of 1 week was too short to induce mucous cell proliferation As a matter of fact, it must be realized that the mucus present in the gill tissue samples is not necessarily a product of the mucous cells in the filaments only. Rich sources of mucus are

present in the nearby buccal and pharyngeal epithelium and this mucus is, at least partly, transported over the gill surfaces. Moreover, it must be emphasized that the general response of the skin epidermis to mercury differs from that of the gill epithelium. The epidermis reacts with an over-all strong hypertrophy and hyperplasia of which the conspicuous recruitment of newly formed mucous cells from undifferentiated cells in the stratum germinativum is only part. In the gill epithelium some cell proliferation takes place but also degeneration and, in general, at the edges of the filaments where the mucous cells are mainly located there is no change in thickness of the epithelium, while the mucous cells retain their typical goblet cell appearance. It is possible, therefore, that in the gill filaments the mucous cells responded to mercury by increasing their secretory activity rather than by becoming more numerous. A difference in behaviour between mucous cells in the skin and mucous cells in the gills in teleosts has been reported before, namely with regard to the effect of the pituitary hormone prolactin. At least in some species, this hormone has been shown to stimulate mucocytes in the skin but not in the gills or *vice versa* (Mattheij and Sprangers, 1969; Mattheij and Stroband, 1971; Marshall, 1976, 1979).

To compare the effects of MC and MMC on the mucus release in the water, the minimal effective doses may be expressed as percentages of the 96-hour $LD_{50}$ values (275 µg Hg/l for MC and 24 µg Hg/l for MMC). For MC, these percentages are 3.6 (4-hour exposure), 3.6 (24-hour exposure) and 2.1 (1-week exposure). For MMC the comparable percentages are 83.2, 62.5 and 25.0 respectively. Thus, on the basis of equitoxic concentrations MC exerts a much stronger effect on mucous secretion than MMC. The results of experiment 3, at least those concerning the 4-hour and 24-hour exposure periods, show that the mucus production induced by MMC reaches a maximum and levels off well below the maxima obtained with MC. This levelling-off occurs approximately

at the lethal concentration of MMC which probably is a direct consequence of the toxic effect. The result of a much stronger mucus-stimulating effect of MC, as compared to MMC, confirms the observations by Olson *et al.* (1973) who stated: "Observations of fish from this and numerous other experiments have shown that methyl mercury is not a greater stimulus to mucus secretion than inorganic mercury and, in fact, the opposite is often the case". A similar conclusion has been reported by Wobeser (1975).

The question arises whether a mercury-induced increased mucus production affects the physiological functions of the trout. One direct consequence of an increased mucus content of the gills might be interference with normal gas exchange as suggested already by Westfall (1945) and more recently demonstrated by Plonka and Neff (1969) and Ultsch and Gros (1979). The results of experiment 2, a comparison between mucus content of the gills and opercular movements, support the notion of a mercury-induced hypoxia. We consider the increased opercular movements to be a compensatory activity related to increased mucus production and aimed at removal of excess mucus and increasing $O_2$ supply and/or release of $CO_2$. Considering this view, it is not surprising that the reduced opercular movements at the highest concentrations of MC and MMC are accompanied by the strongest increases in mucus content.

A second function which could be influenced by enhanced mucification is the process of osmoregulation. If mucus plays a role in this process, as has been suggested in various studies (Pickford *et al.*, 1966, Potts and Evans, 1966; Wittouck, 1975; Marshall, 1976), it would be expected to act at the gill surface, where the major uptake of ions occurs. Van Oosten (1957) suggested that a mucous layer might impede passive ion movement, which in freshwater fish would then reduce the loss of salts from the body fluids. The results of a recent study by Marshall (1978), however, do not support the notion that mucus affects

ion diffusion. Another possibility is that mucus through its ion-binding capacity might serve to concentrate cations in relatively ion-deficient freshwater and thereby promote active uptake of salts (Olson and Fromm, 1973; Kirschner, 1977). At any rate, if mucus plays a role in osmoregulation, the mechanism whereby it contributes to this process remains to be elucidated.

Finally, it is of particular significance that the mucous coating on the fish's body surface acts as a mercury-binding resin (McKone *et al.*, 1971; Olson and Fromm, 1973; Lock, 1975). This capacity of mucus probably causes elimination of part of the mercury compounds, thereby reducing their uptake via the gills. To what extent mercury-induced mucus production constitutes a defense mechanism, resulting in reduced uptake via the gills has, to our knowledge, not yet been investigated.

# CHAPTER 4

## EFFECTS OF MERCURIC CHLORIDE AND METHYLMERCURIC CHLORIDE ON OSMOLALITY OF THE PLASMA, $Na^+$-$K^+$-ATPase ACTIVITY OF THE GILL EPITHELIUM AND WATER UPTAKE BY THE GILLS IN RAINBOW TROUT

### INTRODUCTION

Our preliminary experiments (chapter 1) indicated that in the rainbow trout, mercury at sublethal concentrations causes a reduction of the osmotic value of the blood plasma. Especially the gills are involved in the maintenance of osmolality and ion composition of the blood of freshwater fish by actively taking up ions from the water (Maetz, 1974). Furthermore, as the gill epithelium, which constitutes over 70% of the body surface, is relatively permeable to water, the gills are also the main site of osmotic water movement (Evans, 1969; Motais *et al.*, 1969).

As was demonstrated in chapter 2, mercuric chloride (MC) and methylmercuric chloride (MMC) affect the gill structure, including the chloride cells. It seems, therefore, logical to investigate whether the mercury-induced reduction in the osmolality of the plasma in rainbow trout is caused by a disturbance of the ion uptake mechanism or by a change in the permeability for water.

The chloride cells in particular are responsible for the role of the gills in ion uptake and, therefore, in maintaining the ionic composition of the body fluids. This osmoregulatory process involves the important enzyme $Na^+$-$K^+$-ATPase. Since Skou (1957) proposed a link between $Na^+$-$K^+$-ATPase activity and cation transport, numerous studies have shown the involvement of this enzyme with the active uptake of $Na^+$ in freshwater fish (Maetz, 1974). Within the chloride cells, the $Na^+$-$K^+$-ATPase activity was found to be mainly associated with an extensive tubular membrane system (Karnaky *et al.*, 1976). In freshwater fish $Na^+$ likely enters the chloride cells via $Na^+/NH_4^+$ and $Na^+/H^+$ exchange mechanisms (Maetz, 1973; Evans, 1975). The $Na^+$-$K^+$-ATPase in the tubular membrane system of the cells participates in the further transport

of the $Na^+$ ions to the blood by $Na^+/K^+$ exchange (Kerstetter *et al.*, 1970; Maetz, 1974).

In view of the very strong accumulation of mercury in the gills of trout and the observed damage to the tubular membrane system of the chloride cells (see chapter 2), we decided to investigate whether the reduction in the osmolality of the plasma in these fish was caused by a reduced $Na^+$-$K^+$-ATPase activity in the membrane fraction of the epithelial cells of the gills. In relation to this last question, the distribution of mercury over the various cell fractions was studied.

Besides ion transport, osmoregulation involves the control of water movement. Freshwater fish are continuously faced with the problem caused by water entering osmotically via the gills. In contrast to the gills the skin of teleosts is relatively impermeable for water (Bentley, 1962; Motais *et al.*, 1969). To prevent hemodilution, the osmotic water influx of the gills is maintained at a low level (Evans, 1975). Little is known about mechanisms reducing water movement through the gills, although some investigations indicate a significant role of hormones, notably prolactin, as well as calcium ions in this process (Potts and Fleming, 1970, 1971; Cuthbert and Maetz, 1972; Ogawa *et al.*, 1973; Ogawa, 1974, 1975, 1977).

To investigate the possibility whether the observed decrease in osmolality of the blood is to be attributed to an enhanced permeability of the gill epithelium for water, the effect of MC and MMC on the water uptake by the gills was studied.

## MATERIALS AND METHODS

*Experimental procedure.* Exposure of rainbow trout to either mercuric chloride (MC) or methylmercuric chloride (MMC) at various concentrations for periods of 4 hours, 24 hours or 1 week, was carried out according to the procedure described in chapter 2. At the end of each experiment, fish were removed from the tanks, anaesthetized in a neutralized solution of MS 222, briefly

blotted dry and their caudal peduncle cut off. From the caudal artery of each fish, blood was collected into heparinized hematocrit capillaries. After centrifugation, hematocrit values were measured and the plasma stored at -20 C. Later, the plasma osmolality of a 50-µl sample was measured with a Vogel Micro Osmometer. After appropriate dilution, sodium, potassium, calcium, chloride and phosphate concentrations were determined using a Technicon Autoanalyzer.

For the determination of the $Na^+$-$K^+$-ATPase activity in the microsomal fraction of the gills, four gill arches (two from both sides) were removed, rinsed in a physiological saline solution, the gill filaments removed from the gill arch, and homogenized in an icecold 250 mM sucrose, 25 mM imidazole-HCl buffer (pH 7.4) with an Ultra-Turrax homogenizer. The homogenate was filtered through surgical gauze to remove the cartilagenous gill rays. The filtrate was then centrifuged so as to obtain the following fractions: nuclei (1,000 g,10 min.), heavy mitochondria (10,000 g,10 min.), light mitochondria (20,000 g, 20 min.) and microsomes (100,000 g,60 min.). The microsomal pellet was resuspended in the homogenization buffer and stored at -20 C for determination of ATPase activity. To check whether all mitochondria had been removed after the last centrifugation step, the activity of the mitochondrial marker enzyme, cytochrome c oxidase was determined in the microsomal fraction as described by Cooperstein and Lazarow (1951).

To determine the *in vitro* effect of MC and MMC on the ATPase activity, gill filaments from 14 rainbow trout, 20 - 25 cm long, were pooled, homogenized and filtered as described above. The homogenate was divided equally over two series of 7 tubes each. MC was added to one series and MMC to the other, such that final concentrations in the tubes of both series were 0.02, 0.2, 2, 20, 200 and 2000 ppm Hg. A seventh tube in each of the two series served as a control tube. After an incubation period of 60 minutes at 4 C, the samples were fractionated as described before.

*Subcellular mercury distribution.* In a separate experiment, the mercury content of the various cell fractions of gill tissue was determined. This analysis was made after *in vitro* incubation to MC or MMC of a gill homogenate for a period of 1 hour and after *in vivo* exposure of trout to MC or MMC for a 24-hour period. The various pellets were analyzed for their mercury content after strong acid digestion, according to the procedure described in chapter 2. Mercury concentration of each pellet was expressed as ng Hg per mg protein content.

*Determination of $Na^+$-$K^+$-ATPase activity.* $Na^+$ plus $K^+$ stimulated (ouabain sensitive) ATPase activity was determined as the difference in ATPase activity in two media, one yielding total ATPase activity (medium A) and the other yielding the ouabain insensitive rest ATPase activity (medium E) For composition of both media see Bonting (1970). All determinations were carried out in triplicate

The reaction was started by addition of a 20-µl aliquot of the microsomal fraction of the gills to 400 µl of the assay medium and subsequent incubation at 37 C for 20 minutes. Hereafter, the reaction was stopped by addition of 1.5 ml icecold 8.6% (w/v) solution of trichloroacetic acid (TCA). To each tube 1 5 ml of freshly prepared solution of 9.6% (w/v) $FeSO_4.6H_2O$, 1.15% (w/v) ammonium heptamolybdate in 0.66 M $H_2SO_4$ was added, and after centrifugation at 1,000g for 30 minutes at room temperature, the absorbance at 700 nm was determined on a Zeiss PM2DL spectrophotometer. The amount of ATP ($Na_2ATP$, Boehringer, Mannheim) hydrolyzed can be calculated from the 700 nm absorbance of standard inorganic phosphate solution (0 625 and 1.25 mM $P_1$), treated in the same way. To correct for non-enzymatic phosphate production, endogenous phosphate,and reagent stain, blanks were prepared by incubating 400 µl medium E and adding the 20-µl aliquot after the addition of the TCA solution. Phosphate (as $KH_2PO_4$) concentrations were determined according to the method of Fiske and Subbarow (1925). Protein was estimated by the

method of Lowry *et al.* (1951) with bovine serum albumin (BSA) serving as a standard. Specific activity of the ATPase was expressed as µmol $P_i$/mg protein/hour.

*Determination of water uptake by the gills.* The remaining four gill arches were excised to determine the influence of *in vivo* exposure to MC and MMC on the *in vitro* water uptake by the gills. The rate of water uptake by isolated gills was studied according to the method of Ogawa (Ogawa *et al.*, 1973; Ogawa, 1974), with a modified calculation procedure for the permeability, developed in collaboration with dr. M.A. van 't Hof of the Department of Statistical Consultation.

Permeability (P) is defined as the quantity of water entering per ml initial water content of gill tissue per minute per unit of concentration gradient. This definition implies that permeability is now mainly dependent on the quality of the membrane and not on the external environment or size of the gill.

Isolated gills were rinsed in freshwater teleost saline (containing per liter: 9.9862 g NaCl, 0.1864 g KCl, 0.2646 g $CaCl_2$ and 0.0168 g $NaHCO_3$; total osmotic pressure of this solution is 300 mOsm/l) and were then incubated in a fresh sample of the same oxygenated medium for 20 minutes. This incubation period is necessary to equilibrate the gill tissue to a medium with a known osmotic value. Each gill arch was blotted dry on tissue paper and its wet weight ($W_o$) determined. After transfer to distilled water for $t_i$ = 15, 30 and 45 minutes, its weight was measured again as $W_1$, $W_2$ and $W_3$ respectively. The tissue was then freeze-dried and its dry weight ($W_d$) determined. For this situation the following model was defined: The amount of water entering the gill tissue in a given time span is proportional to the ionic concentration gradient and to the duration of the uptake ($\Delta t$). The constant in this relation is the product of the initial water content of the gill ($V_o$) and its permeability (P) i.e. $PV_o$. The ionic concentration gradient is

the difference between the salt concentration in the gills (s) and that in distilled water (=0), being the total amount of salt in the gill tissue ($sV_o$) divided by the volume at that moment, V(t). This leads to the following equation:

$$V(t + \Delta t) - V(t) = PV_o.sV_o/V(t).\Delta t$$

or $$\frac{dV(t)}{dt} = \frac{P.sV_o^2}{V(t)}$$

or $$V^2(t) = 2PsV_o^2t + V_o^2$$

or $$\frac{V(t)^2}{V_o^2} = 1 = 2Pst$$

This means that the relative change in the quadratic volume is proportional to time. The permeability may be calculated as:

$$P = \left(\frac{V^2(t)}{V_o^2} - 1\right)/2st.$$

To apply this formula in our experimental situation, it is necessary to find the water contents of the gills ($V_i$) at $t_i$ = 0, 15, 30 and 45 minutes of incubation. This is given by:

$$V_i = (W_i - W_d)/sg$$

in which sg is the specific gravity of distilled water. The permeability ($P_i$) at $t_i$ is calculated as:

$$P_i = \left(\frac{V_i^2}{V_o^2} - 1\right)/2st_i$$

$$\text{or} \quad P_i = \left[ \left( \frac{W_i - W_d}{W_o - W_d} \right)^2 - 1 \right] / 2st_i$$

Every $P_i$ is an estimate for the same value of the gill permeability. Therefore, the average, $(P_1 + P_2 + P_3)/3$ is taken as the permeability value for each gill arch. For statistical evaluation of the data Student's t test was used. All tests were two-sided at the 5% significance level.

## RESULTS

*Plasma ion composition.* Osmotic values and electrolyte concentrations of the plasma as well as hematocrit values are presented in tables 4.1 and 4.2.

Tabel 4.1. Plasma osmolality (mOsm/l), electrolyte concentrations (meq/l) and hematocrit values (%) of rainbow trout after continuous exposure to various concentrations of mercuric chloride (MC) for 4 hours (A); 24 hours (B); or 1 week (C). Values are expressed as mean ± S.D.; (n) = number of observations; *p < 0.05.

| | MMC (µg Hg/l) | Osmolality | $Na^+$ | $K^+$ | $Ca^{2+}$ | $Cl^-$ | $PO_4^-$ | Hematocrit |
|---|---|---|---|---|---|---|---|---|
| A | 0 (24) | 307 4· 8 2 | 151 4· 5 9 | 4 1 0 6 | 2 56·0 11 | 115 9· 7 8 | 1 23·0 11 | 34 4 4 3 |
| | 5 (24) | 311 2· 6 4 | 154 7· 6 4 | 4 7 0 3 | 2 5.·0 14 | 119 2· 8 4 | 1 17·0 10 | 35 1·6 1 |
| | 10 (24) | 304 7± 5 8 | 148 2· 6 2 | 3 9·0 5 | 2 45·0 12 | 121 4 6 2 | 1 20·0 14 | 32 2 4 8 |
| | 50 (21) | 300 6· 4 3 | 149 2· 4 8 | 4 0·0 7 | 2 36·0 11 | 109 4· 4 5 | 1 01·0 07* | 37 6 3 7 |
| | 100 (16) | 281 7·11 5* | 128 9· 6 1* | 5 1 0 7 | 2 38·0 19 | 101 4· 5 2* | 1 07·0 10 | 35 3·6 3 |
| | 200 (14) | 264 2·16 2* | 112 6· 9 4* | 5 7·0 4* | 2 44 0 10 | 96 1· 7 1* | 1 14·0 11 | 39 1·7 4 |
| B | 0 (28) | 303 4· 5 7 | 146 7· 7 3 | 3 7 0 4 | 2 64·0 12 | 117 2· 7 4 | 1 31·0 14 | 37 2·4 8 |
| | 1 (28) | 306 8· 6 1 | 151 6· 8 5 | 4 1±0 5 | 2 71·0 14 | 120 3 6 6 | 1 42 0 16 | 39 8·7 3 |
| | 5 (26) | 305 7· 8 4 | 142 1·11 5 | 3 9·0 3 | 2 60·0 18 | 114 9· 8 5 | 1 27 0 12 | 32 9 3 6 |
| | 10 (26) | 297 4· 7 9 | 140 9· 8 9 | 4 1·0 4 | 2 47·0 09 | 112 7· 9 2 | 1 19·0 16 | 33 6·6 1 |
| | 25 (22) | 284 8· 9 1* | 130 9· 7 8* | 4 3·0 5 | 2 43 0 14 | 103 7 5 3* | 1 11·0 13 | 39 2·5 4 |
| | 50 (18) | 264 8±14 1* | 113 4· 9 3* | 4 6·0 6 | 2 59·0 08 | 100 2· 8 0* | 1 14·0 15 | 41 6·6 9 |
| C | 0 (32) | 309 5· 4 6 | 156 6· 8 1 | 4 2·0 5 | 2 49·0 13 | 123 7· 4 8 | 1 23·0 11 | 33 8·2 6 |
| | 1 (32) | 294 6· 7 6 | 147.3· 6.0 | 3 9·0 4 | 2 38 0 12 | 117 6· 8 3 | 1 17·0 15 | 37 7 4 1 |
| | 2 5 (26) | 298 3· 6 9 | 133 8·.0 4* | 4 7·0 5 | 2 36·0 14 | 111 7·11 6 | 1 10·0 12 | 42 3·9 5 |
| | 5 (22) | 282 6· 7 8* | 108 6· 7 1* | 5 1 0 6 | 2 32 0 10 | 89 3· 9 4* | 1 00·0 14 | 44 6·6 1* |
| | 10 (22) | 264 7·14 5* | 101 8·11 8* | 5 8·0 8* | 2 24·0 26 | 81 5· 7 8* | 1 02 0 06* | 45 1 4 8* |
| | 20 (16) | 243 6·19 7* | 91 2·10 2* | 5 6·0 4* | 2 21·0 24 | 90 8·10 6* | 1 16 0 07 | 48 4 7 5* |

Table 4.2. Plasma osmolality (mOsm/l), electrolyte concentrations (meq/l) and hematocrit values (%) of rainbow trout after continuous exposure to various concentrations of methylmercuric chloride (MMC) for 4 hours (A); 24 hours (B); or 1 week (C). Values are expressed as mean ± S.D.; (n) = number of observations; *p < 0.05.

| | MC (µg Hg/l) | Osmolality | $Na^+$ | $K^+$ | $Ca^{2+}$ | $Cl^-$ | $PO_4^-$ | Hematocrit |
|---|---|---|---|---|---|---|---|---|
| A | 0 (16) | 309 2 ±4 6 | 146 2· 6 1 | 4 3·0 7 | 2 86·0 14 | 117 1· 8 3 | 1 29·0 14 | 36 7 4 8 |
| | 50 (16) | 305 7· 9 2 | 143 8· 7 8 | 4 0 0 6 | 2 72±0 13 | 114 2· 9 4 | 1 33·0 11 | 39 3·5 1 |
| | 100 (16) | 311 6·10 8 | 140 9· 9 2 | 4 7·0 6 | 2 64·0 16 | 123 4·.1 7 | 1 44·0 12 | 35 8·2 3 |
| | 500 (14) | 301 4· 8 6 | 142 7· 8 1 | 4 6·0 4 | 2 81·0 19 | 118 6· 9 3 | 1 37·0 16 | 32 6·5 1 |
| | 1000 (12) | 288 6· 8 8* | 132 6 9 4* | 5 1·0 7 | 2 42·0 18* | 110 4·12 3 | 1 48·0 13 | 33 8·4 5 |
| | 2000 (8) | 283 7· 9 6* | 129 7· 8 3* | 5 3·0 4* | 2 12·0 11* | 96 5 7 8* | 1 05 0 10* | 38 3 6 7 |
| B | 0 (14) | 312 6·14 8 | 153 1· 7 6 | 4 1·0 6 | 2 62 0 14 | 114 7· 9 1 | 1 36·0 17 | 37 4·3 1 |
| | 50 (14) | 308 9 12 3 | 148 5± 8 9 | 4 3 0 7 | 2 53·0 14 | 109 6· 8 7 | 1 43·0 12 | 33 9·4 6 |
| | 100 (14) | 306 3·11 1 | 143 2· 9 5 | 4 6·0 5 | 2 46·0 12* | 111 7· 9 2 | 1 28±0 16 | 41 6±7 3 |
| | 250 (14) | 305 6·12 6 | 139 7·14.3 | 4 4·0 6 | 2 29 0 12* | 106 8·11 4 | 1 49·0 14 | 39 2±4 7 |
| | 500 (12) | 281 7·14 2* | 132 1·12 1* | 5 3·0 4* | 2 11 0 14* | 101 7· 6 3* | 1 19·0 11 | 41 2·5 2 |
| | 1000 (12) | 271 2 9 6* | 114 8·10 3* | 5 6·0 5* | 2 07·0 11* | 86 4·11 8* | 1 31·0 15 | 42 3·8 6 |
| C | 0 (18) | 306 7 9 4 | 155 6·11 6 | 4 5·0 7 | 2 81·0 11 | 121 2· 7 6 | 1 31·0 13 | 34 1·2 8 |
| | 10 (18) | 310 1·11 8 | 151 1 9 7 | 4 3 0 6 | 2 72 0 09 | 118 4· 6 2 | 1 44·0 17 | 36 6·4 3 |
| | 25 (20) | 306 4 10 1 | 144 6·10 7 | 3 9 0 5 | 2 68·0 10 | 120 6± 9 1 | 1 56·0 18 | 35 9 5 7 |
| | 50 (16) | 298 6 12 6 | 141 3·11 6 | 4 8 0 4 | 2 62·0 17 | 114 3· 6 5 | 1 08 0 14 | 39 3.2 3* |
| | 100 (16) | 287 2· 8 5* | 131 6· 9 4* | 5 2 0 7 | 2 31 0 11* | 107 2· 6 2* | 1 19·0 10 | 43 6±4 1* |
| | 200 (14) | 261 3·11 8* | 109 7·12 7* | 5 8 0 5* | 2 14 0 12* | 101 1·12 6* | 1 40·0 11 | 46 8·6 5* |

In all six experiments, the dosages MC or MMC that caused a significant decrease in plasma osmolality, also reduced the sodium and chloride concentrations. The data further show a trend for a mercury-induced increase in plasma potassium, although a significant effect was obtained in a few cases only. Plasma calcium concentrations also were mostly reduced in the mercury-treated fish, but significant changes were caused only by mercuric chloride at the highest concentrations used. The phosphate values do not indicate a clear trend, although in those cases where the change is significant, it concerns a reduction. Finally, hematocrit values of fish after a 24-hour exposure period, indicate a trend towards an increase. However, only after a 1-week exposure to the highest concentrations of either mercury compound this increase was significant.

Table 4.3 Distribution of mercuric chloride (MC) over the various fractions of gill tissue homogenate of rainbow trout. Incubation was carried out for 60 min at 4°C in media containing 250 mM sucrose, 25 mM imidazole-HCl (pH 7.4) and indicated mercury concentrations. Data are the mean values of duplicate experiments.

| Fraction | MC concentration in tubes (ppm Hg) | | | | | |
|---|---|---|---|---|---|---|
| | 2000 | 200 | 20 | 2 | 0.2 | 0.02 |
| | Total mercury concentration (ng Hg/mg protein) | | | | | |
| homogenate | 853.1 | 81.4 | 8.88 | 0.83 | 0.11 | 0.02 |
| nuclear $10^3$ g | 982.7 | 76.8 | 10.28 | 0.96 | 0.12 | 0.03 |
| h.mitochondrial $10^4$ g | 821.4 | 72.9 | 10.44 | 0.97 | 0.10 | 0.02 |
| l.mitochondrial $2x10^4$ g | 1045.8 | 81.3 | 10.02 | 0.97 | 0.11 | 0.02 |
| microsomal $10^5$ g | 1092.5 | 92.7 | 10.78 | 1.04 | 0.13 | 0.03 |

Table 4.4. Distribution of methylmercuric chloride (MMC) over the various fractions of gill tissue homogenate of rainbow trout. Incubation was carried out for 60 min at 4°C in media containing 250 mM sucrose, 25 mM imidazole-HCl (pH 7.4) and indicated mercury concentrations. Data are the mean values of duplicate experiments.

| Fraction | MMC concentration in tubes (ppm Hg) | | | | | |
|---|---|---|---|---|---|---|
| | 2000 | 200 | 20 | 2 | 0.2 | 0.02 |
| | Total mercury concentration (ng Hg/mg protein) | | | | | |
| homogenate | 888.4 | 84.3 | 8.38 | 0.86 | 0.09 | 0.02 |
| nuclear $10^3$ g | 1142.6 | 108.6 | 11.22 | 1.14 | 0.11 | 0.02 |
| h.mitochondrial $10^4$ g | 721.3 | 63.5 | 6.82 | 0.65 | 0.06 | 0.01 |
| l.mitochondrial $2x10^4$ g | 683.3 | 74.9 | 8.28 | 0.80 | 0.07 | 0.01 |
| microsomal $10^5$ g | 1266.1 | 138.2 | 14.24 | 1.38 | 0.20 | 0.03 |

*Subcellular distribution.* The results of mercury distribution over the various cell fractions following *in vitro* incubation of the gill homogenates with MC or MMC are presented in tables 4.3 and 4.4 respectively.

The highest concentrations of mercury were found in the microsomal fractions. This was more pronounced in the case of MMC than of MC. The distribution of mercury over the various fractions of gill tissue homogenate, after *in vivo* exposure of the fish to MC or MMC is presented in table 4.5. It appeared that the mercury was not homogeneously distributed throughout the cells. In general, more mercury was bound to the nuclear fractions and the microsomal fractions than to the mitochondrial fractions.

Table 4.5. Distribution of mercury over the various fractions of gill tissue homogenate of rainbow trout after exposure of fish for 24 hours to either mercuric chloride (MC) or methylmercuric chloride (MMC) at indicated concentrations. Data are the mean values of duplicate experiments, based on a total of eight fish per concentration.

| Fraction | MC concentration in water (ppb Hg) | | | | | MMC concentration in water (ppb Hg) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 50 | 100 | 250 | 500 | 1000 | 1 | 5 | 10 | 25 | 50 |
| | Total mercury concentration (ng Hg/mg protein) | | | | | | | | | |
| homogenate | 14.8 | 24.2 | 49.5 | 97.7 | 184.3 | 6.91 | 37.6 | 58.9 | 98.8 | 162.5 |
| nuclear $10^3$ g | 21.3 | 24.6 | 27.2 | 32.9 | 39.7 | 3.96 | 23.5 | 51.7 | 75.7 | 86.6 |
| h.mitochondrial $10^4$ g | 10.7 | 14.2 | 13.6 | 17.5 | 20.9 | 2.28 | 18.3 | 20.3 | 22.8 | 32.6 |
| l.mitochondrial $2x10^4$ g | 10.1 | 13.4 | 16.3 | 18.7 | 24.3 | 1.56 | 17.4 | 13.4 | 14.4 | 16.9 |
| microsomal $10^5$ g | 13.8 | 19.4 | 33.8 | 37.2 | 41.8 | 4.54 | 23.6 | 41.4 | 53.9 | 71.3 |

*Effect on $Na^+$-$K^+$-ATPase and $Mg^{2+}$-ATPase activity.* Initially, short-during *in vivo* experiments were carried out using sublethal concentrations (i.e. below the 96-hour $LD_{50}$ values) of MC and MMC. As no effect on the enzyme activity could then be found, it was decided to first study the *in vitro* effects of

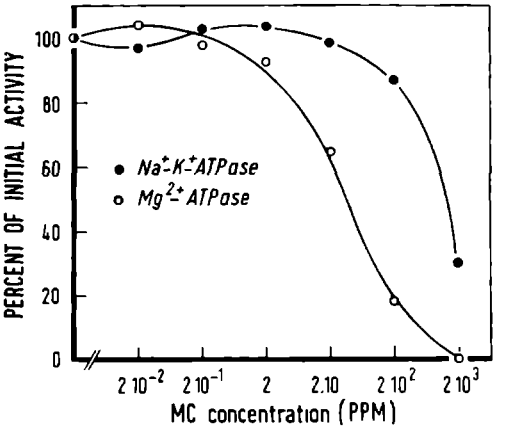



Fig. 4.1. Influence of mercuric chloride (MC) in vitro on the activity of $Na^+$-$K^+$-ATPase and $Mg^{2+}$-ATPase in the microsomal fraction of the gills of rainbow trout. Incubation is carried out for 60 min at 4°C in a medium containing 250 mM sucrose, 30 mM imidazole-HCl (pH 7.4) and mercury at concentrations stated. Data are the mean values of two experiments.

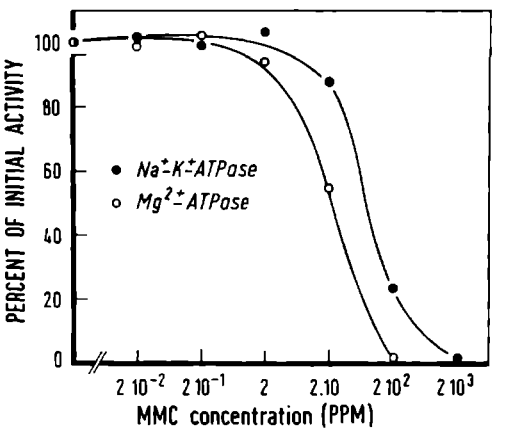



Fig. 4.2. Influence of methylmercuric chloride (MMC) in vitro on the activity of $Na^+$-$K^+$-ATPase and $Mg^{2+}$-ATPase in the microsomal fraction of the gills of rainbow trout. Incubation is carried out for 60 min at 4°C in a medium containing 250mM sucrose, 30 mM imidazole-HCl (pH 7.4) and mercury at concentrations stated. Data are the mean values of two experiments.

the mercury compounds on the ATPase activity of gill homogenates. Via the production of "mercury inhibition curves", and taking into account the known concentration factors for the gills (see chapter 1), it was assessed which concentrations of mercury would be necessary to obtain an effect *in vivo*. The results of the *in vitro* effects of MC and MMC are presented in figures 4.1 and 4.2 respectively. $Mg^{2+}$-ATPase was slightly more sensitive to mercury than $Na^{+}$-$K^{+}$-ATPase, particularly in

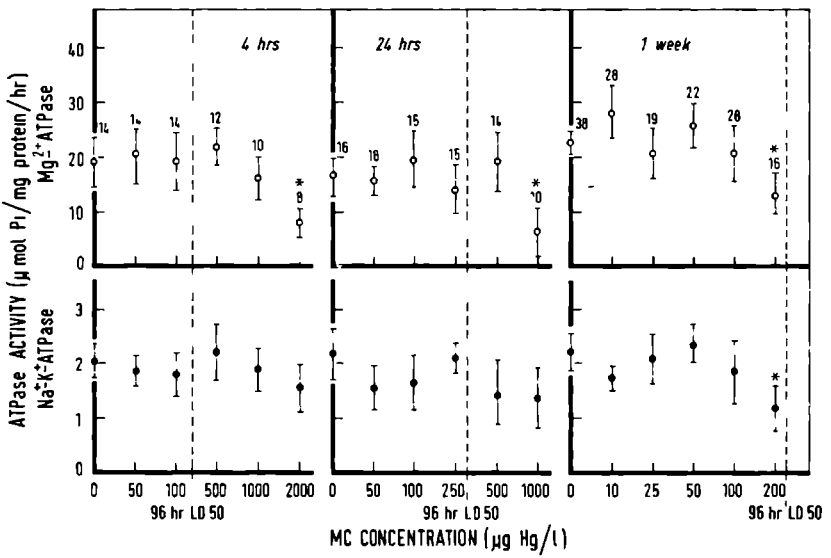



Fig 4.3 Effect of mercuric chloride (MC) on the activity of $Mg^{2+}$-ATPase and $Na^{+}$-$K^{+}$-ATPase in the microsomal fraction of the gills of rainbow trout, after continuous exposure to various concentrations for 4 hours, 24 hours or 1 week. Values are expressed as mean ± S.D , number above bars indicates number of observations, *p < 0 05

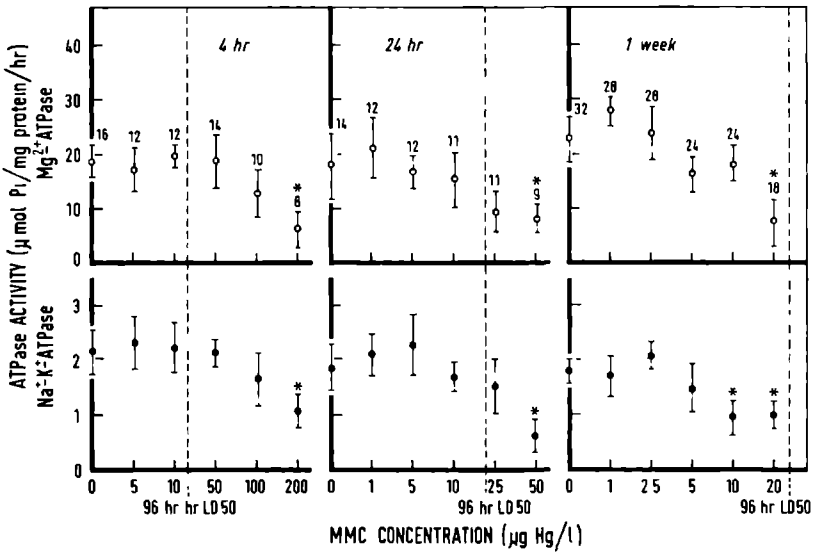



Fig. 4.4. Effect of methylmercuric chloride (MMC) on the activity of $Mg^{2+}$-ATPase and $Na^{+}$-$K^{+}$-ATPase in the microsomal fraction of the gills of rainbow trout after continuous exposure to various concentrations for 4 hours, 24 hours or 1 week. Values are expressed as mean ± S.D.; number above bars indicates number of observations; *p < 0.05.

the case of MC. From the data it was concluded that *in vivo* inhibition of the ATPases might only be obtained by exposing trout to concentrations of mercury approaching or exceeding the 96-hour $LD_{50}$ values. The results of such *in vivo* effects of MC and MMC on the gill ATPase activities are presented in figures 4.3 and 4.4 respectively. Inhibition of $Mg^{2+}$-ATPase was noticed in all six experimental conditions, but only at the highest concentrations of mercury used. $Na^{+}$-$K^{+}$-ATPase, likewise, was inhibited by MMC at the highest concentrations, but MC inhibited only in the 1-week experiment. Only in the 1-week experiments did concentrations lower than the 96-hour $LD_{50}$ value cause a significant decrease in the activity of both enzymes.

*Rate of water uptake by gills.* The measured weight increase and calculated permeability for water of the gill tissue after exposure of the fish to various concentrations of MC or MMC are presented in tables 4.6 and 4.7. The results show that in each of the six experiments only after a critical mercury level in the water had been reached, the permeability for water was enhanced greatly; further increases in mercury concentration did not result in further enhancement of the permeability. As the level of the critical concentration clearly depends on the duration of exposure, it is highly likely that the mercury concentration in the tissue, and not that in the water, is the critical factor.

Table 4.6. Weight increase of isolated gills of rainbow trout after different periods of incubation in distilled water. Fish had previously been exposed to different concentrations of mercuric chloride (MC) for 4 hours (A); 24 hours (B) or 1 week (C). Values are expressed as mean ± S.E.M.; n = 8.

| | MC (µg Hg/l) | Weight increase (%) 15 min | 30 min | 45 min | Permeability* $\times 10^{-4}$ | Significance** |
|---|---|---|---|---|---|---|
| A | 0 | 5.6±0.74 | 12.8±1.37 | 19.4±3.16 | 0.1607±0.0076 | - |
| | 50 | 4.7±0.89 | 15.6±2.06 | 20.1±2.41 | 0.1591±0.0049 | ns*** |
| | 100 | 5.4±1.12 | 17.2±1.35 | 23.6±2.92 | 0.1724±0.0066 | ns |
| | 500 | 8.6±1.23 | 18.9±1.54 | 24.9±2.04 | 0.2798±0.0111 | $p < 0.001$ |
| | 1000 | 11.4±2.19 | 19.4±2.25 | 27.3±2.19 | 0.2818±0.0082 | $p < 0.001$ |
| | 2000 | 14.6±3.36 | 21.8±3.38 | 32.5±4.36 | 0.3003±0.0082 | $p < 0.001$ |
| B | 0 | 6.1±0.93 | 14.7±1.26 | 23.1±2.18 | 0.1696±0.0057 | - |
| | 50 | 7.4±1.14 | 15.4±1.62 | 22.8±3.04 | 0.1636±0.0082 | ns |
| | 100 | 6.7±2.18 | 18.5±2.21 | 25.6±3.17 | 0.1699±0.0068 | ns |
| | 250 | 9.4±0.81 | 20.6±1.87 | 27.3±3.68 | 0.2854±0.0148 | $p < 0.001$ |
| | 500 | 10.5±1.74 | 22.1±1.64 | 26.1±2.08 | 0.2966±0.0080 | $p < 0.001$ |
| | 1000 | 11.3±1.31 | 23.7±2.09 | 25.9±1.91 | 0.2900±0.0085 | $p < 0.001$ |
| C | 0 | 5.8±0.66 | 15.1±1.32 | 19.4±1.91 | 0.1701±0.0119 | - |
| | 10 | 7.7±1.23 | 17.4±1.46 | 22.3±2.84 | 0.1664±0.0104 | ns |
| | 25 | 6.2±0.73 | 16.5±1.58 | 21.9±2.31 | 0.1629±0.0057 | ns |
| | 50 | 7.9±1.05 | 17.9±2.12 | 22.5±2.63 | 0.1821±0.0082 | $p < 0.001$ |
| | 100 | 10.4±2.16 | 21.3±1.69 | 31.1±2.92 | 0.2838±0.0097 | $p < 0.001$ |
| | 200 | 12.1±3.41 | 23.9±2.81 | 34.3±3.54 | 0.2930±0.0150 | $p < 0.001$ |

* ml $H_2O$/ml gill $H_2O$ content/min (Osmotic gradient is 300 mOsm/l)

** relative to difference with control group

*** n s not significantly different from control group

Table 4 7 Weight increase of isolated gills of rainbow trout after different periods of incubation in distilled water Fish had previously been exposed to different concentrations of methylmercuric chloride (MMC) for 4 hours (A), 24 hours (B) or 1 week (C) Values are expressed as mean ± S E M , n - 8

| | MMC (µg Hg/l) | Weight increase (%) 15 min | 30 min | 45 min | Permeability* x10$^{-4}$ | Significance** |
|---|---|---|---|---|---|---|
| A | 0 | 4 7 0 62 | 13 5+1 09 | 21 2+2 32 | 0 1670 0 0032 | - |
| | 5 | 5 3+0 94 | 12 6 1 43 | 19 7 1 82 | 0 1694±0 0017 | ns*** |
| | 10 | 5 2+0 73 | 14 8+1 81 | 22 3 2 08 | 0 1633+0 0030 | ns |
| | 50 | 9 3+1 66 | 20 3_2 09 | 25 9+2 23 | 0 2388 0 0054 | p < 0 001 |
| | 100 | 12 8+2 13 | 19 8 1 88 | 27 2+1 74 | 0 2761_0 0033 | p < 0 001 |
| | 200 | 13 3 2 05 | 21 6 2 11 | 29 5+3 17 | 0 2919+0 0068 | p < 0 001 |
| B | 0 | 6 4+1 12 | 12 7-1 85 | 18 8 2 44 | 0 1616+0 0039 | - |
| | 1 | 5 6+0 88 | 13 9 1 44 | 20 4+3 16 | 0 160b+0 0034 | ns |
| | 5 | 9 1 2 09 | 14 8+2 20 | 21 7+2 18 | 0 1668+0 0028 | ns |
| | 10 | 10 4 2 13 | 21 2_2 64 | 25 7+1 13 | 0 2807+0 0119 | p < 0 001 |
| | 25 | 9 4 2 31 | 16 4+1 92 | 22 6 1 36 | 0 2785+0 0049 | p < 0 001 |
| | 50 | 16 1+2 43 | 28 7+4 08 | 34 2+5 16 | 0 2535+0 0111 | p < 0 001 |
| C | 0 | 5 5+0 97 | 13 9+1 48 | 19 3-2 36 | 0 1585+0 0051 | - |
| | 1 | 7 4 1 02 | 16 2+1 20 | 21 3+1 17 | 0 1580±0 0050 | ns |
| | 2 5 | 8 2 0 80 | 18 7+1 38 | 19 9+1 26 | 0 1596+0 0056 | ns |
| | 5 | 8 6+1 12 | 17 3+1 56 | 24 7+1 91 | 0 2649 0 0102 | p < 0 001 |
| | 10 | 14 2 1 82 | 23 8 2 46 | 29 2+3 21 | 0 2734 0 0113 | p < 0 001 |
| | 20 | 13 0 1 28 | 20 1+1 84 | 27 8 3 56 | 0 2738 0 0119 | p < 0 001 |

* ml $H_2$ ml g 1l $H_2$0 content m n (Osmot c n ad ent s 300 mOsm 1)
** relat ve t d ffe ence w th on ol group
*** n s not s gn f can ly d fferent f om control g oup

## DISCUSSION

Although osmoregulation in freshwater teleosts involves three main organs, namely gills, kidney,and intestine, the first are possibly the most important. For the maintenance of the proper ionic balance in freshwater adapted fish, the gills are concerned with the active uptake of $Na^+$ and $Cl^-$ to compensate for the urinary salt loss. As the gills function as the main route of mercury uptake, the osmoregulatory function of the gills is undoubtedly affected first i e before that of the kidney and the intestinal tract This is especially to be expected in short-term experiments, where the mercury concentration in the gills is initially very high as compared to that in the kidney and the intestine (chapter 1) Indeed, the damage to the tubu-

lar membrane system of the chloride cells (chapter 2) in the mercury-exposed fish points towards an impaired osmoregulatory function of the gills. Thus, it is probable that the decrease in plasma osmolality in the mercury-treated trout was primarily the consequence of an impaired osmoregulatory capacity of the gills. However, the possibility that a compensatory response to this impairment will take place after longer exposure periods,as suggested by O'Connor and Fromm (1975),can not be entirely excluded.

The lowered osmotic value of the plasma obviously is the result of decreased $Na^+$ and $Cl^-$ concentrations. The qualitative effects of the two mercury compounds on the ionic composition were the same with one exception: MC, but not MMC, brought about a reduction in $Ca^{2+}$ concentration. The possible significance of this effect as well as the occasionally observed rise in plasma $K^+$ will be discussed in relation to the effects on water permeability of the gills.

We did not observe a significant inhibitory effect of MC and MMC on the activity of $Na^+$-$K^+$-ATPase except at acute lethal concentrations. The role of this enzyme in active transport of $Na^+$ and $K^+$ across cell membranes, with $K^+$ pumped into the cells and $Na^+$ out of the cells, is now widely accepted (Bonting, 1970; Skou, 1965, 1975). Various studies have reported an inhibitory effect of mercury on $Na^+$-$K^+$-ATPase activity. To our knowledge, in all of those studies, acute lethal concentrations of mercury were used. For example, Bouquegneau (1973a,b) reported elevated $Na^+$ and $Cl^-$ levels in the plasma of seawater adapted eels, *Anguilla anguilla*, following exposure to MC or MMC. Further studies showed that this response was possibly due to the inactivation of $Na^+$-$K^+$-ATPase activity of the gills (Bouquegneau and Disteche, 1973; Bouquegneau, 1977). Inhibition of the same enzyme by MC and MMC has also been demonstrated in a number of other fish (Renfro *et al.*, 1974) and, reportedly, is often accompanied by depressed $Na^+$ uptake.

Instead of using lethal dosages of mercury, as was done in these last studies, in our investigation we employed a wide range of concentrations, including sublethal ones. The results show that both MC and MMC only in lethal concentrations bring about inhibition of ATPase activity. This was confirmed by the results of the *in vitro* analysis of mercury on the gill homogenate. In this last experiment too, inhibitory effects were noted at concentrations which, had they been reached through *in vivo* accumulation, would have required exposure of the living trout to mercury levels exceeding the 96-hour $LD_{50}$ values. The question could be asked whether perhaps the mercury compounds were insufficiently concentrated in the membrane fraction containing the ATPases. This possibility must be rejected as our analysis of the mercury distribution, both *in vivo* and *in vitro*, indicated that, on the contrary, binding of mercury to the membrane (microsomal) fractions was relatively strong.

In this connection, it is important to realize that a demonstrable effect on the $Na^+$-$K^+$-ATPase in the gills required concentrations of mercury higher than those causing measurable changes in the plasma electrolytes. It seems unlikely, therefore, that inhibition of $Na^+$-$K^+$-ATPase in the gills is a direct cause of the decrease in plasma osmolality in mercury-treated trout.

A change in plasma $Cl^-$ concentration in teleosts following mercury treatment has been reported in seawater adapted eel (Bouquegneau and Disteche, 1973; Bouquegneau, 1977). They attributed both the increase in $Cl^-$ and that in $Na^+$ to an impaired $Na^+$-$K^+$-ATPase activity of the gills. It is questionable whether with regard to $Cl^-$ this conclusion is justified. Since Krogh (1939) suggested that freshwater adapted fish independently extract $Na^+$ and $Cl^-$ from the water, numerous studies have confirmed this for almost all species of teleosts investigated so far, including rainbow trout (Maetz and Garcia-Romeu,

1964; Kerstetter *et al.*, 1970; Kerstetter and Kirschner, 1972). Strong evidence has been provided that $Cl^-$ uptake by freshwater adapted teleosts is coupled to the excretion of $HCO_3^-$ to preserve electrostatic neutrality (Maetz, 1974; Evans, 1975). Theoretically, the possibility exists that mercury inhibits carbonic anhydrase which, in turn, would lead to a reduction of $HCO_3^-$ production and, consequently,to decreased $Cl^-$ uptake. The significance of the enzyme carbonic anhydrase for the exchange of $Cl^-$ seems to be limited, however, as injection of its specific inhibitor, acetazolamide, has no effect on $Cl^-$ uptake by the rainbow trout (Kerstetter and Kirschner, 1972).

The main problem freshwater adapted fish are facing is the continuous flow of water entering the gills osmotically and the loss of salts by diffusion. To minimize this danger, freshwater teleosts employ a very low drinking rate, a high urine output combined with an active reabsorption in the kidneys of $Na^+$ and $Cl^-$,and a low ionic and osmotic permeability of the gills. This last permeability is now considered to be under control of the pituitary hormone prolactin, which depresses the $Na^+$ efflux (Maetz, 1974) and water uptake (Ogawa *et al.*, 1973; Ogawa, 1974, 1975, 1977).

Our permeability studies show an increase of water influx into the isolated gills of trout exposed to mercury. Under *in vivo* conditions, such increased water influx may have caused a greater loss of $Na^+$ and $Cl^-$ due to an increased urine flow. The observed increased permeability of the gills for water takes place at concentrations of mercury lower than those affecting the $Na^+$-$K^+$-ATPase activity of the gills. The presumable ion loss via the urine is apparently not compensated by an increased uptake of $Na^+$ and $Cl^-$. Moreover, as no decreased hematocrit values were found, it is likely that *in vivo*, the increased influx of water is offset by an increase in urine flow.

The change in permeability characteristics of the gills could

have been caused by mercury binding to the sulfhydryl groups and other ligands of the cell membrane, altering its structure and function (Passow *et al.*, 1961, Passow, 1970; Rothstein, 1970). Our observation of an increased $K^+$ concentration in the blood after MC exposure suggests that the change in membrane permeability is not limited to the epithelial cells of the gills, but also occurs in other tissues, such as blood cells, which have been shown to have a high affinity for MC as well as MMC (Olson *et al.*, 1973; Olson and Fromm, 1973, Giblin and Massaro, 1975). Evidence in support of this mercury-induced loss of $K^+$ from the cells is provided by studies on the effect of mercury on the permeability characteristics of membranes in isolated cell cultures. For example, Sutherland *et al.* (1967) studying erythrocyte membrane sulfhydryl groups and cation permeability after exposure to various concentrations of mercury, found an increased loss of $K^+$ from the cells, accompanied by a high percentage of hemolysis. Similarly, Passow and Rothstein (1960), studying the effect of mercury on the permeability of yeast cells, found an increase in the general permeability of the membrane resulting in loss of $K^+$ and increased water uptake. Also, the decreased $Ca^{2+}$ concentrations in the plasma of MC exposed trout, as observed in our study, could have contributed to changed permeability characteristics of the cell membranes. Removal of $Ca^{2+}$ from the extracellular fluid is known to cause permeability changes in a variety of cells, resulting in a loss of intracellular $K^+$ (Morrill and Robins, 1967; Steen and Stray-Pedersen, 1975).

To our knowledge, no studies on the effect of mercury compounds on the permeability characteristics at the tissue level have been reported in the literature. The results of our experiments strongly support the suggestion that a change in the permeability characteristics of the cell membrane for water and, possibly , for ions, rather than an inhibition of the $Na^+$-$K^+$-ATPase,

is the primary reason for the decreased ion concentration in the blood and the observed swelling of the epithelial cells of the gills in mercury-treated rainbow trout.

# CHAPTER 5

## GENERAL DISCUSSION

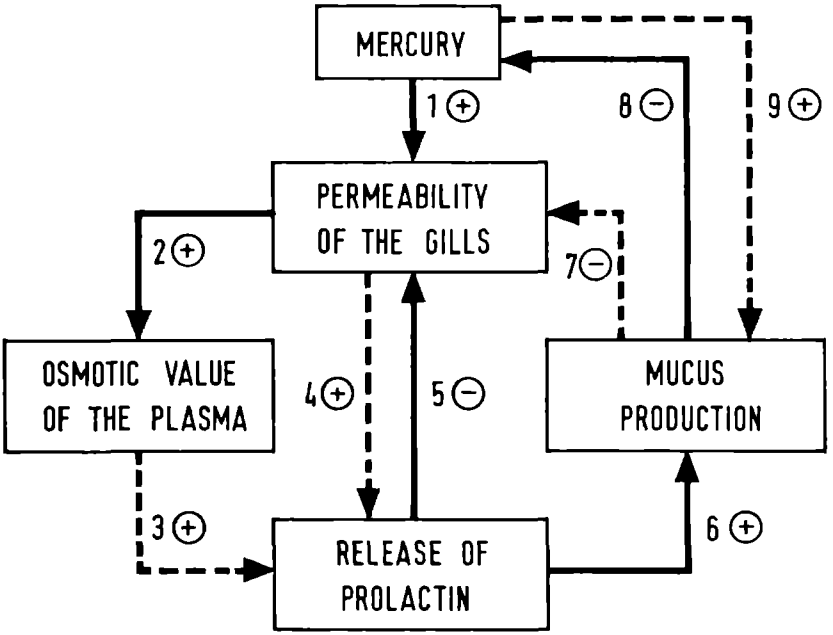
In our investigation, the shortest exposure time after which the various effects of the two mercury compounds were analyzed, was 4 hours. Of the influences of MMC within this period, the release of mucus appeared to be the most sensitive parameter, i.e. changes in mucus release were noted at concentrations lower than those affecting the other physiological activities. These other effects were noticeable only at increasingly higher concentrations in the following order. increased opercular movements; increased mucus content of the tissues and increased permeability for water of the gills, decreased osmolality and altered ion composition of the plasma, decreased activity of $Na^+$-$K^+$-ATPase activity of the gills. Roughly the same sequence in sensitivity was apparent when mercury treatment at lower concentrations was extended to a period of 24 hours or 1 week.

The same conclusion may be drawn for MC. Equitoxic concentrations of the two mercurials exerted roughly the same effects, except with regard to mucus release, in which case, at least in the 4-hour and 24-hour treatments, MC elicited a much stronger response than MMC.

The question may be asked, if, and in what way, there is a relation between the mercury-induced mucus production and the mercury-induced disturbance of the osmoregulatory capacity of the gills. Two points seem relevant One concerns the indication, that mucus plays a role in osmoregulation at sites where ion exchange takes place, such as the gills, either by concentrating cations in ion-deficient freshwater, or by creating an extra barrier, and thus impeding the passive efflux of ions (see chapter 3). In our opinion, the fact that mercury compounds affect both the mucus production and the osmoregulatory activity of the gills, may provide yet another indication for the

existence of a physiological relationship between these two processes. A second point is related to the control of mucus production and osmoregulation in teleost fishes. Since the classic experiment of Burden (1956), who reported atrophy of mucous cells in the gills of hypophysectomized killifish, and subsequent restoration to normal following injection of a pituitary extract, numerous studies have clearly implied the role of prolactin in maintaining the activity of mucous cells. On the other hand, a direct role of prolactin in the gills' osmoregulatory function, particularly their permeability for water, has also been established (Ogawa, 1974, 1977) Furthermore, *in vitro* studies have shown that a reduction of the osmotic value of the incubation medium directly affects synthesis and release of prolactin by isolated pituitaries of teleosts (Ingleton *et al.*, 1973).

Our findings that both mercury compounds affect mucus production and permeability for water of the gills, clearly imply that prolactin plays a role in the sequence of events following exposure to mercury. A pertinent hypothesis with regard to this involvement is the following (see also scheme). Mercury first causes an increase in the permeability of the gills (1), resulting in a lowering of the osmotic value of the plasma (2) which, in turn, may elicit synthesis and release of prolactin (3). There remains the possibility that the change in permeability of the gills affects the prolactin cell activity in some other way (4). Prolactin affects the gills such as to reduce their permeability, either directly (5), or through its stimulation of mucus production (6), indirectly (7). The absence of a measurable change in the permeability of the gills and osmotic value of the plasma at relatively low (sublethal) concentrations of mercury, at some point during the treatment, may well reflect the effectiveness of this compensatory reaction. At higher (lethal) concentrations one would expect the system to break down, resulting in measurable effects.




Included in the scheme is the likelyhood of elimination of mercury on account of the mercury binding capacity of mucus (8) and the possibility that mercury exerts a direct influence on mucous cells (9).

Several steps of the above scheme are now being studied. A recent investigation in our laboratory demonstrated that exposure of rainbow trout to concentrations of MMC of 2.5 µg Hg/l or higher for a period of 24 hours or longer, induced changes in pituitary prolactin cells indicative of increased synthetic activity.

# SUMMARY

Uptake of mercury from the water by freshwater fish almost exclusively takes place via the gills. The highest mercury concentration is therefore found in these organs This observation was the starting-point of a study on the effects of mercury compounds on various physiological functions of the gills in rainbow trout. Mercuric chloride (MC) and methylmercuric chloride (MMC) were compared in view of their different toxicities.

Chapter 1 describes the general aspects on the occurrence of mercury in the aquatic environment and the significance of the bottom sediments for the methylation of this metal. Uptake of both MC and MMC from the water by rainbow trout was studied. Mercury concentrations in the gills by far exceed those of other organs and tissues. This is particularly noticeable for MMC. Following transfer of fish to mercury-free water, the rate of elimination of MMC from the gills was faster than that of MC.

Chapter 2 describes the results of a study on the morphological effects of MC and MMC on the gills. The general effect was studied with the light microscope, while the influence on the chloride cells, which play a crucial role in the process of osmoregulation, was studied electron microscopically. Qualitatively, the effects on the gill structure caused by MC and MMC are similar, but when the degree of the effects is considered, MMC is by far (10 to 20 times) the more toxic of the two. Damage progressed from a slight disarray of the secondary lamellae, followed by hypertrophy and cell proliferation to complete desquamation of epithelial cells. Both mercury compounds also affected the chloride cells causing damage to the tubular membrane system with which $Na^+$-$K^+$-ATPase is associated. Impairment of the osmoregulatory function of the chloride cells seems

likely. It was concluded that the degree of the damaging effect on the gill structure is a function of the mercury concentration in the tissue itself, rather than reflecting a differential effect of the two compounds on account of their chemical structure.

Chapter 3 is a report of a study on the effects of MC and MMC on mucus production. Treatment of rainbow trout with MC or MMC strongly induced mucus secretion. Of the three criteria used, namely mucus content, number of mucous cells and release of mucus into the water, the latter appeared to be the most sensitive. Both mercury compounds caused strong hyperplasia of the epidermis and an increased number of mucous cells after a 1-week exposure. No significant changes in the number of mucous cells of the gill epithelium were observed. In the 4-hour and 24-hour experiments, MC exerted a much stronger effect on the mucus release than MMC at equal concentrations. The physiological consequence of an increased mucus production for the respiratory and osmoregulatory processes is speculated upon.

The study reported in chapter 4 concerns the effects of MC and MMC on osmolality of the plasma, $Na^+$-$K^+$-ATPase activity of the gill epithelium and water uptake by the gills. Both mercury compounds inhibited (*in vivo* and *in vitro*) ATPase only at lethal concentrations. Much lower concentrations of MC and MMC already caused an increased water uptake by the gills. The results strongly support the hypothesis that a change in the permeability of the cell membrane for water and, possibly, for ions, rather than an inhibition of the $Na^+$-$K^+$-ATPase, is the primary reason for the decreased osmotic value of the blood in mercury treated trout.

Chapter 5 is a general discussion of the results. Our findings that MC and MMC affect mucus production and permeability for water of the gills, clearly imply that the pituitary hormone prolactin plays a role in the sequence of events following exposure to mercury. A pertinent hypothesis with regard to this

involvement is presented in a scheme. It is probable that the mercury-induced increase of the permeability of the gills is already at an early stage counteracted by increased prolactin cell activity. It is suggested that prolactin reduces the permeability of the gills either directly, or through its stimulation of mucus production, indirectly. The absence of a measurable change in the permeability of the gills and osmotic value of the plasma at low mercury concentrations, may well reflect the effectiveness of this compensatory reaction.

# SAMENVATTING

In het water voorkomende verontreinigende stoffen, zoals kwikverbindingen, worden door vissen voornamelijk via de kieuwen opgenomen. Dit leidt tot hoge concentraties kwik, het eerst in het kieuwweefsel en daarna ook in andere organen en weefsels. Deze waarneming vormde het uitgangspunt van een onderzoek naar de invloed van kwikverbindingen op enkele kieuwfunkties bij de regenboog forel. Kwikchloride (MC) en methylkwik chloride (MMC) werden hierbij met elkaar vergeleken.

In hoofdstuk 1 wordt een samenvatting gegeven van de literatuur over het voorkomen van kwikverbindingen in aquatische milieu's en de betekenis van het bodemsediment voor de vorming van methylkwik. Vervolgens wordt de stapeling van kwik, inclusief de verdeling over de diverse weefsels en organen geillustreerd. MC maar vooral MMC wordt door kieuwen opgenomen in concentraties die vele malen hoger zijn dan die in andere organen en weefsels.

Hoofdstuk 2 gaat over de invloed van MC en MMC op de struktuur van de kieuwen. Het algemene effekt werd met de licht mikroskoop onderzocht, terwijl de invloed op de chloride cellen, die een beslissende rol in de osmoregulatie spelen, met de elektronen mikroskoop bestudeerd werd. Kwalitatief waren de effekten van MC en MMC op de kieuwstruktuur vergelijkbaar, hoewel de concentraties MMC die daar voor nodig waren, 10 tot 20 maal zo laag waren als die van MC. Beschreven wordt hoe beide verbindingen hypertrofie en proliferatie gevolgd door disintegratie van de epitheel cellen veroorzaken. Electronen mikroskopisch onderzoek toonde aan, dat vooral het tubulair membraan systeem, waarin het $Na^+$-$K^+$-ATPase is gelegen, wordt beschadigd. Een verlaagde osmoregulatorische capaciteit van de chloride cellen lijkt daardoor waarschijnlijk. Geconcludeerd werd dat de mate van beschadiging een gevolg is van de kwik concentratie

in het weefsel en niet direct samenhangt met de chemische struktuur van de kwikverbindingen.

Hoofdstuk 3 is een verslag van het onderzoek over het effekt op de mucusproduktie. MC en MMC verhoogden de mucus sekretie. Van de drie gemeten parameters, namelijk mucus gehalte, aantal mucus cellen en afgifte van mucus aan het water, bleek laatstgenoemde de meest gevoelige. Beide kwikverbindingen veroorzaakten sterke hyperplasie van de epidermis en een daarmee gepaard gaande vermeerdering van het aantal mucus cellen. Geen significante veranderingen werden waargenomen in het aantal mucus cellen van het kieuwepitheel Bij concentraties van overeenkomstige toxiciteit bleek MC, althans in de 4 uur en 24 uur experimenten, een sterker effekt op de slijmafgifte te hebben dan MMC. Over de fysiologische betekenis van een verhoogde mucus produktie voor de ademhaling en osmoregulatie wordt gespeculeerd.

Het onderzoek beschreven in hoofdstuk 4 gaat over het effekt van beide kwikverbindingen op de $Na^+$-$K^+$-ATPase aktiviteit van het kieuwepitheel en op de permeabiliteit van dit weefsel voor water. Zowel MC als MMC remden (*in vivo* en *in vitro*) het ATPase slechts bij lethale concentraties. Aanmerkelijk lagere concentraties van beide kwikverbindingen veroorzaakten reeds een verhoogde wateropname van het kieuwweefsel. Een en ander leidt dan ook tot de conclusie, dat de reeds door relatief lagere concentraties kwik geïnduceerde daling van de osmolaliteit van het bloedplasma eerder wordt veroorzaakt door een verhoogde permeabiliteit van de kieuwen dan door een verstoring van de $Na^+$-$K^+$-ATPase aktiviteit.

In een algemene discussie (hoofdstuk 5) is getracht om onze gegevens in een samenhangende hypothese samen te vatten. Deze hypothese stelt ons in staat om de verschillende aspekten van de relatie tussen een verhoogde mucus produktie en een verstoorde osmoregulatie beter te begrijpen. Geconcludeerd werd dat de

door kwik veroorzaakte verandering van de permeabiliteit van de kieuwen reeds in een vroeg stadium gecompenseerd wordt door een verhoogde afgifte van prolactine Dit hormoon verlaagt de permeabiliteit van de kieuwen hetzij direct, ofwel indirect via een verhoogde mucus produktie. Het feit dat lage concentraties kwik nog geen meetbare verandering in de permeabiliteit van de kieuwen en van de osmotische waarde van het plasma veroorzaakten, zou de doeltreffendheid van een dergelijke compensatie van prolactine weerspiegelen

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# CURRICULUM VITAE

R.A.C. Lock werd op 4 juni 1942 te Hilversum geboren. Na het behalen van het diploma van de Gemeentelijke Middelbare Handelsdagschool (1961, Amersfoort) vervulde hij de militaire dienstplicht tot 1963. Na verkrijging van het diploma van de Rijks Hogere Landbouwschool (1966, Deventer) volgde emigratie naar Canada. Tot augustus 1967 was hij werkzaam als analist aan het Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada. In september 1967 werd met de biologiestudie aan laatstgenoemde universiteit begonnen. In april 1970 werd de graad van Bachelor of Science (Biology), en in april 1974 de graad van Master of Science (Biology) behaald. Vanaf september 1974 tot heden was hij aangesteld als wetenschappelijk medewerker aan de afdeling Dierfysiologie van de Faculteit der Wiskunde en Natuurwetenschappen van de Katholieke Universiteit te Nijmegen, waar het onderzoek voor dit proefschrift werd verricht. Daarnaast werd een bijdrage geleverd aan het onderwijs voor prekandidaats- en doctoraal studenten biologie.

# STELLINGEN

## I

Bij de bepaling van de permeabiliteit voor water van de kieuwen in vissen verdient de *in vitro* methode volgens Ogawa de voorkeur boven de methode van Potts & Fleming, waarbij de permeabiliteit *in vitro* met behulp van getritieerd water gemeten wordt

Ogawa, M 1974 Comp Biochem Physiol 49A, 545-553
Potts, W T W & Fleming, W R 1971 J Exp Biol 54, 317-327

## II

De door Harding *et al* gebruikte voedselzoek-test is ongeschikt om bij knaagdieren een verondersteld anomiserend effekt van zinksulfaat aan te tonen

Harding, J W, Getchell, T V & Margolis, F L 1978 Brain Res 140, 271 285
Schoots, A F W, Cruslo, W E & Van Abeelen, J H F 1978 Physiol Behavior 21, 779-784

## III

De "klassieke" opvatting dat dormante zaden gekenmerkt worden door een geblokkeerde RNA- en eiwitsynthese is onjuist

Hecker, M & Bernhardt, D 1976 Biochem Physiol Pflanzen 169, S 417-426

## IV

De conclusie van Uzzell *et al*, dat het morfologische fenotype de beste indicator is voor de genetische constitutie van de door hen bestudeerde kikkers, valt niet uit hun resultaten af te leiden

Uzzell, T Gunther & Berger, L 1977 Proc Acad Nat Sci Phil 128, 147 171
Berger, L & Rogushi, H 1978 Folia Biol (Krakow) 26, 231-248

## V

De in de literatuur gebruikte begrippen oligo-, meso- en eutrofe systemen zijn inadequaat om de grote variatie in aquatische plantengemeenschappen weer te geven

VI

Om aan te tonen of een toxisch agens de funktie van een enzym dan wel de synthese ervan remt, dient het effekt van het agens zowel *in vivo* als *in vitro* biochemisch onderzocht te worden.

VII

De conclusie van Coleman *et al.* dat, bij onderzoek over het effekt van pesticiden op de kieuwfunktie, parameters gebaseerd op metingen van enzymatische aktiviteit gevoeliger zijn dan parameters gebaseerd op veranderingen in de ultrastruktuur van chloride cellen, is onvoldoende gefundeerd.

Coleman, R Yaron, Z & Ilan, Z. 1977.
J. Fish Biol 11, 589-594.

VIII

Er bestaan nog steeds onvoldoende gegevens om het orgaan van Hoyle bij de cephalapoden als "hatching-klier" te erkennen.

IX

De veronderstelling dat wetenschappelijke onderzoekers minder ontvankelijk zijn voor nieuwe ideeen naarmate ze ouder worden is ongegrond.

Hull, D.L., Tessner, P.D. & Diamond A.M. 1978. Science 202, 717-723.

Nijmegen, 30 november 1979 R.A.C. Lock