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Effects of Calcium Removal from Dilute Sea Water on the Salinity Tolerance and Blood Constituent Levels of Goldfish with a Note of Mucus Release from the Gills

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Abstract

Effects of calcium removal from artificial dilute sea water on the salinity tolerance, serum electrolyte levels, osmolarity and blood moisture content were investigated in a fresh-water fish, *Carassius auratus*. The mucus of the gill filaments and in ambient water was also examined histochemically and chemically.

The fish can not survive long immersion in 1/4 Ca-free sea water. This was the same case with the pre-acclimated specimens to 1/4 normal sea water for 7 days. Their death was characteristically preceded by an unusual dispersal of mucus from the gills, and by an unbalance in serum sodium to chloride concentration: the sodium decreased while the chloride increased. Serum osmolarity showed a consistent increase despite the reduction in sodium concentration. Potassium and calcium levels remained unchanged during the 6-hr experimental period. Blood moisture content decreased more greatly in the control group than in the Ca-free group.

Considering no significant elevation in these electrolyte levels of the Ca-free group in comparison with those of the control group, the dispersal of mucus does not seem to cause an increase in ion invasion through the gills, resulting in the death of the fish.

Calcium ions in environmental water have an important role in the osmoregulation of teleosts, especially in their adaptation to a new environment of different salinity. The rich addition of calcium to fresh water enable some stenohaline marine fishes to survive in a markedly hypoosmotic environment for a long period[1,2]. By contrast, even sea-water-adapted eels die within a few days, if calcium is removed from the sea water[3]. These effective roles of calcium in osmoregulation have been accepted in relation to the fact that calcium is a controller for the permeability of biological membranes to ions and water. Recently the branchial fluxes of sodium proved to depend upon external calcium in both fresh-water and marine fishes[4-6]. The chloride uptake of *Oryzias latipes* is also affected by dissolved calcium[7]. In spite of these several studies, however, we have no knowledge about the blood chemistry of the fish which fail to adapt to a new environment of different salinity owing to a low concentration of calcium in the environment.

The present study was undertaken to elucidate the effects of calcium removal

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from dilute sea water on the salinity tolerance, serum electrolyte levels, osmolarity and blood moisture content of goldfish. The mucus release from the gills was also examined histochemically and chemically in the Ca-free environment.

Material and Methods

Goldfish were obtained from a commercial dealer and maintained for acclimation in aerated glass aquaria for intervals of not less than one week before use. They were fed on pellets *ad libitum* but fasted on, and after, the 3rd day before experiment. They were kept at 24±1°C throughout the acclimating and experimental periods.

Van't Hoff's artificial sea water was prepared, diluted to 1/4 sea water by deionized water, and used as ambient media. Ca-free sea water was obtained by removing the calcium from the artificial sea water. Virtually, a medium obtained of 1/4 Ca-free sea water contained approximately 0.06 mEq Ca/l.

The salinity tolerance of goldfish in dilute sea water with or without calcium was examined by using 20 specimens of 41.2±11.7 g (mean±standard deviation) in body weight. Ten individuals were directly transferred from fresh water to 1/4 Ca-free sea water, and the remainders to 1/4 normal sea water as controls. After transfer, their survival rate was investigated versus time. A judgment on death was made at the time when the fish apparently showed no response to mechanical stimuli. The salinity tolerance was similarly reexamined using 10 individuals which had been beforehand acclimated to 1/4 normal sea water for 7 days. As controls, other 10 fish were simultaneously transferred from fresh water to the same Ca-free experimental aquarium. These specimens were 9.8±0.9 g in body weight.

Changes in concentrations of serum sodium, potassium, calcium and chloride were examined versus hours after transfer to 1/4 Ca-free sea water. Blood moisture content and serum osmolarity were also determined. Blood was collected from the caudal vessels into capillary tubes and centrifugalized. The separated sera were used for these determinations within one day after collection, during which time the samples were stored at 0°C. Sodium, potassium and calcium were determined by a flame photometer (Hitachi 139–0400). Chloride and osmolarity were measured with a chloride meter (Evans) and an osmometer (Osmette 2007), respectively. Blood moisture content was determined after Kuroda8). Serum sodium, potassium, calcium and blood moisture content were determined using the same blood samples from 45 individuals of 26.4±7.2 g in body weight. For determining serum chloride and osmolarity, however, other samples from 35 individuals of 29.4±10.7 g in body weight were used. In the case of calcium determination, phosphate and anion interferences were abolished by diluting the samples with a solution of 0.1% lanthanum chloride.

The mucus release from the gills (and other body surfaces) in 1/4 Ca-free sea water were examined histochemically and chemically. The histochemical observation was made by using the gill specimens dissected from the same individuals as used for the blood analyses. The gills were fixed in Bouin's fluid, embedded in paraffin by the routine method, and sectioned transversely at 8 μ. The sections were submitted to the periodic acid-Schiff (PAS) reaction, and the mucus of the gill filaments was observed.

The amount of mucous substances accumulated in the Ca-free medium was also determined in comparison with that in other ambient media. Forty fish of 24.3±5.8 g in body weight were divided into 4 groups on the basis of body weight, and each group was kept for 6 hours in a non-filterated and aerated aquarium containing one of the following media: deionized water, fresh water, 1/4 normal sea water and 1/4 Ca-free sea water. These media were analysed for sugars and related substances by the calorimetric method of Dubois et al.[9]

Results

Survival time

Goldfish can not survive long immersion in 1/4 Ca-free sea water (Fig. 1): When 10 fish were transferred from fresh water to the Ca-free medium, they lived in good condition at least during the first 2 hours. Thereafter, however, they

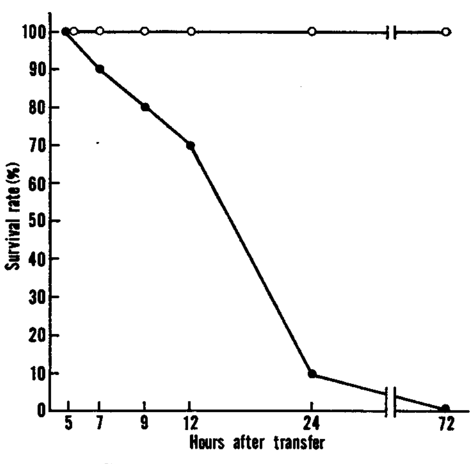

Fig. 1. Survival rate of goldfish transferred to 1/4 normal or Ca-free sea water. •—•: 1/4 Ca-free sea water (281.0 mOsm/l); ○—○: 1/4 normal sea water (307.4 mOsm/l). Each group consists of 10 individuals.

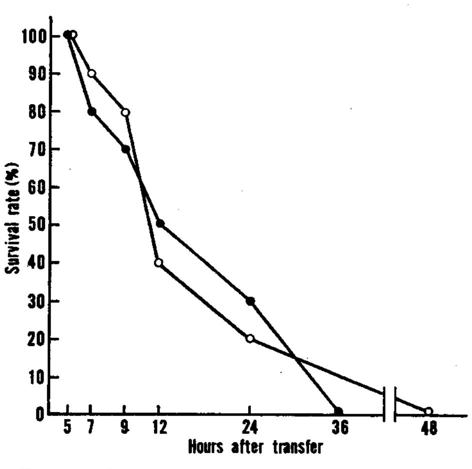

Fig. 2. Survival rate of goldfish transferred to 1/4 Ca-free sea water (288.5 mOsm/l) after exposure to 1/4 normal sea water (302.2 mOsm/l) for 7 days. •—•: the fish pre-exposed to 1/4 normal sea water; ○—○: the fish transferred from fresh water. Each group consists of 10 individuals.

became inactive in movement in comparison with the control fish which were simultaneously transferred to 1/4 normal sea water. About 5 hours after transfer, 3 fish lost their balance and the others stayed quietly at the bottom of the aquarium. At a period of 5 to 7 hours after transfer, one fish died and the survivors hardly responded to mechanical stimuli. At this time the mucous films and scum originating from the body surfaces floated in and on the Ca-free medium which became whity in color. At a period of 7 to 9 hours, one fish died and all of the remainders lay down sinking to the bottom or floating on the surface. Another fish died at a period of 9 to 12 hours. Six other fish died at periods of 12 to 24 hours and the last one about 72 hours after transfer. Eventually, all experimental fish died within 72 hours after transfer to 1/4 Ca-free sea water. No fish died in the control aquarium throughout this experimental period (Fig. 1).

In order to see whether or not the death of the fish in the Ca-free medium was caused by a failure in initial adaptation to the saline environment, the survival rate was reexamined by using the specimens pre-acclimated to 1/4 normal sea water for 7 days. The results are presented in Fig. 2. No difference was found in survival time between the pre-acclimated specimens and the controls which were directly transferred from fresh water.

Blood constituents

Sodium: Changes in concentration of serum sodium are presented in Fig. 3. The sodium concentration of the fresh-water specimens was 121.1 mEq/l on the average of 5 individuals. When the fish were transferred to 1/4 normal or Ca-free

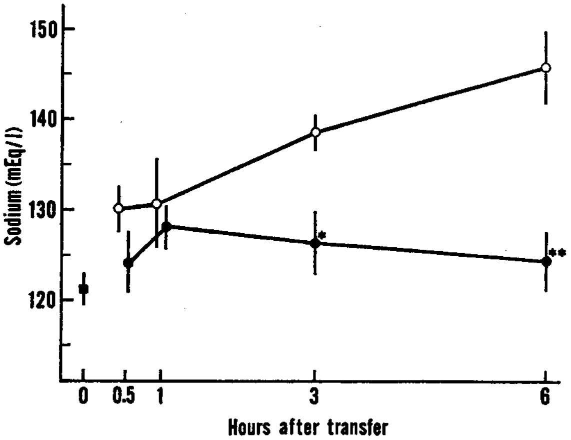

Fig. 3. Changes in concentration of serum sodium in goldfish transferred to 1/4 normal or Ca-free sea water. Each of plotted values represents mean±SE for 5 or 6 animals. •: fresh water; •—•: 1/4 Ca-free sea water (263.7 mOsm/l); ○—○: 1/4 normal sea water (276.4 mOsm/l).
*$P<0.05$ **$P<0.01$

sea water, their serum sodium increased during the first one hour following transfer. Thereafter, however, the sodium in the Ca-free group characteristically showed a gradual decrease to a concentration of 124.7 mEq/l 6 hours after transfer, while that in the normal sea-water group increased consistently to a level of 145.5 mEq/l ($P<0.01$).

Potassium: The results are shown in Fig. 4. The serum potassium of the fresh-water specimens was 2.45 mEq/l on the average of 5 individuals. In the Ca-free group, the serum level of potassium was fairly constant throughout the experimental period. In the normal sea-water group, the potassium concentration was somewhat variable showing an elevation and a successive recovery 3 and 6 hours after transfer, respectively. No significant difference in potassium concentration, however, was found between the two groups at any examination time.

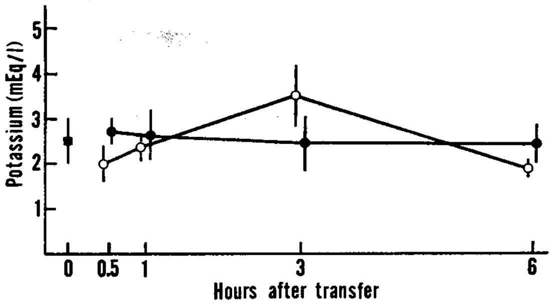

Fig. 4. Changes in concentration of serum potassium in goldfish transferred to 1/4 normal or Ca-free sea water. Each of plotted values represents mean±SE for 4 or 6 animals. ▪: fresh water; •—•: 1/4 Ca-free sea water (263.7 mOsm/l); ○—○: 1/4 normal sea water (276.4 mOsm/l).

Calcium: The results are given in Fig. 5. Calcium concentration in the serum of the fresh-water specimens was 4.99 mEq/l on the average of 5 individuals. An increase in concentration followed exposure to 1/4 normal sea water. Transferred to 1/4 Ca-free sea water, however, they showed no change in calcium concentration maintaining a calcium level similar to that of the fresh-water specimens. Thus serum calcium was significantly higher in the control group than in the Ca-free group ($P<0.05$).

Chloride: Changes in concentration of serum chloride are shown in Fig. 6. The chloride concentration of the fresh-water specimens was 102.4 mEq/l on the average of 5 individuals. In the fish transferred to 1/4 normal or Ca-free sea water, their chloride levels were elevated even one hour after transfer, and remained elevated throughout the experimental period of 6 hours. No significant difference was found in chloride concentration between the Ca-free and normal sea-water groups.

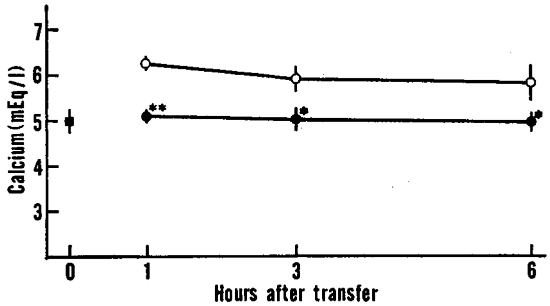

Fig. 5. Changes in concentration of serum calcium in goldfish transferred to 1/4 normal or Ca-free sea water. Each of plotted values represents mean±SE for 4 or 5 animals. ▪: fresh water; •—•: 1/4 Ca-free sea water (263.7 mOsm/l); ○—○: 1/4 normal sea water (276.4 mOsm/l).
*P<0.05 **P<0.01

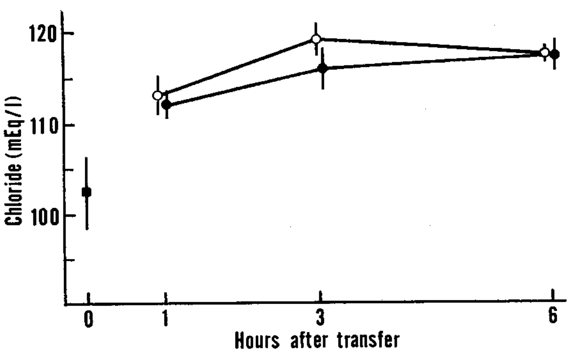

Fig. 6. Changes in concentration of serum chloride in goldfish transferred to 1/4 normal or Ca-free sea water. Each of plotted values represents mean±SE for 4 or 5 animals. ▪: fresh water; •—•: 1/4 Ca-free sea water (260.5 mOsm/l); ○—○: 1/4 normal sea water (268.8 mOsm/l).

Osmolarity: The results are given in Fig. 7. The serum osmolarity of the fresh-water specimens was 264.0 mOsm/l on the average of 5 individuals. Transferred to 1/4 Ca-free or normal sea water, they showed an elevation in serum osmolarity even one hour after transfer. Further elevations followed for 2 subsequent hours. After then, the osmolarity of the control group decreased rather, while that of the Ca-free group continued to increase to a high level of 290.8 mOsm/l at the end of the 6-hr experimental period despite a steady decrease in concentration of their serum sodium as aforementioned. There was, however, no significant difference in serum osmolarity between the two groups at any examination time.

Blood moisture: Changes in blood moisture content of the fish transferred to 1/4 normal or Ca-free sea water are shown in Fig. 8. In the fresh-water specimens, the moisture content was 88.4% on the average of 5 individuals. After transfer

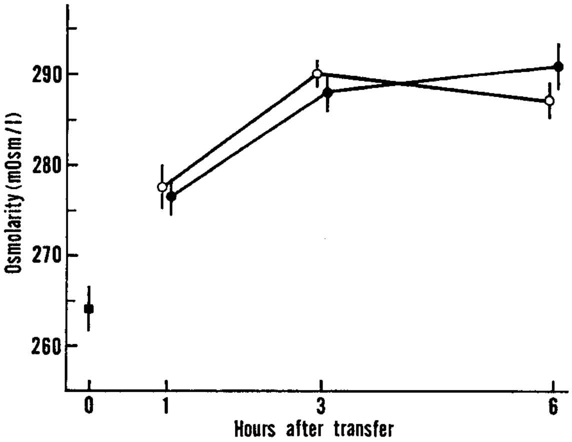

Fig. 7. Changes in serum osmolarity of goldfish transferred to 1/4 normal or Ca-free sea water. Each of plotted values represents mean±SE for 4 or 5 animals. ■: fresh water; ●—●: 1/4 Ca-free sea water (260.5 mOsm/l); ○—○: 1/4 normal sea water (268.8 mOsm/l).

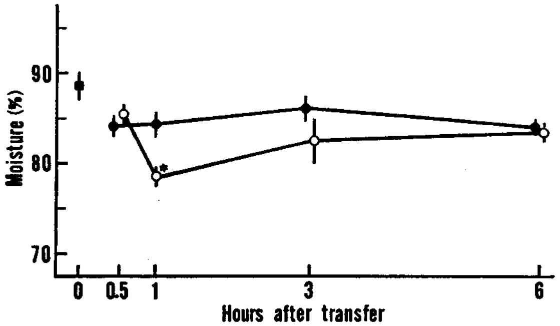

Fig. 8. Changes in moisture content of blood in goldfish transferred to 1/4 normal or Ca-free sea water. Each of plotted values represents mean±SE for 4 or 5 animals. ■ : fresh water; ●—●: 1/4 Ca-free sea water (263.7 mOsm/l); ○—○: 1/4 normal sea water (276.4 mOsm/l).
$^*P<0.01$

to 1/4 normal or Ca-free sea water, the content decreased more heavily in the control group than in the Ca-free group, resulting in a significant difference between the two groups one hour after transfer ($P<0.01$). Hereafter the decreased moisture content of the control group recovered gradually to a level equal to that of the Ca-free group.

Mucous substances

Mucus in ambient media: Fig. 9 shows the amount of sugars and related substances in the ambient media where the fish were kept during 6 hours. There was no wide difference in amount of these substances among the bathing media of fresh water, deionized water and 1/4 normal sea water. In 1/4 Ca-free sea water, however, these substances were detected in a large quantity, especially in the fluid sample containing the scum accumulated on the surface of the medium (Fig. 9).

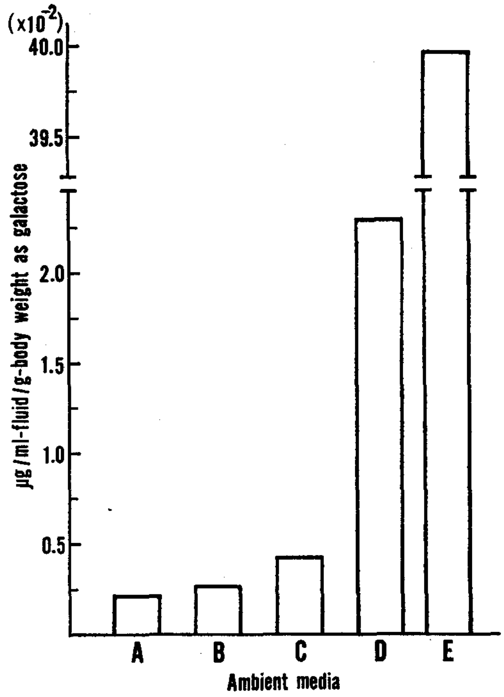

Fig. 9. Sugars and related substances of ambient media in aerated and non-filtrated aquaria in which goldfish were kept during 6 hours. A: deionized water; B: fresh water; C: 1/4 normal sea water; D: 1/4 Ca-free sea water; E: a sample pipetted from the surface of the Ca-free medium where much scum floated.

Histochemical observations: In the gill filaments of the fresh-water specimens, a large number of mucous cells stand in a line just under the free surface of the epithelium (Fig. 10). The cells are circular or oval in shape, and are stained strongly and uniformly with the PAS-reagent. The surface of the gill filaments is covered with a film of mucous substances which may be secreted by the mucous cells (Fig. 10). After transfer to 1/4 normal sea water, the film became more strongly positive to the PAS-reagent, while the cells began to stain more weakly and irregularly (Fig. 11). These changes may be due to the possible release of mucus from the cells. In the fish transferred to 1/4 Ca-free sea water, these signs for mucus secretion were still more conspicuous. The surface of their gill filaments, however, was not covered with the mucous film (Fig. 12).

Discussion

Although goldfish, a stenohaline fresh-water species, survive in a hypersaline environment to plasma[10,11], they can not tolerate even a still more diluted environment of 1/4 sea water, if calcium is removed from the sea water. A similar situation was the case with the pre-acclimated specimens to 1/4 normal sea water for 7 days. In a Ca-free hyperosmotic environment (approximately 330 mOsm/l) of mannitol, however, no fish died within 72 hours after transfer from fresh water (unpublished data). These facts suggest that their death in the Ca-free environment may be due not to a well-known pattern of failure (salt loading) in adaptation to a saline environment, but to an abnormal (unbalanced) change in ion movement, e.g. through the gills.

Cuthbert and Maetz5) have shown that the removal of calcium from the gill surface of goldfish with chelating agents increases the branchial influx of sodium twofold. From this result, the present study was expected to show a characteristic increase in serum sodium of the Ca-free group because of no intrinsic effect of calcium removal on the outflux5). A steady decrease in a once-elevated level of sodium, however, was the case with the group as the fish were debilitated. The replacement of cellular potassium by extracellular sodium may not be expected for explaining this reduction, because the serum potassium of this group showed no change in concentration throughout the experimental period. This discrepancy will be explained by further studies on the renal sodium excretion, and the sodium space of goldfish in a Ca-free saline environment.

The ionic composition of body fluids, especially in their relative rather than absolute values, are major determinants in regulating the intensity and pattern of biochemical pathways12). When a fresh-water fish is transferred to a saline environment, it is a general pattern that a rise in plasma sodium is accompanied by an equivalent increase in plasma chloride12). In 1/4 normal sea-water-adapted goldfish, such a correlation was obtained between the sodium and chloride concentrations. In the case of goldfish transferred to 1/4 Ca-free sea water, however, a once-elevated level of serum sodium showed a gradual decrease to that of the fresh-water specimens, while the chloride level continued to increase, resulting in a conspicuous unbalance in serum sodium to chloride concentration. This disturbance in ion balance may be one of the important factors for reducing the salinity tolerance of the fish in the Ca-free sea water. In company with this reduction in concentration of serum sodium, serum osmolarity was also expected to decrease to the same extent as the sodium. An increase in osmolarity, however, was the case with the Ca-free group. This fact shows that certain substances which are osmotically significant appeared into the blood to compensate a sodium-induced reduction in osmolarity. Potassium, chloride and calcium are not involved in this osmoregulatory compensation, because these ions showed no special increase at the serum level in comparison with those of the control group. A buildup of yet unidentified intermediate metabolic products may be responsible for a compensatory increase in serum osmolarity for a decrease in serum electrolytes in cold-acclimated goldfish13). Further studies are needed to make clear the nature of these compensatory substances in the serum of the Ca-free group.

Ozaki14) supposed that the effective role of calcium in salinity tolerance was probably related to an internal level of calcium. Pickford et al.15) also discussed that the effect of environmental calcium on salinity tolerance would be of an indirect nature, possibly related to the maintenance of the circulating levels of serum calcium. Adapted to 1/4 normal sea water, goldfish showed an elevated level of serum calcium. No prolongation, nevertheless, was found in survival time after their transfer to 1/4 Ca-free sea water. The mature specimens of female goldfish which had a higher level of serum calcium than the immature ones also died within 48 hours after transfer to the Ca-free medium (unpublished data), showing no increase in salinity tolerance in comparison with the immature specimens. These facts indicate that an elevation of serum calcium is not effective for goldfish to survive in a Ca-free saline environment.

It is well known that the mucus on the body surfaces has a protective effect on the osmoregulation of fishes[16,17]. Mashiko and Jozuka[18] have shown that the transfer of carp to 45% Ca-free sea water causes a liquefaction of mucus from the body surfaces, resulting in the direct exposure of the gill epithelium to the external medium. They concluded that this might be one of the most important factors for reducing the salinity tolerance of the fish. Such a liquefaction of mucus was also confirmed histochemically and chemically in the present study. At the level of serum electrolytes, however, no evidence was found in favor of a special increase in ion invasion in the mucus-liquefied goldfish. Although the removal of external calcium increases branchial permiability to ions[4-7] and water[19,20], mucus itself might not be so important as a barrier for their movements through the gills[21].

Acknowledgments

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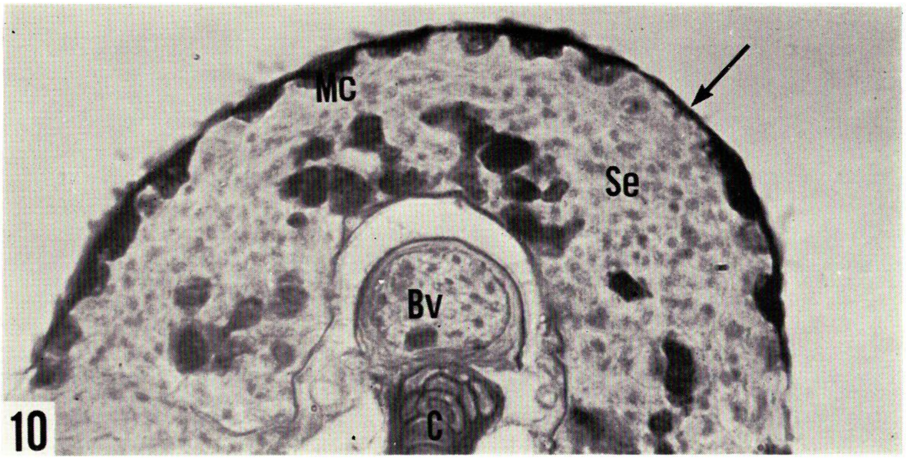
Explanation of Plate

Figs. 10–12. Cross sections of the gill filaments of goldfish kept in various media. Bv: blood vessel; C: cartilage; Mc: mucous cell; Se: stratified epithelium. Arrows indicate mucous films on the filaments. PAS-reaction. ×450. 10. A fresh-water specimen. 11. A specimen kept in 1/4 normal sea water for 6 hours. 12. A specimen kept in 1/4 Ca-free sea water for 6 hours.



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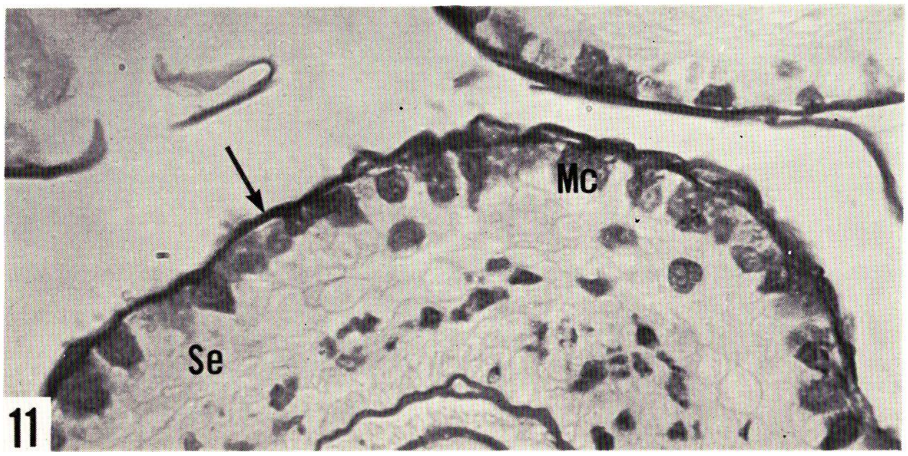

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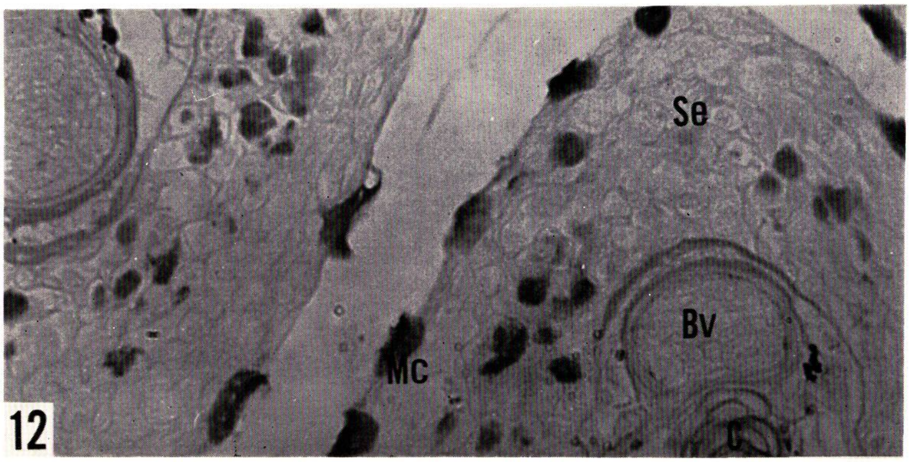

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ARAYA & MUGIYA: Goldfish in Ca-free dilute sea water