Fluoride Inhibition of Enolase from Antarctic Organisms*

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INTRODUCTION

The finding by Soevic and Braekhan (1979) of abnormally high concentrations of fluoride in the Artarctic Krill, *Euphausia superba* Dana, led to the conclusion that "krill in any form, even peeled, failed to comply with requirements for human consumption". This provoked a great biologial interest as well as the stimulation of food technology studies as applied to krill production.

Bone and Manthey (1983) established the anatomical distribution of F^- within various body segments and organs of Antarctic krill. They found that the F^- content of the cephalothorax to be 3782.6 μg/g freeze-dried and 3805 μg/g freeze-dried in the abdomen, the exoskeleton alone concentrating as much as 50% of the total F^- content of the abdomen. Previously, the F^- content of Antarctic marine animals caught off Elephant Island, among them 6 species of fish and a number of benthic animals was assayed by Oehlenschläger and Manthey (1982).

A very important phenomenon was then established by Christians and Leinemann (1980, 1983) and Christians *et al.* (1981) which was the discovery that fluoride migrates from the shell into the muscle flesh of dead krill. This process is not interrupted by a frozen stage period, the intensity of migration depending on the temperature, the time spent in the frozen state, and the method of preparation of the krill. The F^- content in the muscle flesh of the krill rose considerably at –20°C.

According to Christians *et al.* (1983), krill products derived from normal processing contained 200–250 mg $F^-.kg^{-1}$, an amount which cannot be tolerated by man due to the known deleterious effects of ingestion of high amounts of fluoride.

Although these facts raised a most important problem for the krill processing technology, it was sufficiently attractive to call the attention of biologists. This was mainly due to the direct relationships existing in the Antarctic ecosystem between living organisms, such as krill and penguin, the former concentrating F^- which is fed to the latter, krill being its main source of food. In connection with this, Prof. D. Adelung* from the Institut für Meereskunde, Kiel, carried out, at the Arctowski Polish Antarctic Station, during the summer of 1984-1985, a very important experiment on F^- metabolism in penguins.

In order to have an insight at the molecular level of this problem, Bacila *et al.* (1985) investigated the relationships between fluoride and enolase from tissues and organs

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of Antarctic organisms. Fluoride is an inhibitor of important enzymes, one of them being enolase, a well known enzyme of the glycolytic pathway, which generates phosphoenolpyruvate, the substrate of pyruvatekinase. Also, F^- is known to inhibit alkaline phosphatase and cytochrome oxidase a terminal enzyme of the respiratory chain.

MATERIALS AND METHODS

Enolase was partially purified from three different organisms: liver from a gentoo penguim (*Pygoscelis papua*) and the Antarctic fish *Notothenia coriiceps neglecta* and flesh muscle of the Antarctic krill, *Euphausia superba* Dana. A preparation of purified yeast enolase was also used for comparative purposes.

Liver from an adult gentoo penguin, as well as that from *Notothenia coriiceps neglecta* caught in Admiralty Bay, were homogenized in a Potter-Elvehjem homogenizer in 3 volumes of TRIS-HCl 0.05M buffer, pH 7.4, containing 1 mM EDTA and 1 mM β–mercaptoethanol (β–ME). The homogenate was spun down 10 min at 3000 rpm in an angular Sorval centrifuge at 0–3°C. The sediment was discarded and the supernatant centrifuged 20 min at 10,000 g. In both supernatants, salting out of enolase was carried out by adding $(NH_4)_2SO_4$ up to 0.3 sat. with gentle stirring. After leaving 30 min at room temperature, the suspension was spun down 20 min at 10,000 rpm, the supernatant collected and the saturation of $(NH_4)_2SO_4$ increased to 0.60 using the same technique. The sediment was collected by centrifugation 20 min at 10,000 rpm and then dissolved in the same TRIS-HCl 0.05M pH 7.4 buffer containing 1 mM EDTA and 1 mM β–ME.

Preparation of enolase from krill flesh muscle followed essentially the same steps as described above using krill caught at Admiralty Bay, and kept frozen in the laboratory. For preparative purposes, a batch of frozen krill was thawed and then homogenized with the same TRIS-HCl-EDTA- –ME buffer. The salting out procedure for the krill enolase was similar to that used for the liver enzyme.

Al steps of enolase preparation, beginning from the initial homogenates, were monitored by measurement of enzyme activity and protein concentration in the different preparations. Enolase was assayed by the direct method of Warburg and Christian (1942), following phosphoenolpyruvate formation, spectrophotometrically at 240 nm. The assay medium contained 1 mM substrate (2–phosphoglycerate), 1 mM $MgSO_4$, 0.2 M KCl, and 50 mM imidazole buffer with final pH 7.0. Protein was assayed by the method of Lowry *et al* (1951).

All reagents used throughout this work were from reliable sources. Purified yeast enolase, used for comparative purposes, was from Sigma Chemical Co. St. Louis. Mis, USA.

RESULTS

The effect of F^- on enolase activity was assayed in experiments (Fig. 1) in which of activity enolase purified from penguin and fish liver and from krill, was assayed after preincubation with F^- in concentrations varying from 1 mM up to 6 mM. For comparative purposes, a purified yeast enolase was also used in a similar experiment.

According to Figure 1, enolase from both penguin and krill is not inactivated by F^- in concentrations up to 1 mM, both preparations maintaining 75% of the total activity in the presence of 2 mM F^-, and 20 to 25% in the presence of 4 mM F^-. On the other hand, enolase from *Notothenia* liver is much more sensitive to F^-, losing about 68 and 80% of the original activity in the presence of 1 mM and 2 mM F^- respectively. The residual activity of the fish liver enolase is about 5% when 4 mM F^- is present in the system.

DISCUSSION

It is now a known fact that the Antarctic krill is a very efficient concentrator of fluoride from its environmental sea-water, accumulating abnormally high amounts in different parts of the body, mainly in the exoskeleton of the abdomen (Soevik and Broekkan, 1979; Booene and Mathey, 1983). Lesser amounts of fluoride are concentrated in the hepatopancreas (7.6 μg/g or 0.1% of the total); and in the plen muscle (70 μg/g or 2.1% of the total) of the krill. In dead krill, by an unknown mechanism, F^- migrates from the shell into the muscle flesh, (Christian and Leinemann, 1980, 1983). This phenomenon is

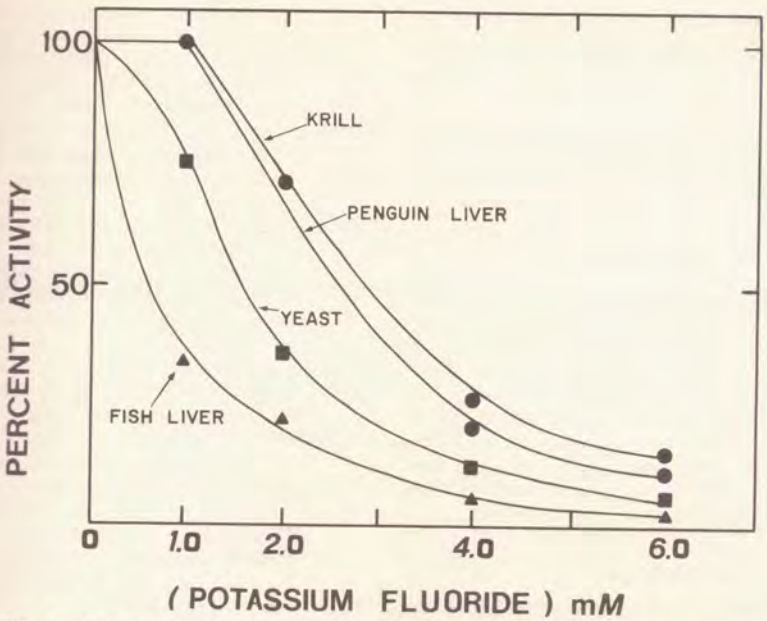

Fig. 1 – *Effect of different concentrations of fluoride on the activity of enolase from Antarctic organisms.* System: imidazole buffer, pH 7.0, 50 mM; $MgSO_4$, 1 mM KCl, 0.2 mM; substrate (2–phosphoglycerate) 1 mM; enzyme preparation: krill, 18.8 mIU; penguin liver, 12.5 mIU; fish liver, 18.3 mIU; yeast, 17.0 mIU. Each system was preincubated 10 min with KF prior the addition of substrate to begin the reaction. Enzyme activity was followed spectrophotometrically at 240 nm by monitoring the formation of phosphoenolpyruvate.

meaningful, considering the high quantities of F^- stored in krill and the fact that penguins eat large amounts of this crustacean, being from 800 to 1000 g per day on the average. In *Notothenia coriiceps neglecta* Oehlenschläger and Manthey (1982), found the following concentrations of F^- in mg/kg: 3.7 in muscle, 865 in vertebra, 6 in the gastric tract, and 3.7 in the stomach. In the case of *Notothenia gibberifrons*: 1.3 in muscle, 1.156 in vertebra and in that of *Notothenia rossi marmorata*: 2.2 in muscle and 964 in vertebra.

Thus in having to deal with such abnormally high quantities of F^-, both the krill which concentrates it, and the penguin, which ingests it through the krill used as its main natural food, one might expect that suitable biological mechanisms of protection against the deleterious effect of F^- should have been developed in both animals.

The finding in the present paper that enolases from penguin liver and from krill are less sensitive to the inhibitory effect of F^- than that from *Notothenia* liver, might indicate that enolase underwent either a conformational change in the shape of the protein molecule or developed a peculiar structure at the active site. This would allow the cell to resist to higher concentrations of F^- without affecting the normal operation of the glycolytic pathway, helping in such a way to maintain the homeostasis of the entire animal in spite of the abnormal ingestion of fluoride.

Increases in blood F^- concentrations have no effect whatsoever on the red blood cell behaviour or metabolism in penguins. According to Rosa *et al* (1985a, b) penguin erythrocytes possess a defective glucose metabolism, the whole blood glucose being partitioned mainly in plasma (327 mg/100 ml in both *Pygoscelis antarctica* and *Pygoscelis papua*) and almost none in red blood cells. The following values were found by Rosa *et al.* (1985b) for enolase activity in erythrocytes and somatic tissues of *P. papua*, as mIU/mg protein: erythrocytes, 2.87; encephalous, 193.47; leg muscle, 421.26; heart muscle, 150.16; breast muscle, 826.82; liver, 296.41.

The erythrocyte defective glucose metabolism in penguins might not be due to F^- but mainly to a biochemical lesion in the site of the hexokinase activity which is

extremely low and also on the nature of the organic phosphate compounds present in the erythrocytes, which are now being investigated by us.

Other mechanisms of protection against the deterious effects of fluoride might exist in such organisms.

SUMMARY

In view of the high concentration of fluoride content in the body of the Antarctic krill *Euphausia superba* Dana, a central organism in the food chain of the Antarctic ecosystem; and because of the increase interest in the use of this crustacean for human consumption, an investigation was carried out in order to establish the kinetics of the F^- inhibition of enolase isolated from different animal sources, namely penguin liver, krill muscle flesh, and fish liver (*Notothenia coriiceps neglecta*), as an approach for a better insight of this important problem at a molecular level.

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