CALCIFICATION IN FISH AND SHELL-FISH—IV.
THE DIFFERENCES IN NITROGEN CONTENT BETWEEN
THE TRANSLUCENT AND OPAQUE ZONES OF
OTOLITH IN SOME FISH*

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The mechanism of otolith formation in fish has not yet been clearly explained, though fisheries scientists have estimated the age of fish by counting the number of the two concentric rings or zones, opaque and translucent, found in the otolith. For instance, the problem concerning the factors producing differences between the opaque and translucent zones of otolith still remains unsolved. Although many studies dealing with the otolith of fish have hitherto been reported, most of them are mainly concerned with the determination of the age of fish. So, investigations made concerning differences in chemical components and crystal texture between the opaque and translucent zones of otolith have been very few¹⁾⁻³⁾. However, IRIE⁴⁾ has recently demonstrated the differences in both the size of the crystal grains of calcium carbonate, aragonite and the content of organic substance between the opaque and translucent zones in the otolith of perch.

In a previous paper, the author⁵⁾ reported that, in the otolith fluid of fish whose otolith has a distinct ringed structure of the opaque and translucent zones, both the seasonal occurrence of an additional prealbumin fraction and the seasonal increase of the protein concentration are found at a certain time corresponding to the period of opaque zone formation in the otolith. It is probable that the proteins contained in this fluid will form the organic matrix of the otolith and, at the same time, determine its nature, that is, whether it is opaque or translucent. However, no study has yet been made on the quantitative determination of the proteins of otolith in relation to its formation, especially taking into consideration the possible influence of these substances upon the zone formation. The present experiments were undertaken to detect a difference in nitrogen content between the opaque and translucent zones of otolith in some fish by means of the micro-Kjeldahl method.

Materials and Methods

The following nine species were used in the present study: flatfish *Limanda*

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herzensteini, *Kareius bicoloratus* and *Cleisthenes pinetorum herzensteini*, bullhead *Hemilepidotus gilberti*, black rockfish *Sebastes taczanowskii*, cod *Gadus macrocephalus* and *Theragra chalcogramma*, goldfish *Carassius auratus*, and rainbow trout *Salmo gairdnerii irideus*. Of these, the first seven species are marine and the last two fresh-water forms. The specimens, both goldfish and rainbow trout, were reared in the ponds of the author's laboratory, and, in the case of the marine forms, those of both flatfish and black rockfish were caught in Hakodate Bay and the others off the coast of Kushiro, Hokkaido, Japan.

The otoliths obtained from these specimens were classified into three types on the basis of their characteristics of opaque and translucent zones. Type A: otoliths of flatfish, bullhead and black rockfish are rather flat on both inner and outer sides and their ringed structure of the opaque and translucent zones is easily visible to the naked eye. Type B: otolith of goldfish is somewhat globular in shape, and its zones can not be observed superficially but are disclosed with the ground preparation. Type C: otoliths of cod and rainbow trout appear homogeneously milky or glassy, not showing any alternate patterns of opaque and translucent zones even with the ground preparation. Accordingly, to determine the nitrogen content of the otoliths, only those obtained from the fish belonging to type A were used for separating into the two different parts of opaque and translucent zones.

For the separation of the two different zones, the following method was satisfactorily used, though a little contamination was inevitable. The otolith, which had been preserved in a mixture of glycerin-water-neutral formalin (10:9:1 v/v)⁶⁾, was cleaned with distilled water, and, in order to make further treatment easier, one tip of it was fixed within a mass of dental modelling compound (Zishi Chemical Ind.) which is free from nitrogen. Next, a so-called "dental electric engine" equipped with a diamond disk of 7mm in diameter and 0.2mm in width was applied to the margin of the otolith to grind down its respective opaque and translucent zones to powder or small pieces, and then these shavings were carefully collected. In this process of grinding the otolith and collecting the shavings, either binocular microscope or microscope was used when needed as a precautionary measure against any contamination of one zone by the other. In the case of black rockfish, whose otolith is somewhat globular on its inner side only, not showing so clear zone as on the outer side, the otolith was polished on this surface to such a thickness that the ringed structure was clearly visible to the naked eye. And then the procedure mentioned above was followed. Usually, several otoliths of 400 mg or so were used for each determination.

Each sample of both the pieces and powder was washed again several times with distilled water, dried at $100^{\circ}\text{C} \pm 1$ for 20 minutes (cf. Text-fig. 1), and weighed in a Mettler analytical balance with an accuracy of 0.1 mg.

The total nitrogen of the calcified otolith was directly determined by the micro-

Kjeldahl method of KING⁷⁾ but slightly modified as follows in order to eliminate any measuring errors caused by the precipitation of calcium sulfate during the digestion with sulphuric acid; that is, otolith of about 400 mg is digested with 4 ml (2 ml in the

Text-fig. 1. Effects of drying time on the weight of wet otoliths, heated at $100^{\circ}\text{C} \pm 1$.

Table 1. Influences of the co-existence of CaCO_3 in the measurement of total nitrogen in the sera of both rainbow trout and horse, using the micro-Kjeldahl method.

Species	Sample	50 % - H_2SO_4 (ml)	Nitrogen (g/dl)
Rainbow trout	serum (0.2 ml)	2	0.550
	serum + CaCO_3 (0.2 ml) + (400 mg)	2	0.439
	serum + CaCO_3 (0.2 ml) + (400 mg)	4	0.554
Horse	serum (0.2 ml)	2	0.957
	serum + CaCO_3 (0.2 ml) + (400 mg)	2	0.643
	serum + CaCO_3 (0.2 ml) + (400 mg)	4	0.954

original method) of 50% sulphuric acid containing 1% selenium dioxide. By this procedure, the otolith is decalcified and its nitrogen is turned into ammonium sulphate. The digested mixture is transferred into a distillation apparatus and the ammonia is liberated by adding 20 ml (10 ml in the original) of 40 % sodium hydroxide. Table 1 shows that the method modified thus is satisfactory to use for the purpose of the direct determination of nitrogen even in calcified otolith.

Results and Discussion

The results obtained are summarized in Table 2. Generally, the nitrogen content of the otoliths of the fish used is considerably low, ranging from 0.167–0.229%, compared with that of the other hard tissues such as bone, dentine, pearl, shell of shell-fish and others but excepting enamel. However, it is highly characteristic that, in the case of all the otoliths separated into the two different parts of opaque and translucent zones, the former zone contained about 1.4–1.6 times as much nitrogen

Table 2. Nitrogen contents of the opaque and translucent zones of the otolith in some fish.

Species	Parts of otolith	No. of samples examined (No. of otoliths used)	% Nitrogen	
			Range	Mean
<i>Kareius bicoloratus</i>	whole	3 (93)	0.186—0.203	0.195
	opaque	3 (195)	0.222—0.263	0.243
	transl.	3 (195)	0.133—0.173	0.157
<i>Limanda herzensteini</i>	whole	2 (60)	0.179, 0.197	0.188
	opaque	2 (126)	0.220, 0.248	0.234
	transl.	2 (126)	0.141, 0.177	0.159
<i>Cleisthenes pinetorum herzensteini</i>	whole	2 (60)	0.177, 0.203	0.190
	opaque	2 (120)	0.212, 0.225	0.219
	transl.	2 (120)	0.138, 0.170	0.154
<i>Hemilepidotus gilberti</i>	whole	2 (32)	0.201, 0.219	0.210
	opaque	2 (60)	0.239, 0.284	0.262
	transl.	2 (60)	0.169, 0.181	0.175
<i>Sebastes taczanowskii</i>	whole	2 (8)	0.186, 0.221	0.204
	opaque	1 (10)	0.231	0.231
	transl.	2 (20)	0.157, 0.171	0.164
<i>Carassius auratus</i>	whole*	1 (128)	0.197	0.197
<i>Salmo gairdnerii irideus</i>	whole**	6 (300)	0.141—0.195	0.167
<i>Gadus macrocephalus</i>	whole**	5 (5)	0.205—0.242	0.229
<i>Theragra chalcogramma</i>	whole**	5 (5)	0.196—0.244	0.222

* Type B: globular otolith; ** Type C: homogeneously glassy or milky otolith (cf. classification of the otoliths examined, p. 867)

as the latter. Moreover, the glassy otolith of rainbow trout contains quite a little less nitrogen than the milky one of each species of cod. As indicated in Table 2, no marked difference in the nitrogen content of otolith is found between the species of marine and fresh-water forms.

As mentioned earlier, it was reported by the author⁵⁾ that, in the otolith fluid of a flatfish, both the seasonal occurrence of an additional fraction of prealbumin and the seasonal increase in the protein concentration are found at a time corresponding to the period of the formation of otolith in the opaque zone, while these seasonal

changes can not be detected in the fluid of rainbow trout whose otolith is homogeneously glassy. Considering these seasonal changes in protein contained in the otolith fluid, the present data suggest a possibility that, in the course of calcification of the otolith, the soluble protein in this fluid is taken into the otolith itself and turned into an insoluble form in its organic matrix, just like the proteins of the extrapallial fluid in the shell formation in mollusca^{8,9)}. However, a circulatory system of otolith fluid (endolymph of a sacculus) in teleostei has not yet been clarified. Possibly, by such a process, and also corresponding to the difference of the protein concentration in the otolith fluid, the organic matrix of the opaque zone of the otolith might contain more protein than that of the translucent one.

DANNEVIG³⁾ has reported in the chemical analysis of cod otolith that the organic substance, conchiolin, is contained in both the center of the otolith, which is opaque, and the opaque zone, whereas the translucent zone entirely lacks this substance, though calcium compounds are deposited in all parts of the otolith. However, IRIE⁴⁾ has demonstrated by electron microscope observation on the otolith of perch, *Lateolabrax japonicus*, that, in the opaque zone, much protein fills the groove between the micro-crystal grains of calcium carbonate, while, in the translucent zone, a very little protein fills the groove. In the present study, more or less nitrogen was found not only in the opaque zone but also in the translucent zone of the otoliths used, though both zones may possibly be contaminated with some from each other in the process of grinding and separating them. This result agrees with Irie's observation that a little protein is contained even in the translucent zone. Accordingly, proteins contained in the otolith are considered to be an important factor which determines the difference between the opaque and translucent zones of the otolith. At the same time, these proteins might be one of the main components forming the organic matrix of the otolith on which inorganic materials deposit.

Summary

The nitrogen content of both the opaque and translucent zones of the otolith was determined by the micro-Kjeldahl method of KING slightly modified, using seven marine and two fresh-water fish.

(1) In the case of the otolith which is flat on both its inner and outer sides, two different parts of the opaque and translucent zones of it can be successfully separated by use of a dental excavator equipped with a small diamond disk.

(2) The nitrogen content of the otoliths measured was very small, ranging from 0.17 to 0.23% of their total weight. However, the opaque zone was found to contain more nitrogen than the translucent one, ranging from 0.22 to 0.26% and from 0.15 to 0.18%, respectively.

(3) Accordingly, the amount of protein deposited on otolith is considered to be an important factor which determines the difference between the opaque and trans-

lucent zones of the otolith.

References

- 1) LUNDE, G.: *Biochem. Zeitschr.*, **206**, 436-439 (1929).
- 2) IRIE, T.: *Jour. Fac. Fish. Anim. Husb. Hiroshima Univ.*, **1**, 1-8 (1955).
- 3) DANNEVIG, E. H.: *Jour. du Cons.*, **21**, 156-159 (1956).
- 4) IRIE, T.: *Jour. Fac. Fish. Anim. Husb. Hiroshima Univ.*, **3**, 203-225 (1960).
- 5) MUGIYA, Y.: *This Bull.*, **30**, 955-967 (1964).
- 6) DANNEVIG, A.: *Rep. Norw. Fish. Mar. Invest.*, **4**, 1-145 (1933).
- 7) KING, E. J.: *Micro-Analysis in Medical Biochemistry*, J. and A. Churchill Ltd., London, 46-49 (1951).
- 8) WILBUR, K. M.: *Physiology of Mollusca*, Vol. I, Academic Press Inc., New York, 243-282 (1964).
- 9) KOBAYASHI, S.: *Biol. Bull.*, **126**, 414-422 (1964).