CALCIFICATION IN FISH AND SHELL-FISH—III.
SEASONAL OCCURRENCE OF A PREALBUMIN FRACTION IN THE
OTOLITH FLUID OF SOME FISH, CORRESPONDING TO THE PERIOD
OF OPAQUE ZONE FORMATION IN THE OTOLITH*

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From the fisheries' point of view, age determination of fish is one of the most important aids to analyzing their population dynamics. Fisheries scientists have estimated age by counting the number of concentric rings or zones of the hard tissues such as otolith, scale, centrum of vertebra, and others. In the case of the otolith, it has been generally considered that in the course of a year two zones, opaque and translucent, are formed in it. However, in spite of the fact that more than half a century has passed since REIBISCH¹⁾ suggested the possibility of estimating the age of fish on the basis of the number of zones in the otolith, the mechanism of their formation has not yet been clarified. For instance, in opposition to IMMERMANN's hypothesis²⁾ that the ringed structure of the translucent and opaque zones is due to differences in the texture of the inorganic constituents, HICKLING³⁾ and DANNEVIG⁴⁾ were of the opinion that it is due to differences in the organic components, the inorganic ones having only a supporting function. Recently, IRIE⁵⁾ has demonstrated by electron microscope observation on the otolith of a perch, *Lateolabrax japonicus*, that there is a difference in both the size of the crystal grains of calcium carbonate, aragonite, and the protein content between the translucent and opaque zones. That so little information is available on the mechanism of the otolith formation in fish may be due to the fact that previous investigations have concentrated only on the nature of the otolith itself.

A sagitta, the largest otolith, which has been used for the age determination of fish, is enclosed in a sacculus together with endolymph, that is, otolith fluid. This fluid is considered to contain precursors of the organic matrix, calcium carbonate in the state just prior to its deposition, carbon dioxide, and other substances. At the same time, it is probable that the chemical composition and physico-chemical characteristics of the fluid will determine the nature of the organic matrix and the crystallization of calcium carbonate in the otolith.

In addition to the otolith, the author's study has been concerning the otolith

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fluid. As far as the author is aware, there have been only a few reports on this fluid in fish, and these have been concerned with sharks from the medical point of view⁸⁾⁻¹⁰⁾. Accordingly, we have no knowledge about the protein components of the otolith fluid in relation to otolith formation. The present experiments were undertaken as an initial study in this field, to detect the seasonal correlation between the proteins of the otolith fluid and the zone formation of the otolith by means of paper and cellulose acetate electrophoresis and also by the micro-Kjeldahl method.

Materials and Methods

The following four species of fish were used in the present study: flatfish *Kareius bicoloratus* (age of the specimens: 2-3 years) and *Limanda herzensteini* (2-3 years), goldfish *Carassius auratus* (3 years) and rainbow trout *Salmo gairdnerii irideus* (2-3 years). The specimens of both rainbow trout and goldfish were reared in the ponds of our laboratory, and those of flatfish were caught in Hakodate Bay, Hokkaido, Japan. In addition to these materials, the fry of rainbow trout were used for histological observation on the sacculus in order to make sure of the method for the collection of otolith fluid as described below.

The characteristics of the otoliths in these three types of fish are different from one another. In each species of flatfish, the otolith is rather flat on both inner and outer sides, and the ringed structure of its opaque and translucent zones is easily visible to the naked eye (Fig. 1). In goldfish, the otolith is somewhat globular in shape, and its zones are not observable superficially but disclosed with the ground preparation (Fig. 2). In rainbow trout, however, the otolith appears homogeneously glassy, not showing any alternate pattern of opaque and translucent zones even with the ground preparation, though an opaque nucleus is seen at the central part (Fig. 3).

For the collection of otolith fluid, the following method was devised. As a first precaution, the tail of the fish is cut to draw out the blood in order to avoid contamination with the otolith fluid; the serum is prepared from this blood sample. Next, as shown in Text-fig. 1A, the upper part of the head is cut off horizontally, and then the exposed brain is taken out gently. Thus, two sacculi are seen, one on each side, situated approximately under the cerebellum. Fluid attached around the sacculus is blotted out with filter paper. The needle-like tip of a fine glass capillary is inserted into each sacculus to suck out the otolith fluid with the aid of gentle aspiration through a rubber tube fixed on the other end (Text-fig. 1B). After cutting the tip of the capillary, one end is sealed in the flame from a burner (Text-fig. 1C). The otolith fluid and serum contained in the capillaries are centrifuged, and each supernatant is used for the test. The amounts of otolith fluid collected from both sacculi of an individual were very small, ranging approximately from 10-20 μ l in flatfish, 0.8-1.0 μ l in goldfish, and 5-13 μ l in rainbow trout. Consequently, the fluid of from 2-22 individuals was pooled for a test according to the species and the size

Text-fig. 1. Schematic diagrams showing the method of collecting the otolith fluid. A: horizontal cutting of the upper part of the head; B: insertion of the needle-like tip of a glass capillary into the sacculus to suck out the fluid; C: the tip of the capillary is cut and one end is sealed with a flame for both centrifugation and preservation of the sample contained in it.

of fish.

The total proteins of the otolith fluid and serum were determined by the micro-Kjeldahl method of KING.¹¹⁾

For paper electrophoresis, a modified type of GRASSMANN-HANNIG's apparatus was used. Most of the procedures were performed according to the recommendations made by KOBAYASHI¹²⁾ for this apparatus. The experiment was made under the following conditions: HOLT's barbital buffer, pH 8.5 and ionic strength 0.045; constant voltage 11.5 V/cm length of the strip; cooling temperature for the apparatus 5–6°C; duration of run 3 hours; Toyo No. 51 filter paper of 12×26 cm (four simultaneously run); amount of the otolith fluid 10 μ l, and 7 μ l of both fish and human sera. After completion of the electrophoresis, the strips were dried at about 110°C. They were stained for 20 minutes in 0.05% bromphenol blue in 1% mercuric chloride and 2% acetic acid. After passing through the paraffin solution heated to about 130°C, each strip was scanned with a Natsume-type densitometer at 460 m μ .

Cellulose acetate electrophoresis was according to SMITH,¹³⁾ under the following conditions, using an apparatus of Jyôkô Ind. Inc. in Japan: LAURELL's barbital buffer, pH 8.6 and 0.06 M (0.07 M for impregnation of the strips); constant current intensity 0.5 mA/cm width of the strip; cellulose acetate strip (Oxoid) of 1.6×9.0 cm; and amount of the otolith fluid 7 μ l, and 5 μ l of both fish and human sera. The dried strips were stained with either 0.2% light green in 5% acetic acid or 0.4% Ponceau 3R in 3% aqueous trichloroacetic acid.¹⁴⁾ These strips were washed in 5% acetic acid until the background became white.

Both the determination of the total protein and the electrophoresis for the otolith fluid and serum were conducted within 10 hours after collection, during which time the samples were stored in a refrigerator at about -18°C .

Preparation of the ground otolith was made according to KOBAYASHI *et al.*¹⁵⁾ but slightly modified in order to make the identification of opaque and translucent zones easier. Each otolith is embedded within a mass of methacrylate of suitable hardness, and then ground to about 300μ thickness so that the polished plane is perpendicular to its longitudinal axis across the center. Preparation of methacrylate is as follows. To a mixture of 1 ml methylmethacrylate (Ooken Kôgyô, Ltd.) and 4 ml n-butylmethacrylate (O. K.) is added 100 mg of Luperco CDB (Lucidol Division).

Results and Discussion

A photomicrograph of the inner ear of a fry of rainbow trout is presented in Fig. 4. An otolith is seen fixed with fibrous elements to the inner side of the sacculus situated approximately under the cerebellum. Accordingly, the otolith fluid can be collected satisfactorily by inserting the tip of a capillary obliquely into the sacculus. Around the otolith is seen a thin membrane which may possibly be concerned with otolith formation just like a pearl-sac in pearl formation. Studies on this membrane are in progress in the author's laboratory.

As indicated in Table 1, the protein concentration of the otolith fluid in each species was found to be much lower than that of the serum, though it has not been determined yet in the case of goldfish, there being too little otolith fluid, as already mentioned. It was highly characteristic that in the otolith fluid of flatfish a seasonal increase of the protein concentration occurred at a time distinctly corresponding to

Table 1. Total proteins of the otolith fluid and serum in flatfish and rainbow trout.

Species	Sample	No. of samples examined	% Protein		Time sacrificed
			Range	Mean	
<i>Limanda herzensteini</i>	otolith fluid*	2	1.61, 1.89	1.75	August
	serum	7	3.12-4.11	3.65	
	otolith fluid*	2	0.58, 0.64	0.61	January
	serum	4	2.84-3.36	3.05	
<i>Salmo gairdnerii irideus</i>	otolith fluid*	4	0.92-1.25	1.17	August
	serum	12	3.03-4.61	4.01	
	otolith fluid*	2	1.11, 1.11	1.11	January
	serum	5	3.01-4.22	3.89	
Human	serum	3	7.43-7.95	7.77	

* Otolith fluids pooled from 10-13 specimens in flatfish and from 15-20 specimens in rainbow trout were needed for each determination.

the period of opaque zone formation in the otolith. For instance, as is seen in Table 1, the protein concentration of the fluid in the fish sacrificed in August when the opaque zone formation was occurring in its otolith was measured to be about 2.7 times as much as that of January, the period of the translucent zone formation. However, no distinct seasonal change of the protein concentration was detected in the otolith fluid of rainbow trout nor in the sera of both the flatfish and rainbow trout.

The electrophoretic patterns of the otolith fluid for each species are presented in Figs. 5-8, together, with those of the serum. Generally, the migration rates and number of the protein fractions in both the otolith fluid and serum were closely similar to each other, though in flatfish a prealbumin fraction was found to occur in the serum but not in the otolith fluid. However, it is noteworthy that an additional fraction of prealbumin was found to occur seasonally in the otolith fluid of both flatfish and goldfish corresponding to the period of opaque zone formation, while such a seasonal occurrence of a prealbumin fraction was not detected electrophoretically throughout the year in the otolith fluid of rainbow trout nor in all the sera of the species examined (Figs. 5-8). That is, in the otolith fluid of flatfish, an additional fraction of prealbumin was found invariably to occur for the five consecutive months of May to September; the migration rate of this fraction on cellulose acetate electrophoresis was about 1.5 times (1.3 times on paper) greater than that of human serum albumin (Figs. 5 and 8). The additional fraction was recognized at first to occur faintly on May 26th by means of cellulose acetate electrophoresis, but became so distinct as to be easily detectable in July even by paper electrophoresis. Around the beginning of September, this additional prealbumin fraction became gradually indistinct again, and finally could not be detected in October even by cellulose acetate electrophoresis. The otolith fluid of goldfish collected during the period of June to September was found to contain a similar additional prealbumin fraction (Fig. 6). As already stated, in the serum of each species of flatfish, a prealbumin fraction was found to migrate on cellulose acetate electrophoresis at the position about 1.2 times (1.1 times on paper) greater than human serum albumin throughout the year (Figs. 5 and 8). However, such a prealbumin fraction was not found in the otolith fluid throughout the year. Consequently, occurrence of the additional prealbumin fraction of the fluid was recognized unmistakably in the flatfish.

In the case of rainbow trout, the proteins of the otolith fluid separate into three fractions on paper electrophoresis, the fastest one migrating to the position almost similar to that of human serum albumin (Fig. 7). This pattern was unchanged throughout the year. The cellulose acetate electrophoresis revealed a better separation into five fractions (Fig. 8). However, the pattern obtained by this method also was unchanged throughout the year.

These findings suggest that both the seasonal occurrence of an additional fraction

of prealbumin and the seasonal increase of the protein concentration in the otolith fluid may favor the formation of the organic matrix, or conchiolin,⁴ of the opaque zone of the otolith, at least in the flatfish and goldfish. However, as the monthly determination of the total protein in the latter fish is not yet completed, the data are not presented here.

Polysaccharides of the otolith fluid also are considered to play an important role in otolith formation. Studies made on these substances will be published in the near future.

Summary

The protein concentration and distribution of the protein fractions of the otolith fluid (endolymph of the sacculus) were determined seasonally by the micro-Kjeldahl method and by paper and cellulose acetate electrophoresis, using three types of fish, flatfish, goldfish and rainbow trout, which have differing otolith characteristics.

(1) The otolith fluid was successfully collected from the sacculus by use of a fine glass capillary. The average amount of this fluid collected from one medium-sized individual was about 15 μ l in flatfish, 1 μ l in goldfish, and 7 μ l in rainbow trout.

(2) An additional fraction of prealbumin was found to occur seasonally in the otolith fluid of both flatfish and goldfish at a certain time corresponding to the period of opaque zone formation in their otoliths, while such a seasonal occurrence of a prealbumin fraction was not detected electrophoretically throughout the year in the otolith fluid of rainbow trout whose otolith is homogeneously glassy, nor in all the sera of the species examined.

(3) In the otolith fluid of flatfish, the protein concentration was found to increase seasonally corresponding to the period of opaque zone formation in the otolith, while such a seasonal change of the protein concentration was not found in the otolith fluid of rainbow trout, nor in the sera of both flatfish and rainbow trout.

(4) Accordingly, both the seasonal occurrence of an additional prealbumin fraction and the seasonal increase of the protein concentration in the otolith fluid may favor the formation of the organic matrix of the opaque zone in the otolith.

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Explanation of Plate I

Figs. 1-3. Photomicrographs showing the ground preparations of the otoliths in three types of fish. 1. Otolith of flatfish, *Kareius bicoloratus*, caught in July—the periphery is opaque. 2. Otolith of goldfish sacrificed in August—the periphery is opaque. 3. Otolith of rainbow trout sacrificed in July, not showing any alternate pattern of opaque and translucent zones.

Fig. 4. A photomicrograph of a transverse section of the head of a rainbow trout fry showing a sacculus within which are enclosed an otolith and the otolith fluid (endolymph). Mallory azan triple stain. B: brain; M: thin membrane around the otolith; O: otolith; S: sacculus. $\times 140$.

PLATE I

Explanation of Plate II

Fig. 5. Paper electrophoretic patterns and their scanning diagrams showing the protein fractions of both the otolith fluid and serum in flatfish, *Kareius bicoloratus*. A: otolith fluid collected from an individual caught in July; B: serum obtained from the same individual. In the scanning diagrams, a solid line indicates otolith fluid, a broken line fish serum, and a dotted line human serum. Arrow indicates the origin of electrophoresis.

Fig. 6. Paper electrophoretic patterns and their scanning diagrams showing the protein fractions of both the otolith fluid and serum in goldfish. A: otolith fluid pooled from 10 individuals sacrificed in August; B: serum obtained from those individuals. In the scanning diagrams, a solid line indicates otolith fluid, a broken line fish serum, and a dotted line human serum. Arrow: origin.

PLATE II

Explanation of Plate III

Fig. 7. Paper electrophoretic patterns and their scanning diagram showing the protein fractions of both the otolith fluid and serum in rainbow trout. A: otolith fluid collected from an individual sacrificed in July; B: serum obtained from the same individual. In the scanning diagrams, a solid line indicates otolith fluid, a broken line fish serum, and a dotted line human serum. Arrow: origin.

Fig. 8. Negative contact prints from uncleared cellulose acetate strips showing the electrophoretic patterns of the protein fractions of both the otolith fluid and serum in flatfish, *Kareius bicoloratus*, and rainbow trout. A: otolith fluid of flatfish collected from an individual caught in July; B: serum obtained from the same individual; C: otolith fluid of rainbow trout collected from an individual sacrificed in July; D: serum obtained from the same individual. Alb: relative migration distance of human serum albumin; arrow: origin.

PLATE III