HISTOCHEMICAL STUDY ON THE CHANGE OF 17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN THE OYSTER DURING THE STAGES OF SEXUAL MATURATION AND SPAWNING

HISTOCHEMICAL STUDY ON THE CHANGE OF 17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN THE OYSTER DURING THE STAGES OF SEXUAL MATURATION AND SPAWNING

By

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Introduction

In the mammalian tissues, the Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-enzyme) and 17β-hydroxysteroid dehydrogenase (17β-enzyme) have been studied biochemically and histochemically, because these enzymes are considered to be responsible for the metabolism of steroid hormones (1-6). In the invertebrate tissues, however, no reports had ever been known on the presence of these enzymes, until the present authors (7, 8) demonstrated it histochemically in the tissues of maturing oysters. They proved that the Δ^5-3β-enzyme activity exists in the elongated epitheloid tissues adjacent to the visceral ganglion and adductor muscle, and in the interstitial cells in the glycogen-bearing tissues of the oysters kept in the high temperature condition after their spawning (7). And then, in the oysters at their natural maturing season, they proved that the activity exists in the interstitial cells of the gonad and those of the glycogen-bearing connective tissue between the gonad and digestive tracts (8). The 17β-enzyme activity was observed in the epithelia of the nephridium, digestive diverticulum and intestine of oysters collected during the natural maturing season (8). The reliability of the histochemical techniques used in these studies was confirmed by the control experiments and also by those on the rat tissues.

The aforementioned results obtained in oyster tissues strongly suggest the possibility that the metabolism of steroid sex hormones exists in the marine bivalve. This possibility has also been suggested by Botticelli et al. (9) who reported the presence of estrogens and progesterone in the pecten.

This paper deals with the investigation on the change of the 17β-enzyme activity in the tissues of the oyster during the stages of sexual maturation and spawning to clarify the presence of the metabolism of steroid hormones associated with the sexual maturation.

Materials and Methods

The experiments were carried out from May to December in 1965. Two groups of two year old oysters, *Crassostrea gigas*, which differ in their rate of sexual maturation and accordingly in their spawning period, were used as the materials. One group of oysters had been cultured in Onagawa Bay of Miyagi Prefecture since 1963 by the raft-culture method. The other was transplanted from Onagawa Bay to Matsushima Bay on May 13 in 1965. As indicated in the preceding papers (10, 11), the oysters in Matsushima Bay showed more massive gonad formation than those in Onagawa Bay, and their spawning commenced earlier and occurred repeatedly during a season, mainly due to the environmental conditions such as higher temperature and better nourishment. After investigation of the mortality rate, observations on the gonadal condition of oysters were made mainly macroscopically. The fresh tissues containing the nephridium, digestive diverticulum and intestine of three to four individuals were sampled regularly for the histochemical observation of the 17β-enzyme reaction. In a few cases, seven or more oysters were examined. For the demonstration of the enzyme activity, the histochemical methods employed in the previous paper (8) were used. Only estradiol-17β was used as a substrate in the incubation medium. The control media, i.e., NAD without estradiol-17β and estradiol-17β without NAD, were also used in all of the incubation experiments. Parallel sections were sometimes incubated for the diaphorase reaction using $NADH_2$ (final conc., 0.61mM) for 60 minutes.

Observation

I. Seasonal change of the 17β-enzyme activity in the tissues of oysters cultured in Onagawa Bay (Table 1).

On May 13, when the present study was started, the mantles appeared white, and the digestive diverticula were surrounded by a milky white layer of connective tissues. Sexes were not distinct in most oysters. In the epithelia of the

Table 1. Seasonal change of 17β-hydroxysteroid dehydrogenase

Organ and Tissue \ Sampling / Sex		May 13	June 11		
		?	♀	♂	?
Nephridium		±* & −	+*~±	+*~±	±* & −
Intestine		−	±	−	−
Digestive diverticulum	Duct	−	−~±	−~±	−
	Tubule	−	±~+	±~++	−

* The enzyme activity was observed sporadically.

nephridium, a very faint enzyme reaction was found in a few cells located sporadically (Fig. 1). In the digestive tracts, no activity was observed (Fig. 2).

On June 11, the sexes were distinct in a considerable number of oysters, though they were still at the early stage of maturation. In oysters whose sexes were distinct, the enzyme activity of partially high intensity was observed in the epithelia (mainly in the light cells) of tubules of the digestive diverticulum. A weak enzyme reaction was also observed in the epithelia of the nephridium, intestine and ducts of the digestive diverticulum. In oysters whose sexes were not distinct, the enzyme activity was scarcely demonstrated in the epithelia of the digestive tracts. However, a very faint enzyme reaction was found in a few cells located sporadically in the nephridium.

On July 9, the gonads developed considerably and the sexes of all oysters were distinct even by the smearing method. In the nephridium, there were a large number of sporadical parts showing an intense enzyme activity, though a faint reaction was observed all over the tissue (Fig. 3). Most of these strongly reactive parts consisted of several cells (Figs. 4, 5). The epithelia (mainly the light cells) of the tubules of the digestive diverticulum showed a weak reaction. A faint reaction was also observed in the epithelia of the intestine and ducts of the digestive diverticulum. The diaphorase activtiy was intense in these organs.

On July 24, the sexual maturation of the oysters was found to have made rapid progress. The genital canals and gonoducts appeared clear in most individuals. The mantles remained creamy-white. In the nephridium, a strong enzyme reaction was found all over the tissue (Fig. 6). The reaction in the digestive tracts was about the same as that of July 9, except that a stronger enzyme activity was observed partially in the epithelia (mainly light cells) of the tubules of the digestive diverticulum. The diaphorase activity was considerably intense in all tissues.

On August 19, oysters were found to have large numbers of sexual cells, and their genital canals and gonoducts were very clear. Symptoms indicative of spawning were not observed in any individual, though the mantles were pretty translucent in

activity in the tissues of oysters cultured in Onagawa Bay.

July 9		July 24	Aug. 19	Sept. 27		Nov. 24
♀	♂	♀	♀	♀	♂	?
⧺* & ±	+～⧺	⧺～⧺	⧺	±* & −	±* & −	±* & −
±	−	−～±	±～+	−	−	−
±	−～±	−～±	±～⧺	−～±	−	−
±～+	±～+	±～⧺	+～⧺	±～+	±	−

a few oysters. The enzyme reaction in all tissues examined was stronger than that of July 24. As is shown in Fig. 9, a strong reaction was observed in the light cells of the tubules of the digestive diverticulum.

On September 27, the spawning was already finished and the mantles appeared partially translucent and watery in most oysters. But, sexual cells still remained in considerable numbers, therefore the sexes were distinctly separable in all individuals. A marked decline in the enzyme activity was observed in all tissues. In the nephridium, the reaction was the same as that of May 13.

On November 24, the gills and layer of connective tissues surrounding the digestive diverticula as well as the mantles were almost transparent and watery in many oysters, while the mantles were slightly white in a a few individuals. The sexes were not distinct in most oysters. In some cases, the epithelia of the nephridium showed a very faint enzyme activity, but no reaction was observed in the digestive tracts such as the digestive diverticulum and intestine.

No enzyme reaction was observed in the control media.

II. Seasonal change of the 17β-enzyme activity in the tissues of oysters transplanted to Matsushima Bay (Table 2).

On May 31, the digestive diverticula were completely covered with male or female sexual cells in most oysters. The genital canals and gonoducts, however, were not clear, indicating that the sexual maturation was just beginning. The mantles were completely creamy-white. A fairly strong reaction of the 17β-enzyme activity was observed in the epithelia of the nephridium. The enzyme reaction was also found in the digestive diverticulum and intestine, though it was generally much weaker than in the nephridium.

On July 7, oysters contained a very large number of sexual cells, and the genital canals and gonoducts were clearly seen. The mantles were partially translucent in many oysters. A partial spawning had occurred in a few individuals. In the nephridium, there were a few sporadical parts which showed a very strong

Table 2. Seasonal change of 17β-hydroxysteroid dehydrogenase

Organ and Tissue \ Sampling		May 13	May 31		July 7	July 24	
	Sex	?	♀	♂	♀	♀	♂
Nephridium		±* & −	++	++	+++* & +	+++	++~+++
Intestine		−	−	−~±	−~±	−~±	−~±
Digestive diverticulum	Duct	−	±	±~+	−~±	−	−
	Tubule	−	+	±~+	±~+	−~±	−

* The enzyme activity was observed sporadically. ** Rare Case

enzyme activity, though a weak reaction was observed all over the tissue. However, such sporadical parts were so scarce that the enzyme activity in the tissue as a whole appeared weaker than that of May 31. The reaction in the digestive tracts was as strong as that of May 31.

On July 24, an increased gonad formation was observed in most oysters. In the nephridium, an intense enzyme reaction was observed all over the tissue (Figs. 7, 8). In the digestive tracts, however, only a slight and partial activity was observed. The duct of the digestive diverticulum showed no reaction in most individuals. The positive diaphorase reaction was observed in all organs.

The mortality had been almost negligible in this group of oysters until the loss of about 23% was found on August 18. In the majority of surviving oysters, the digestive diverticula were covered with extremely large amounts of sexual cells, and the genital canals and gonoducts remained clear, while the mantles were almost transparent and watery. Five oysters in seven showed no enzyme activity in any tissue examined (Figs. 10, 11). In two other oysters, only a faint reaction was found partially in the epithelia of the nephridium.

On September 8, it was clearly recognized that the main spawning was completed in most oysters. The total loss due to the mass mortality up to this time was about 46%. In the majority of surviving oysters, the mantles and gills were almost transparent and watery. As some sexual cells remained undischarged, the sexes were separable. The enzyme reaction was found in the nephridium again. It was considerably strong in a few cases. However, no reaction was observed in the digestive tracts. The diaphorase activity was observed in all organs.

On September 27, no additional deaths were recorded. It was, therefore, presumed that the mass mortality had come to an end. In most oysters, the mantles, gills and layer of connective tissues surrounding the digestive diverticula appeared translucent and watery. The sexes were distinct in all oysters. A few oysters had a considerable number of eggs unspawned. In the nephridium, only a few cells showing a weak enzyme reaction was observed sporadically. In most cases, no reaction was found in the digestive tracts. In a few cases, however, a

activity in the tissues of oysters transplanted to Matsushima Bay.

Aug. 18		Sept. 8		Sept. 27		Nov. 17		Dec. 16
♀	♂	♀	♂	♀	♂	?	♂**	?
−	−	++	+	+*～−	±*～−	±*～−	++* & ±	±* & −
−	−	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−
−	−	−	−	−	−～±	−	−	−

very faint reaction was found in the epithelia of the tubules of the digestive diverticulum.

On November 17, the mantles became slightly white again, indicating that they had entered into the fattening stage. The sexes were indistinguishable, except in rare case in which some sperms remained unspawned. In a few oysters whose sexes were not distinct, a very faint enzyme activity was observed in a few cells located sporadically in the nephridium. In a male oyster, a weak reaction was found all over the nephridium, and a fairly strong one was observed sporadically. No activity was observed in the digestive tracts such as the intestine and digestive diverticulum of all oysters examined.

On December 16, the mantles were partially white in most oysters. The digestive diverticula were surrounded by a thick and milky white layer of connective tissues. In a few cases, a very faint enzyme activity was found in a few cells located sporadically in the nephridium. No activity was observed in the digestive tracts. The intense diaphorase activity was found in all tissues.

No reaction was observed in the control media.

Discussion

The studies on the hormones and reproduction in fishes have been reported and reviewed by several authors during the past ten years (12). As Hoar said in his recent review (12), it is clear that the gonadal hormones are universal in broad outline among the vertebrates; those in fishes are also steroid in nature and those synthesized by the testis are androgenic and those synthesized by the ovary are estrogenic. In the invertebrates, on the other hand, there have been few reports concerning the presence of sex control mechanisms by the steroid hormones, but material with estrogenic activity demonstrable by bioassay in rodents has been found in tissues of diverse groups of marine invertebrate (13). Recently, Botticelli et al. (9) and Lisk (14) have identified progesterone, estradiol-17β, and some other estrogens in the ovaries of marine invertebrates including a marine bivalve, pecten. However, estrogens are also well known in the plant world (12), so there is a possibility that those in the marine invertebrates may be taken in through their mouth together with the foods. Accordingly, the mere presence of a gonadal steroid is no evidence for the biosynthesis of the steroid sex hormone and its hormonal function. Very recently, the present authors (7, 8) demonstrated histochemically the presence of the Δ^5-3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase activities in the tissues of maturing oysters. These results led us to the conclusion that the biosynthesis of steroid hormones might exist in the marine bivalve. However, it has not been proved whether this steroid metabolism is actually associated with sexual maturation.

In the present study, the oysters both in Onagawa Bay and in Matsushima

Bay showed an increase of the 17β-enzyme activity in the epithelia of the nephridium and the digestive tracts as sexual maturation proceeded, and a decline of the activity after spawning. However, certain differences in the occurrence of the enzyme activity were noticed between the oysters in two bays. The increase of the activity in the nephridium appeared earlier, and the increase and decline appeared repeatedly from May to September, in those transplanted to and cultured in Matsushima Bay where sexual maturation proceeded more rapidly than in Onagawa Bay and maturation and spawning occurred repeatedly during a season (10, 11).

These results in the epithelia of the nephridium and digestive tracts strongly suggest that the seasonal change of 17β-hydroxysteroid dehydrogenase activity, namely, the active steroid hormone metabolism closely related to sexual maturation, does exist in the oyster, though it is a little too early to conclude that the sexual maturation in the oyster is controlled by steroid hormones in the same way as in vertebrates.

As has been well known, the presence of reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$) which is the coenzyme of hydroxylase is necessary for the biosynthesis of steroid hormones, because the hydroxylation is involved in many stages including the side-chain splitting of cholesterol (15). It has been suggested, in mammalian tissues, that the oxidative mechanism of pentose phosphate pathway for the metabolism of carbohydrate is responsible for the production of $NADPH_2$, therefore the glycogen breakdown is necessarily activated at the beginning of the steroid hormone biosynthesis (16–18). Mori et al. (10, 11) carried out the biochemical and histochemical studies on the change in the glycogen content of the oyster during the stages of sexual maturation and spawning. They used two groups of oysters kept in Onagawa Bay and Matsushima Bay separately. The results of the studies showed that a decrease in the glycogen content occurred in both bays as sexual maturation proceeded and the glycogen content fell to the bottom at the period of spawning. However, in oysters transplanted to and cultured in Matsushima Bay where body growth and sexual maturation proceeded more rapidly and maturation and spawning occurred repeatedly, the decrease was more marked than in Onagawa Bay. It seems to be evident from these observations that the active breakdown of glycogen is closely associated with the sexual maturation in the oyster. They (10) also reported that the seasonal change of the glycogen content was in parallel with that of the physiological activity, suggesting that glycogen is one of the main energy sources in the oyster. Thus, it may be of great significance for the energy metabolism to attempt to find the relation of the glycogenolysis to the metabolism of steroid sex hormones.

It is interesting that only a very slight or no activity of 17β-enzyme was found already on July 24 especially in the epithelia of the digestive diverticulum in

Matsushima Bay, because we (19) observed the atrophy of the digestive diverticulum in summer. It is also noticeable that the activity was not shown in any tissue of the majority of oysters (five in seven) in the bay, on August 18 just before they spawned in large quantities (Figs. 10, 11). Such phenomena were not observed in Onagawa Bay where the decline in the glycogen content during sexual maturation was less and the summer mortality was almost negligible (10, 11). In Matsushima Bay, the total loss due to the mortality was estimated as about 23% on August 18, and about 46% on September 8 after they spawned in large quantities. These observations indicate that the marked decline in the 17β-enzyme activity occurred just before the mass mortality or in its early stage when the glycogen content fell to the bottom (10, 11). From these results, it can be assumed, concerning the correlation between the extreme fall in the glycogen content and the unusual fall in the 17β-enzyme activity, that a rapid decrease of production of $NADPH_2$ due to the abnormal glycogenolysis exceeding the limit for the normal sexual maturation might cause the unusual drop in the enzyme activity, namely, a disturbance in the metabolism of steroid hormones. Further investigations on these phenomena will be required in connection with the mass mortality.

Summary

1. The seasonal change of the 17β-hydroxysteroid dehydrogenase activity in the Japanese oyster, *Crassostrea gigas*, kept in Onagawa Bay and Matsushima Bay separately was followed histochemically from May to December in 1965 to clarify the presence of the metabolism of steroid hormones associated with the sexual maturation.

2. The oysters both in Onagawa Bay and in Matsushima Bay showed an increase in the enzyme activity in the epithelia of the nephridium and the digestive tracts as sexual maturation proceeded, and a decline after spawning.

3. In Matsushima Bay, sexual maturation proceeded far more rapidly than in Onagawa Bay, and maturation and spawning occurred repeatedly during a season. Also, an increase of the enzyme activity in the nephridium was seen earlier in Matsushima Bay than in Onagawa Bay. The increase and decline of the activity were observed repeatedly from May to September in Matsushima Bay.

4. These results strongly suggest that the seasonal change of the 17β-hydroxysteroid dehydrogenase activity, namely, the active steroid hormone metabolism closely related to sexual maturation, exists in the oyster.

5. An unexpected decline in the enzyme activity in the tissues of the oyster in Matsushima Bay before the enormous spawning was discussed in connection with the occurrence of the mass mortality.

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Plate 1

Explanation of the Figures

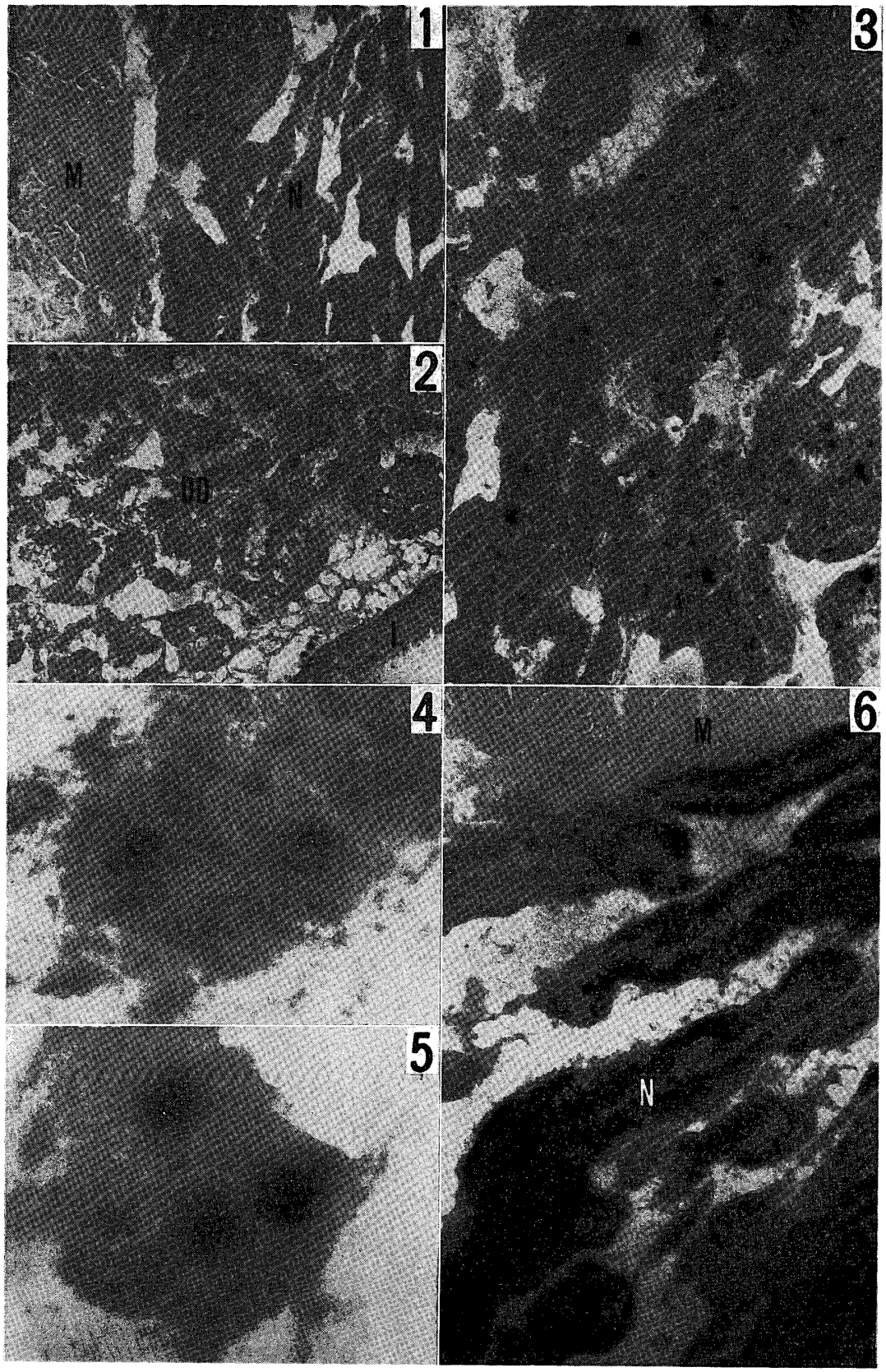
All microphotographs were taken with 12μ fresh frozen sections of oyster tissues cut in a cryostat, and fixed in 10 per cent neutral formalin after incubation for 120 minutes at 37°C. The incubation medium contained estradiol-17β (final concn., ca. 5mM), Nitro-BT (0.16 mM), NAD (0.68 mM) and phosphate buffer, pH 7.1 (0.057 M). The sections were stained by Kernechtrot solution for nuclear differentiation.

Fig. 1. The 17β-enzyme reaction in the epithelia of the nephridium (N) adjacent to the adductor muscle (M) of an oyster collected on May 13 in Onagawa Bay. A very faint enzyme reaction is found in some cells located sporadically. ×160.

Fig. 2. The section from the tissue containing the digestive diverticulum (DD) and intestine (I) of an oyster collected on May 13 in Onagawa Bay. No reaction was observed. ×160.

Figs. 3–5. The 17β-enzyme reaction in the epithelia of the nephridium of an oyster collected on July 9 in Onagawa Bay. There are a large number of sporadical parts showing an intense enzyme activity, though a faint reaction is observed all over the tissue (Fig. 3). Most of these strongly reactive parts consist of several epithelial cells (Figs. 4, 5). Fig. 3, ×160, Figs. 4, 5, ×640.

Fig. 6. The nephridium (N) of an oyster collected on July 24 in Onagawa Bay. A strong reaction is found all over the tissue. ×160.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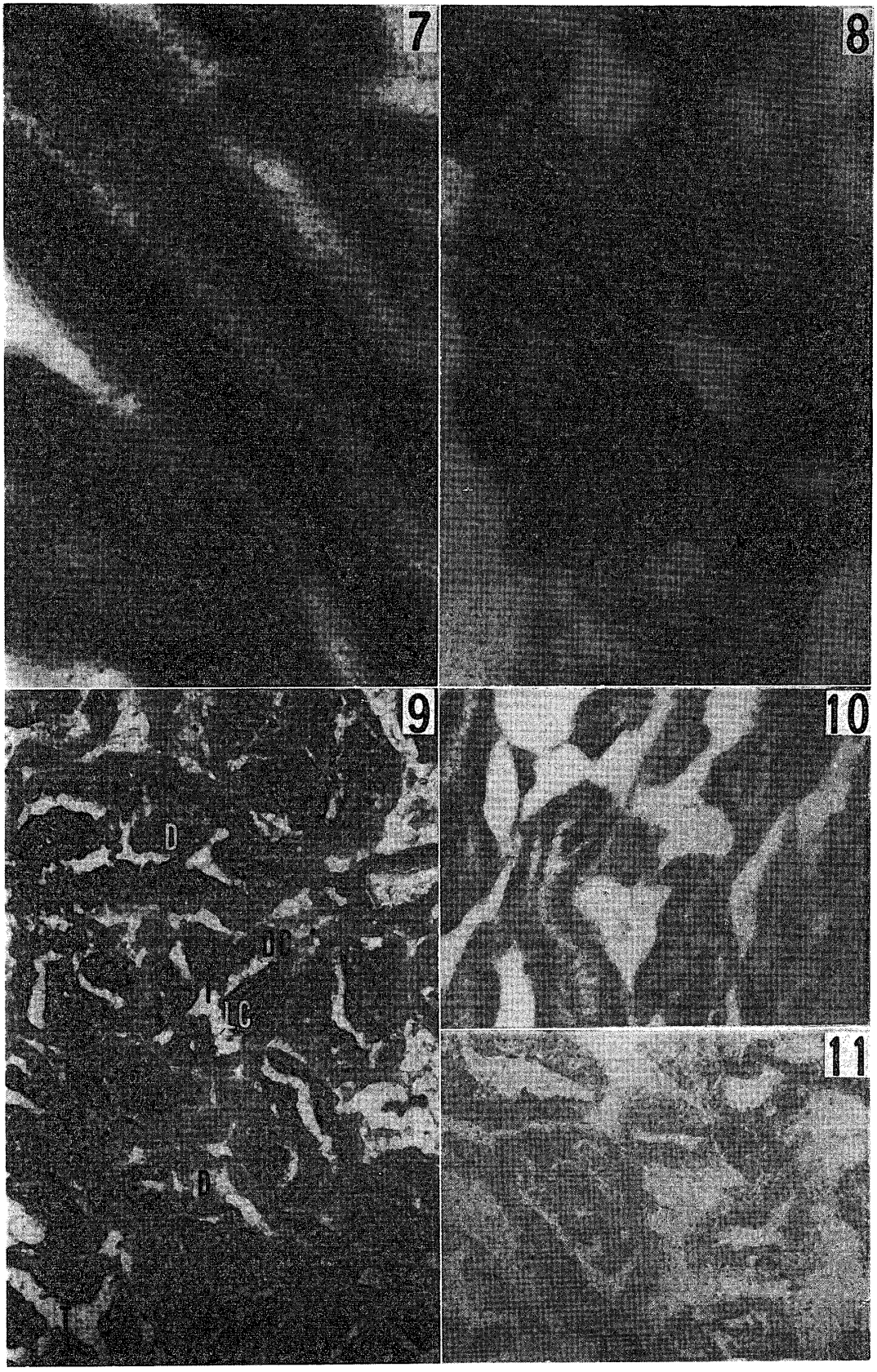
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Plate 2

Explanation of the Figures

Figs. 7 & 8. The 17β-enzyme reaction in the epithelia of the nephridium of an oyster collected on July 24 in Matsushima Bay. An intense enzyme reaction is observed all over the tissue. $\times$640.

Fig. 9. The digestive diverticulum of an oyster collected on August 19 in Onagawa Bay. A strong reaction is observed especially in the light cells (LC) of tubules (T). A reaction is also detectable in the dark cells (DC). A rather strong reaction is seen in the ducts (D). $\times$160.

Fig. 10. The nephridium of an oyster collected on August 18 in Matsushima Bay. Only the nuclei are strongly stained by Kernechtrot with no deposits of formazan. $\times$160.

Fig. 11. The digestive diverticulum of an oyster collected on August 18 in Matsushima Bay. No enzyme activity is observed. $\times$160.