HISTOCHEMICAL STUDY ON THE LOCALIZATION AND PHYSIOLOGICAL SIGNIFICANCE OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE SYSTEM IN THE OYSTER DURING THE STAGES OF SEXUAL MATURATION AND SPAWNING

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Introduction

The glycolytic system of Embden, Meyerhof and Parnas and the TCA cycle of Krebs have been regarded as the main pathways for matabolism of carbohydrate. However, it has been recently known that the pentose phosphate pathway participates in production of reduced nicotinamide-adenine dinucleotide phosphate ($NADPH_2$) which is necessary for biosyntheses of organic compounds including nucleic acid, amino acids and lipids (1). Glucose-6-phosphate (G-6-P) produced through glycogenolysis enters this pathway *via* a NADP mediated dehydrogenation to gluconolactone-6-phosphate, with reduction of NADP to $NADPH_2$. Gluconolactone-6-phosphate is changed to 6-phosphogluconate by gluconolactonase. 6-Phosphogluconate is in turn oxidatively decarboxylated, again using NADP as an electron acceptor, with production of ribulose-5-phosphate and $NADPH_2$.

It has been proved that $NADPH_2$ is concerned with a number of steps in steroidogenesis including the ring closure and hydroxylation (1, 2). From biochemical (3) and histochemical (4, 5) investigations on the glucose-6-phosphate dehydrogenase (G-6-P enzyme) system, it has been suggested that the adrenal cortex known as the site of active production of steroid hormones is one of the few mammalian tissues which utilizes the oxidative mechanism of pentose phosphate pathway for carbohydrate metabolism in preference to the more usual glycolytic system and TCA cycle. On the other hand, our histochemical studies on the presence of activities of steroid dehydrogenases (6, 7) and on the seasonal change of 17β-hydroxysteroid dehydrogenase activity (8) strongly suggest that the active steroid metabolism which is closely connected with sexual maturation exists in the oyster.

This paper deals with the histochemical localization of glucose-6-phosphate dehydrogenase system in the oyster at the maturing stage, and with the comparison

between the enzyme activity at this stage and that after the spawning season, to clarify a physiological significance of the enzyme system.

Materials and Methods

Japanese oysters, *Crassostrea gigas*, which had been cultured for two years in Matsushima Bay by the raft-culture method, were used as the materials. The experiments were carried out on July 7 and September 20 in 1966. Seven oysters were sampled in each experiment. The fresh tissues containing the nephridium adjacent to the visceral ganglion which is situated in a shallow depression between the two divisions of the adductor muscle, and those of the digestive diverticulum, intestine, glycogen-bearing connective tissue and gonad of both sexes were studied.

The blocks of fresh tissues were placed in a cryostat (temperature, –20°C) for two hours. They were then sectioned at 12μ. Before incubation, the sections mounted on cover glasses were rinsed in a cold (4°C) acetone for 10 minutes to remove lipids. They were then immersed in 0.1M veronal buffer (pH 7.1) at room temperature for five minutes to remove acetone and soluble endogenous substrates. They were transferred to the substrate solution designed to demonstrate the activity of G-6-P enzyme system.

This solution was a modification of that used by Cohen (4). It contained barium salt of G-6-P (final conc., ca. 5mM), Nitro-Blue Tetrazolium (Nitro-BT: 0.16 mM), NADP (0.56 mM), potassium cyanide (5mM) and veronal buffer, pH 7.1 (0.057M). Potassium cyanide was added as an inhibitor of tissue cytochrome system. In this incubation medium, G-6-P enzyme transfers electrons from the substrate to NADP; in turn the reduced coenzyme is oxidized by $NADPH_2$ diaphorase, which transfers the electrons to Nitro-BT. Reduction of Nitro-BT results in deposition of a dark blue, granular pigment, namely, a formazan, at site of enzyme activity. The following control media were used; G-6-P without NADP, and NADP without any substrate.

In order to study the specificity of localization and a metabolic signiñcance of G-6-P enzyme system, the comparison of activities of this enzyme and the succinate (S-enzyme) and malate (M-enzyme) dehydrogenases of TCA cycle, was carried out histochemically using parallel tissue sections. The 12μ sections mounted on cover glasses were first treated with cold acetone for 10 minutes and 0.1M phosphate buffer (pH 7.1) for five minutes. They were then transferred to the incubation media of Nachlas et al. (9) for S-enzyme activity and Hess et al. (10) for M-enzyme activity. Each control medium lacked the specific substrate. The substrate without coenzyme medium was also used in the control incubation for M-enzyme.

The incubation time was 60 minutes at 37°C in all media. After incubation,

the sections were fixed for five minutes in 10 per cent neutral formalin and counter-stained with Kernechtrot solution for five minutes after washing in distilled water. They were then dehydrated through a series of ethanol and xylene, and finally mounted on slide glasses with Canada balsam.

Observation

I. Localization of G-6-P enzyme system in oysters sampled on July 7.

Most oysters (five in seven) contained a large number of sexual cells, and the genital canals and gonoducts were clearly seen. The color of mantles was creamy-yellowish. The mantle response to a mechanical stimulus was strongly positive, roughly indicating that oysters were healthy. From these observations,

Table 1. Histochemical demonstration of the activities of glucose-6-phosphate, succinate and malate dehydrogenases in the tissues of maturing oysters (Incubation time: 60 minutes at 37°C)

Substrate and coenzyme / Organ and tissue		Glucose-6-phosphate*1		NADP without substrate	Succinate*3 without coenzyme	L-Malate*3 plus NAD*4
		plus NADP*2	without NADP			
Nephridium		++	−	−	++～+++	+～++
Connective tissue around the nephridium		−	−	−	+	++～+++
Adductor muscle		−	−	−	+	+～++
Visceral ganglion		−～±	−	−	±	+
Cerebro-visceral connective		±&++	−	−	±	+
Intestine		±～+	−	−	−～++	++
Digestive diverticulum	Duct	++～+++*5	−	−	++～+++	+～++
	Tubule	−～±	−	−	+	+～++
Glycogen-bearing connective tissue		−	−	−	−～±	+++
Gonad	Egg	−	−	−	++++	++
	Sperm	−	−	−	++++	
	Genital canal	−～+	−	−	±	++
	Gonoduct	−～++	−	−	±	+～+++

*1. barium salt
*2. NADP=nicotinamide-adenine dinucleotide phosphate
*3. sodium salt
*4. NAD=nicotinamide-adenine dinucleotide
*5. weaker or no reaction in the basal part of the epithelium

it was suggested that sexual maturation was actively proceeding in these oysters.

A partial spawning had occurred in two other individuals, whose mantles were partially translucent. The mantle response was strongly positive also in them.

In maturing oysters, a strong reaction of G-6-P enzyme (second column, Table 1) was observed in the epithelium of the duct of digestive diverticulum (Figs. 1, 2). A considerably intense reaction was found in the epithelium of the nephridium (Figs. 3, 4). Such a reaction was partially demonstrable in the cerebro-visceral connective (Figs. 5, 6) and gonoduct. A weak activity was observed in the visceral ganglion and in the epithelia of the intestine, tubule of digestive diverticulum and genital canal. The enzyme activity was not detectable in the connective tissue around the nephridium. The adductor muscle, glycogen-bearing connective tissue, eggs and sperms showed no formazan deposition. In oysters which showed a symptom of partial spawning, the enzyme reaction was not demonstrated in the nephridium with a few exceptions, though other tissues showed about the same intensity of reaction as in maturing oysters. All experimental tissues of oysters showed no demonstrable reaction in two control media; G-6-P without NADP, and NADP without any substrate (third and fourth columns, Table 1).

S-enzyme reaction was widely demonstrated in tissues of all oysters examined (fifth column, Table 1). The greatest deposition of formazan was found in the eggs and sperms (Fig. 7). The epithelia of the nephridium and duct of digestive diverticulum showed less formazan deposition. A considerably strong activity was partially found in the epithelium of the intestine. M-enzyme activity was also widely observed in tissues of all oysters examined (sixth column, Table 1). A strong activity was found in the connective tissue around the nephridium and in the glycogen-bearing connective tissue. A partial intense reaction was demonstrated in the gonoduct. The intestine, eggs and genital canal showed a fairly strong reaction. The nephridium, adductor muscle and digestive diverticulum showed about the same or less formazan deposition. The reaction of both S- and M-enzymes was negative in all control media.

II. Localization of G-6-P enzyme system in oysters sampled on September 20.

The surface epithelia of most oysters were so transparent that the dark brown color of underlying digestive organs was fairly visible through the thin and watery tissue, indicating that spawning was already finished. As a small number of sexual cells remained undischarged, sexes were distinct in all oysters. The mantle response to a mechanical stimulus was strongly positive.

G-6-P enzyme activity (second column, Table 2) was not demonstrated in the nephridium (Fig. 8), though a faint reaction was partially found in a few oysters. The intestine and tubule of digestive diverticulum were also negative in most cases. In the epithelium of the intestine, however, a considerable strong reaction was found

Table 2. Histochemical demonstration of the activities of glucose-6-phosphate, succinate and malate dehydrogenases in the tissues of oysters after the spawning season (Incubation time: 60 minutes at 37°C)

Substrate and coenzyme / Organ and tissue		Glucose-6-phosphate		NADP without substrate	Succinate without coenzyme	L-Malate plus NAD
		plus NADP	without NADP			
Nephridium		−	−	−	++	++
Connective tissue around the nephridium		−	−		−~±	+~++
Adductor muscle		−	−	−	+	+~++
Visceral ganglion		−~±	−	−	±	
Cerebro-visceral connective		−~±	−	−	±	±
Intestine		++*1 & −	−	−	−~++	++
Digestive diverticulum	Duct	−~++	−	−	++~+++	+
	Tubule	−	−	−	+	+
Glycogen-bearing connective tissue		−	−	−	−	+~++
Gonad	Egg	−	−	−	+	−~±
	Sperm	−	−	−	±	
	Genital canal	±~++ & −*2	−	−	±	++
	Gonoduct	+~+++ & −*2	−	−	±	++

*1. Rare case
*2. Negative in three oysters in seven

in rare cases. The cerebro-visceral connective and the epithelium of the duct of digestive diverticulum (Fig. 9) showed less formazan deposition on this date than on July 7. The visceral ganglion showed a faint reaction. The enzyme reaction was not observed in the genital canal and gonoduct of three oysters in seven. However, the reaction was stronger in these tissues of four other female individuals which had comparatively many sexual cells unspawned, than in those of July 7 (Figs. 10, 11). Other tissues showed no formazan deposition. No reaction was found in the control media (third and fourth columns, Table 2).

With both S- and M-enzyme reactions (fifth and sixth columns, Table 2), there was no wide difference in intensity between the tissues of oysters of July 7 and September 20 with a few exceptions. In the sexual cells, these enzyme reactions were far weaker on this date than on July 7. They were also weaker in the connective tissue around the nephridium (Fig. 12) and in the glycogen-bearing

connective tissue. No reaction was observed in the control media of S- and M-enzymes.

Discussion

In the present study, the activity of glucose-6-phosphate dehydrogenase system was histochemically demonstrated in the epithelia of the nephridium, digestive diverticulum and intestine of maturing oysters (second column, Table 1). Since this enzyme system is a potential provider of $NADPH_2$ which is necessary for steroid synthesis, demonstration of its presence in these tissues where active steroid synthesis is presumed to occur (7, 8) is reasonable and of considerable interest. The activity was also detectable in the visceral ganglion, cerebro-visceral connective, genital canal and gonoduct, but there are no available data concerning the metabolic significance of its presence. It was not observed in the connective tissue around the nephridium, adductor muscle, glycogen-bearing connective tissue, egg or sperm, suggesting that the oxidative mechanism of pentose phosphate pathway for metabolism of carbohydrate is not utilized in these tissues or its function is too low to be demonstrable by the present procedure.

The pattern of formazan distribution observed in the procedure for demonstrating the activity of succinate or malate dehydrogenase (fifth or sixth column, Table 1) differed from that for G-6-P enzyme. The tissues which were negative with G-6-P enzyme procedure were positive with S- and M-enzyme procedures. This was evident in the sexual cells, connective tissue around the nephridium, adductor muscle and glycogen-bearing connective tissue. Such a non-parallelism of formazan distribution is also found between $\triangle^5$-3β- or 17β-hydroxysteroid dehydrogenase (6,7) and TCA cycle enzymes (in the present study). These results with oyster tissues may indicate that the enzymes which are involved in steroid metabolism, including G-6-P enzyme system, are limited in distribution as has been known with mammalian tissues (4, 11). A wider distribution of S- and M-enzymes may be due to a wider participation of these enzymes in a number of fundamental metabolic systems.

With both S- and M-enzyme reactions, there was no wide difference in intensity between the tissues of oysters of July 7 and September 20 with a few exceptions (fifth and sixth columns, Tables 1 and 2). In contrast, a decline in intensity after spawning was observed with G-6-P enzyme reaction in the nephridium, intestine and digestive diverticulum. The decline was also found in the cerebro-visceral connective. These observations strongly suggest that the G-6-P enzyme system which is closely related to sexual maturation exists in the oyster as was anticipated in the preceding paper (8).

It has been suggested that it may be of great significance for energy metabolism to attempt to find the ralation of glycogenolysis to steroid metabolism

in the oyster (8). On the basis of the present and previous observations (6–8, 12), it may be possible to propose the following hypothesis. During the stage of sexual maturation, the stimulated steroid metabolism acts on the tissues such as the nephridium and duct of digestive diverticulum to cause an activation of the G-6-P enzyme system to produce $NADPH_2$, a reductant important in steroid synthesis. Glycogen is the source of G-6-P, therefore glycogen breakdown is necessarily activated in steroid biosynthesis (13, 14). On the other hand, glycogen is one of the main energy sources in the oyster (12). Accordingly, a marked sexual maturation causes a decline in physiological activity. With this hypothesis, it seems to be rather easy to account for the observation that the decline both in physiological activity and in glycogen content was far more marked in oysters transplanted to and cultured in Matushima Bay where sexual maturation proceeded far more markedly, than in those kept in Onagawa Bay (12).

It has been well known that $NADPH_2$ is also necessary for biosynthesis of lipids such as fatty acid or neutral fat (1). Mori et al. (15) reported that total lipids or sudanophilic substances were found to deposit in the epithelia of the digestive diverticulum and intestine of oysters during the stages of sexual maturation and spawning, and to show a drop in amount after spawning. This deposition of lipids was more marked in oysters of Matsushima Bay where sexual maturation proceeded far more markedly, than in those of Onagawa Bay. In this study, G-6-P enzyme system was found to show the activity in the epithelia of these digestive tracts during the stages of sexual maturation and partial spawning, and to show a drop in activity after spawning. These results indicate that the lipid biosynthesis which is connected with sexual maturation exists in the epithelia of digestive tracts of the oyster. Further investigations, however, will be required to clarify the physiological role of this lipid deposition.

On July 7, the genital canal and gonoduct showed a weak or partial intense activity of the G-6-P enzyme system both in maturing oysters and in those which showed a symptom of partial spawning. On September 20 when the spawning season was over, no reaction was found in these tissues of three oysters in seven. However, the enzyme reaction was stronger in these tissues of four other female individuals which had comparatively many sexual cells unspwaned, than in those of July 7. These results seem to suggest that these tissues may show an increase in the activity of G-6-P enzyme system at the late stage of or just after spawning. However, no data are now available on the metabolic significance of this increase in activity.

Summary

1. Glucose-6-phosphate dehydrogenase activity was demonstrated histochemically in the Japanese oysters, *Crassostrea gigas*, which had been cultured for two

years in Matsushima Bay and were collected on July 7 and September 20 in 1966. The comparison of activities of this enzyme and the succinate and malate dehydrogenases of the TCA cycle was carried out using parallel sections.

2. In maturing oysters, G-6-P enzyme activity was observed in the epithelia of the nephridium, digestive diverticulum and intestine. It was also found in the visceral ganglion, cerebro-visceral connective, genital canal and gonoduct. These results suggest that the oxidative mechanism of the pentose phosphate pathway for metabolism of carbohydrate exists in the oyster. It was not detectable in other tissues; the connective tissue around the nephridium, adductor muscle, glycogen-bearing connective tissue, egg or sperm.

3. The distribution of G-6-P enzyme system was far more restrictive than that of TCA cycle enzymes.

4. A decline in intensity after spawning was observed with G-6-P enzyme reaction in the nephridium, intestine and digestive diverticulum. The decline was also found in the cerebro-visceral connective. These observations strongly suggest that the G-6-P enzyme system which is closely related to sexual maturation exists in the oyster as was anticipated in the preceding paper (8).

5. A hypothesis was proposed concerning the glycogenolysis, steroidogenesis and decline in physiological activity of the oyster during the stage of sexual maturation.

6. The result of the present study together with that of our previous one (15) indicated that the lipid biosynthesis which is connected with sexual maturation exists in the epithelia of digestive tracts.

7. It was suggested that the epithelia of the genital canal and gonoduct may show an increase in the activity of G-6-P enzyme system at the late stage of or just after spawning.

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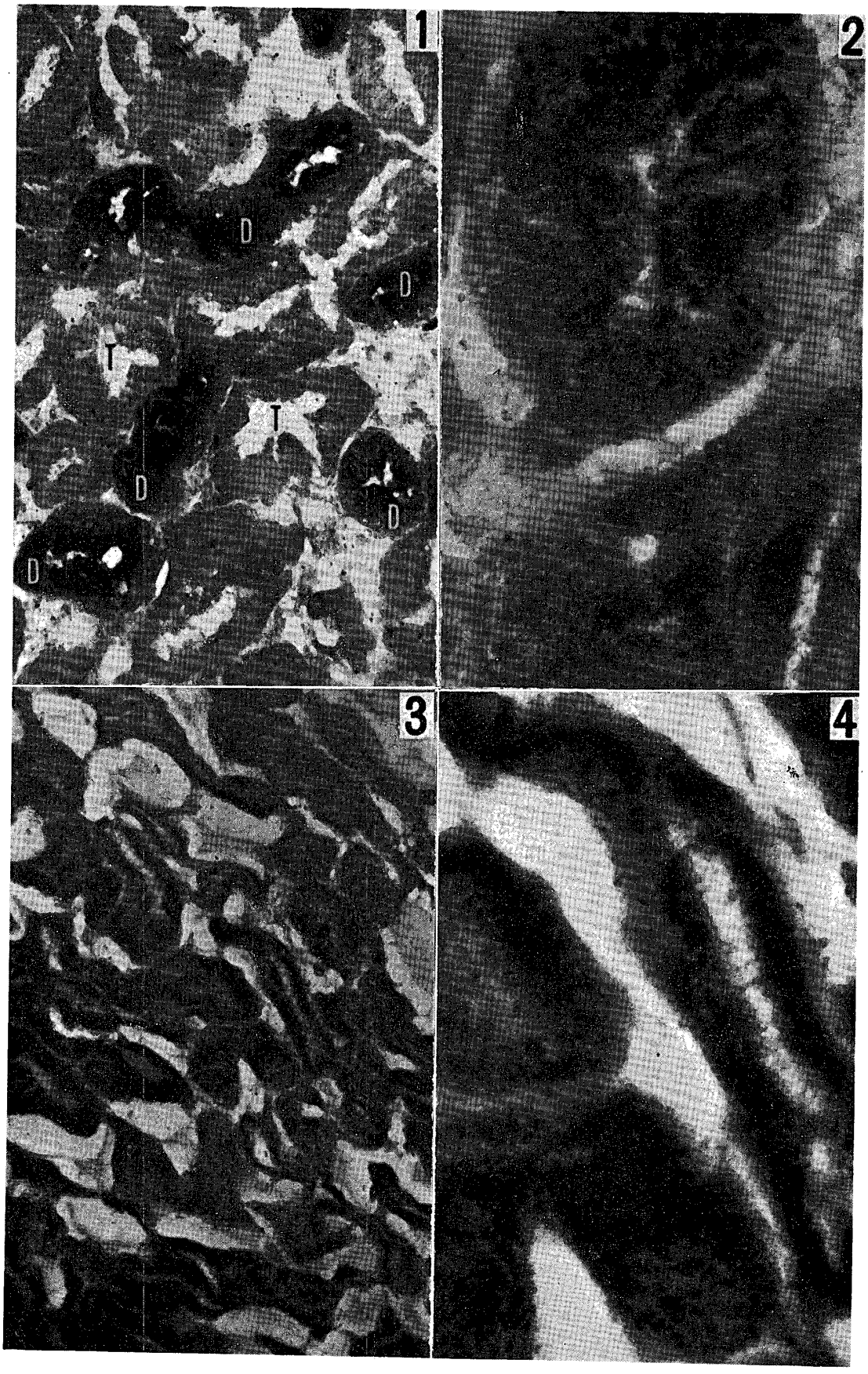
Plate 1

Explanation of the Figures

Figs. 1 and 2. G-6-P enzyme reaction in the digestive diverticulum of a maturing oyster sampled on July 7. A strong reaction is observed in the epithelium of the duct (D). A weak enzyme activity is found in the epithelium of the tubule (T). Fig. 1, × 150. Fig. 2, × 600.

Figs. 3 and 4. G-6-P enzyme reaction in the nephridium of a maturing oyster sampled on July 7. A considerably intense reaction is found in the epithelium. Fig. 3, × 150. Fig. 4, × 600.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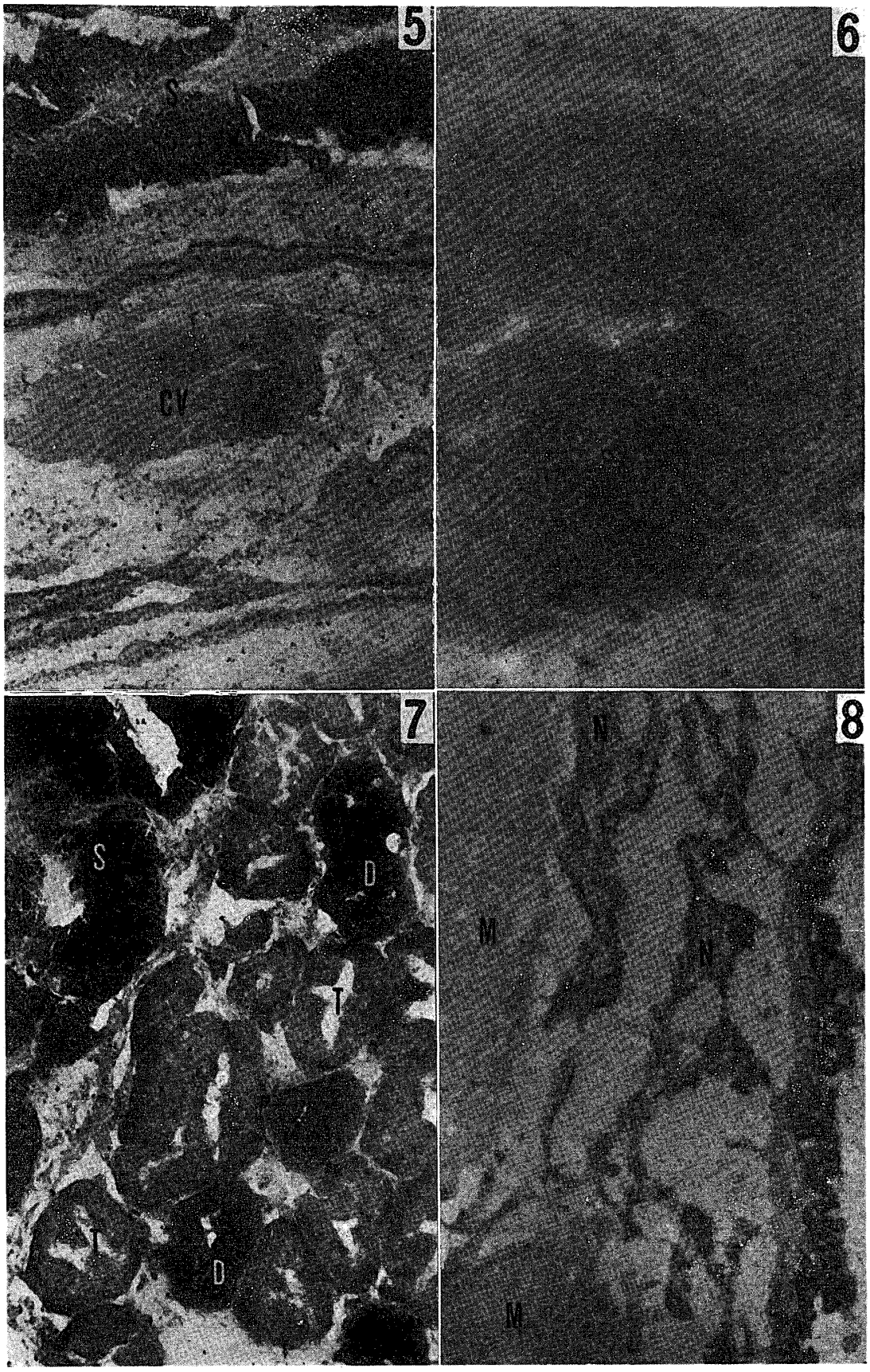
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Plate 2

Explanation of the Figures

Figs. 5 and 6. G-6-P enzyme reaction in the cerebro-visceral connective of a maturing oyster sampled on July 7. A considerably intense reaction is partially observed. CV — cerebro-visceral connective. S—sperms. Fig. 5, × 150. Fig. 6, × 600.

Fig. 7. S-enzyme reaction in the spermary and digestive diverticulum of a maturing oyster sampled on July 7. The greatest deposition of formazan is found in the sperms (S). The epithelium of the duct of digestive diverticulum (D) shows less formazan deposition. A weak reaction is observed in the epithelium of the tubule (T). × 150.

Fig. 8. G-6-P enzyme reaction in the nephridium of an oyster sampled on September 20 when the spawning season was over. No enzyme activity is found. M—adductor muscle. N—nephridium. × 150.

Plate 3

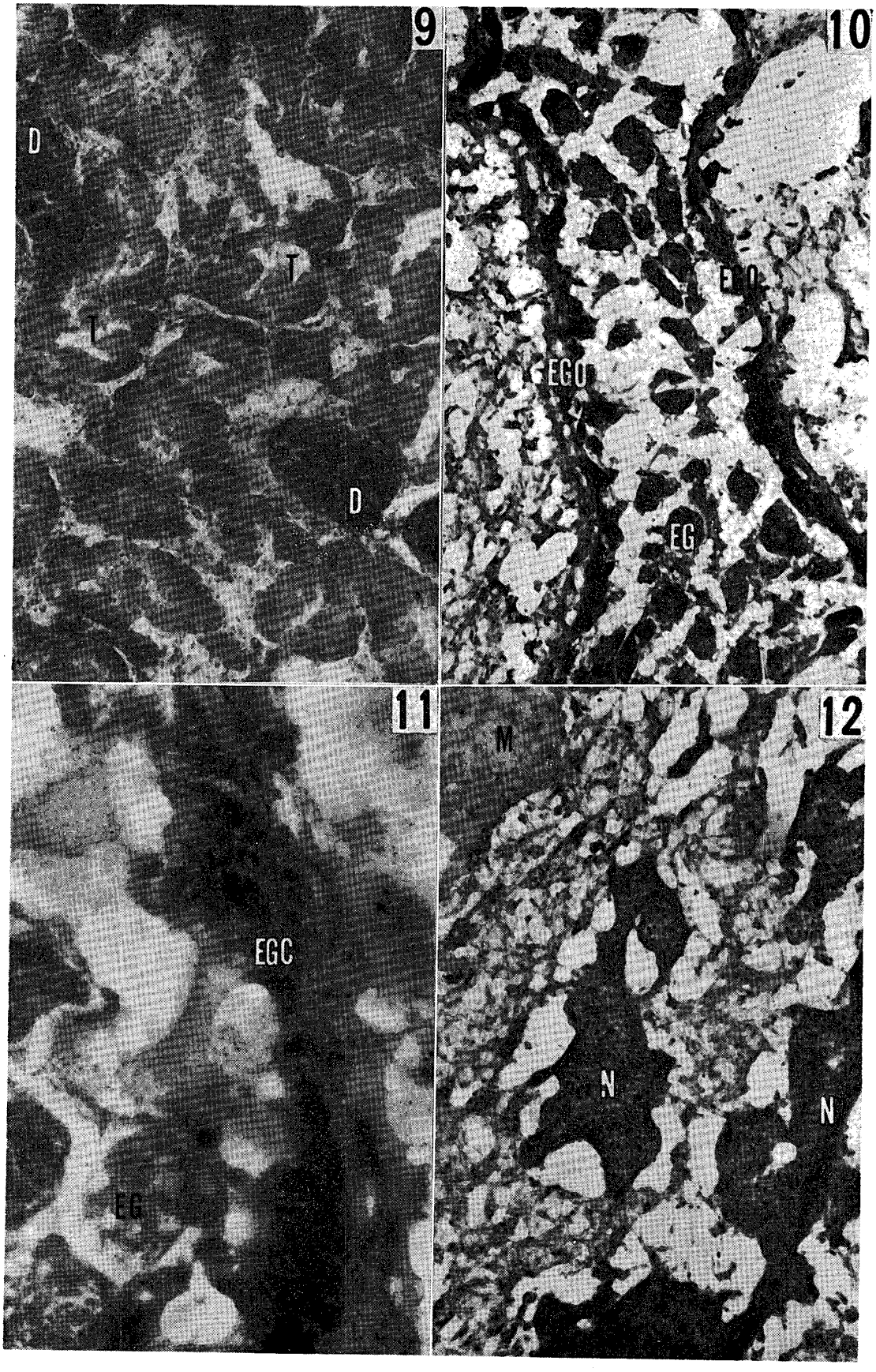
Explanation of the Figures

Fig. 9. G-6-P enzyme reaction in the digestive diverticulum of an oyster sampled on Sept. 20. The epithelium of the duct (D) shows less formazan deposition on this date than on July 7 (Figs. 1 and 2). No reaction is found in the tubule (T). × 150.

Fig. 10. G-6-P enzyme reaction in the gonoduct of an oyster sampled on Sept. 20. A strong reaction is partially observed in the epithelium of the gonoduct (EGO). EG—eggs. × 150.

Fig. 11. G-6-P enzyme reaction in the genital canal of an oyster sampled on Sept. 20. A fairly strong reaction is partially found in the epithelium of the genital canal (EGC). × 600.

Fig. 12. M-enzyme reaction in the nephridium and adductor muscle of an oyster sampled on Sept. 20. A considerably intense reaction is found in the epithelium of the nephridium (N). Such a reaction is partially observed in the adductor muscle (M) and connective tissue around the nephridium. × 150.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