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Summary

A pathogenetic investigation on the mass mortality of oysters in Matsushima Bay was carried out and the following results were obtained.

1) A heavy physiological involution due to the rapid sexual maturation and spawning of the oyster was confirmed by the study on the seasonal change in the morphology of digestive diverticula.

2) A few nodules and abscesses resulting from colonies of both Gram-positive and Gram-negative bacteria, were found in a few specimens regardless of the season. The occurrence of colonies of Gram-negative bacteria, however, seemed to be very rare.

3) It is considered to be most probable that bacteria invaded through the mouth, because their colonies were found most numerously in the mucous membrane of the digestive tracts and in the connective tissues around them. In addition, it was indicated that the bacteria moved to various parts of the body through the blood vessels and through the spaces between the glycogen-bearing cells.

4) Serious pathological changes such as enteritis and multiple abscess were observed only when oysters showed physiological involution in the spawning season. However, the occurrence of such serious changes were rather rare and we could not regard them as the main causative agent of the mass mortality.

5) It was proved that our observations reported as an invasion of the egg by small amoebocytes were similar or identical to the amoebiasis described by Sindermann. We could not regard the amoebiasis as the main cause of the mass mortality, because its frequency of occurrence among egg cells was extremely low.

6) A probable pathogenesis of the mass mortality of the oysters in Matsushima Bay was discussed and it was shown diagrammatically.

7) In conclusion, we assume that the mass mortality of the oyster in Matsushima Bay was caused primarily by a physiological disorder and a metabolic disturbance of the animal as a result of intensive growth and maturation of the gonad under the conditions of high water temperature and very high nutrition, rather than by an infectious diseases caused by a bacterial infection or amoeboid parasites.

The mass mortality of oysters, *Crassostrea gigas*, in Matsushima Bay, Miyagi Prefecture, Japan, has occurred annually (in late summer) since 1961. Such mass mortality has been reported in many parts of the world in the past several decades (1–3). In Japan also, the mass mortality occurred in several important beds of cultivated oysters particularly in the western region (3). However, the causative agents in the mass mortality of oysters in Japan have never been clarified, though it was assumed to be due to either unfavorable environmental conditions or certain infectious pathogens.

We have carried out the ecological, physiological and pathological studies on the mass mortality of the oyster in Matsushima Bay since 1962, with the cooperation of the Tohoku Regional Fisheries Research Laboratory in Shiogama and the Miyagi Prefectural Fisheries Experimental Station in Ishinomaki (4–9). Here we will report the results of the pathogenetic analysis of the mass mortality of oysters in Matsushima Bay.

Materials and Methods

Two year old oysters, *C. gigas*, cultivated in Matsushima Bay by the hanging method, were used for the study. Eight micron paraffin sections crossing the middle part of the body were prepared with the oysters fixed in 10 per cent buffered formalin, alcohol-formalin (1:1 or 9:1) or Helly's fluid. The sections were stained with hematoxylin-eosin, periodic acid-Schiff (PAS), carbol-thionin, Gram's stain of Weigert and Kopeloff-Beerman, acrolein-Schiff, Feulgen and pyronine-methyl green.

Results of Observations and Discussion

Seasonal Change in the Morphology of Digestive Diverticula

It was clearly shown in our physiological study (8) that the digestive diverticula played an important role in the metabolic function of the oyster. In the pathological study (9), marked pathological changes were frequently observed in the digestive diverticula. Therefore, we attempted in this study to follow the seasonal changes in the morphology of the digestive diverticula, in detail, for the period of from May to October in 1965.

In May when oysters were at the early stage of sexual maturation, the tubules consisted distinctly of two kinds of cells, namely, the dark cells and the light cells (Fig. 1). The cytoplasms of the dark cells were well stained with basic dyes such as hematoxylin. In the light cells, the cytoplasm contained numerous granules of a yellowish brown color. The former cells had a lower height than the latter.

In July when oysters were at the peak of sexual maturation, yellowish brown granules in the light cells increased in number. Besides, eosinophilic, acrolein- and PAS-positive globular granules were observed in many of the cells. In a few cases,

some of these globular granules were thrown off as apocrine secretion into the lumen of diverticula.

In August when the oysters were spawning, atrophy of the epithelia was observed in a good many tubules and a marked activation was noticed in the interstitial cells around the tubules (Fig. 2).

In September when oysters had just finished their spawning, a conspicuous activation of the interstitial cells was noticed. Vacuoles of various sizes were observed in the epithelia of the tubules. In a part of the ducts and tubules, the epithelia showed an evagination and some of them were isolated, showing degenerative necrosis (Fig. 3).

In October when the viscera appeared translucent and watery in most oysters, the lumina of the tubules were very much enlarged and most of the cells in the epithelia became flat (Fig. 4). Necrosis was observed in a few cells. Also a marked activity in the interstitial cells was noticed.

These pathological observations indicate that an inflammation of the digestive diverticula occurs during and after spawning, namely from August to October. The mortality rate of the remaining oysters in the group used in this study was 0 per cent in July, 23 per cent in August, 16 per cent in September and 8 per cent in October. The total mortality was nearly 50 per cent. From these results, it can be seen that the inflammation of the digestive diverticula and the mass mortality of oysters occur simultaneously. However, according to our previous study (9), the simple inflammation of the digestive diverticula could not be regarded as pathological, but as the normal physiological involution due to the sexual maturation and spawning of the oyster. We have shown evidence indicating such cyclic physiological involution in the activities of the oyster (7, 8).

Pathological Changes in the Oysters Infected with Bacteria

We (9) reported previously the occurrence of multiple abscess, caused by a bacterial infection in the oysters cultivated in Matsushima Bay. The observations, however, were made on a very few cases. Therefore, in the present study, an attempt was made to detect in detail the appearance of bacterial colonies in the oysters for a period of from April 1965 to March 1966.

Colonies of both Gram-positive and Gram-negative bacteria, nodules and abscesses were observed regardless of the season except for May through July. Colonies of Gram-negative bacteria, however, were observed very rarely. Table 1 shows the seasonal change in the frequency of the occurrence of bacterial infections detected mainly by the Gram's stain of Weigert. No definite coincidence was observed between the bacterial infection and the oyster deaths.

Table 2 shows the relative frequency of the occurrence of the bacterial colonies found in various tissues. It is clear from the table that the colonies are found most numerously in the mucous membranes (lamina propria mucosae) of the digestive

TABLE 1. *Seasonal Change in the Rate of Bacterial Infection in the Oysters Cultivated in Matsushima Bay*

Item	1965 Apr.	May	Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	1966 Jan.	Feb.	Mar.
No. of oysters examined	20	10	20	40	40	30	20	10	10	10	10	10
No. of oysters infected	1	0	0	0	1	5	4	1	1	3	0	2
Rate of infection (%)	5	0	0	0	2.5	16.7	20	10	10	30	0	20
Rate of mortality (%)	0	0	0	0	23	16	8	0	0	0	0	0

TABLE 2. *Relative Frequency of Occurrence of the Bacterial Colonies in Various Tissues of the Oysters Cultivated in Matsushima Bay*

Organ and tissue		1965 Apr.	Aug.	Sep.	Oct	Nov.	Dec.	1966 Jan.	Mar.
Stomach and intestine	Epithelium	−	−	+	−	−	−	−	−
	MM and SCT	+	+	++	++	+	+	+	++
Digestive diverticula	Epithelium	−	−	+	−	−	−	−	−
	MM and SCT	+	+	++	+	−	−	+	+
Gonad	Germ cell	−	−	+	+	−	−	−	−
	Interstitium	+	+	+	+	−	+	+	+
Blood vessel	Lumen	+	−	+	+	−	−	+	+
	SCT	+	−	+	+	−	−	+	+
Epidermis and mantle		−	−	+	+	−	−	−	+

MM-Mucous membrane. SCT-Surrounding connective tissue.

tracts and the surrounding connective tissues as compared to other parts of the body. Therefore, we carried out detailed pathological observation mainly on the digestive tracts and their surrounding tissues, in order to trace the route of the bacterial infection.

Figs. 5 and 6 are the pictures showing a general view of the intestine, blood vessel and glycogen-bearing tissues of a normal, uninfected oyster sampled in January. The cilia of the intestinal epithelia could be clearly seen. The glycogen-bearing cells in the connective tissues were well-fattened. Among these cells, aggregations of amoebocytes were observed though there were no bacterial colonies (Fig. 5), which seems to indicate that the space between the connective tissue cells may serve as a pathway for blood.

In September and October, nearly ten per cent of the oysters showed the following pathological changes. Bacterial colonies were found among the epithelial cells of the stomach where an intense infiltration of amoebocytes was observed surrounding the colonies (Fig. 7). In such a stomach, the epithelial cilia fell off

partially and a compression atrophy was recognized in the pseudostratified epithelia where the bacterial colonies existed. Besides, there were found colonies of bacilli in the connective tissue surrounding the intestine and nodular amoebocytic infiltration was found around the colonies, together with a partial exudation (Fig. 8). As is shown in Fig. 9, multiple abscesses were observed in the glycogen-bearing connective tissue near the blood vessel. It can be presumed from the pictures in Figs. 9 and 10 that the bacterial invasion occurred through a pore-like space in the wall of blood vessel (indicated by an arrow in Figs. 9 and 10), and that the bacteria moved into the connective tissue through the blood pathway in the space of the glycogen-bearing cells. Multiple abscesses were also observed in the mucous membrane of the stomach and in the connective tissues surrounding the digestive diverticula (Figs. 11 and 12). In the case of the stomach (Fig. 11), diffused infiltration of amoebocytes and diffused colonies of bacteria were observed in the lamina submucosa, together with a proliferation of amoebocytes around the abscesses. In addition, the falling-off of the cilia of the stomach and the decrease in the height of the epithelium were clearly recognized. In the digestive diverticula (Fig. 12), a remarkable infiltration of amoebocytes was found in the connective tissue where the glycogen had been spent.

From these observations, we can assume the route of bacterial infection in the oysters. The bacterial invasion takes place through the mouth, because the colonies were found most numerously in the mucous membrane of the digestive tracts and in the connective tissues around the tracts. Then, they move into various parts, through the blood vessel and the space between the glycogen-bearing cells, and there, they multiply.

From our observations, the existence of bacterial colonies was always followed by an infiltration of amoebocytes. In a few cases, however, no infiltration of amoebocytes was recognized around the bacterial colonies among the epithelial cells of the stomach or in the connective tissues surrounding the intestines, though a compression atrophy was seen in some cells around the colonies. Such different reactions to the infiltration of amoebocytes seem to suggest that there is a certain discriminative character toward the amoebocytosis or the phagocytosis in oysters.

In the previous study (9), such serious pathological changes as ulcerative or purulent inflammation were also observed in the epithelia of the intestine in a few oysters sampled in September and October.

No serious pathological changes such as multiple abscess and enteritis described above were observed in oysters sampled except in September and October. Such facts seem to indicate that these serious pathological changes occur only at the stage when the oysters suffer from the physiological involution as a result of rapid sexual maturation and spawning.

An Amoeboid Parasite

Sindermann (10, 11) has recently described the presence of amoeboid parasites in Matsushima Bay oysters collected in August, 1966. According to him, the parasite is cytozoic in blood cells and eggs, and is characterized by a small (1μ) nucleus with a rather thick membrane and a 0.5μ centrally located endosome which is surrounded by a thin clear zone. The overall diameter of the parasite is approximately 5μ to 8μ. The organism appears to undergo budding within eggs to produce inclusions containing 2 to 6 small amoebae. Moreover, he observed a massive infiltration of leucocytes and abscesses formed in infected tissues. However, the frequency of the parasitic invasion of the eggs in the infected oyster was not made clear and the fate of the invaded eggs was not traced in detail.

The morphological characteristics of the amoeboid parasite described by Sindermann seems to be what we have reported as an invasion of the egg by a small type of amoebocyte (9). Therefore, we carried out the following investigations, in order to confirm Sindermann's observation and to investigate whether the parasite could be a causative agent in mass mortality of oysters in Matsushima Bay, or not.

The inclusion body in the egg observed in the present study was a round cell of about 7μ in diameter (Figs. 13–15). It had a round nucleus smaller than 2μ. It was detected singly or as a group of two or three in the cytoplasm of the egg. No clear-cut distinction could be made between the inclusion cell in the egg and a small type of amoebocyte in the connective tissue could not be distinguished clearly in sections stained with ordinary dyes such as hematoxylin-eosin or PAS.

TABLE 3. *Frequency of the Invasion of Eggs by the Amoeboid Cells in the Oysters Cultivated in Matsushima Bay.*

Year	Date	No. of oysters examined	No. of oysters invaded	Percentage of egg cells with parasite in invaded oysters*1
1962	Aug. 13	8	3	3.9% 1.5 0*2
	Aug. 24	5	3	1.2 0 0
	Total	13	6(46.2%)*3	
1964	May 8	6	1	0
	Jun. 2	4	0	
	Jul. 2	3	0	
	Jul. 15	4	0	
	Jul. 25	5	1	0
	Aug. 5	4	2	0 0
	Aug. 12	8	4	1.0 0 0 0
	Aug. 19	4	2	5.0 0
	Total	38	10(26.3%)	

*1. No. of eggs examined, 200.

*2. No. invaded egg was counted in 200 eggs examined, but one or two were detected in all the gonad of a section.

*3. Frequency of the invasion of oysters by the amoebocyte-like cells.

However, while the nucleus of the common amoebocyte was well stained with Feulgen, that of the parasite was not. Therefore, these two cells should be considered to be different.

Then, we investigated the frequency of the invasion of the egg by the amoebocyte-like cell, assuming that the cell is identical with the amoeboid parasite described by Sindermann. The result is given in Table 3. Amoebiasis was detected in 46.2 per cent of oysters sampled in 1962 and in 26.3 per cent in 1964. However, the percentage of eggs invaded by parasites was extremely small, 5 per cent at the most as is shown in Table 3. In many of the cases, no invaded egg was found in 200 eggs examined.

From these observations it is confirmed that the amoebocytic invasion reported in our previous paper (9) is similar to or identical with the amoebiasis described by Sindermann (10, 11). However, we are not able to regard the amoebiasis as a causative agent in the mass mortality of oysters in Matsushima Bay, because its frequency in eggs was extremely low (fifth column, Table 3).

Pathogenesis in Matsushima Oyster

In the present study, we confirmed that the physiological involution occurs following the sexual maturation and spawning of the oyster, and we also made pathological observations on the oysters infected with bacteria and an amoeboid parasite. From the results thus far obtained in the present study together with those of the previous one (9), we will be able to permit a diagram illustrating the

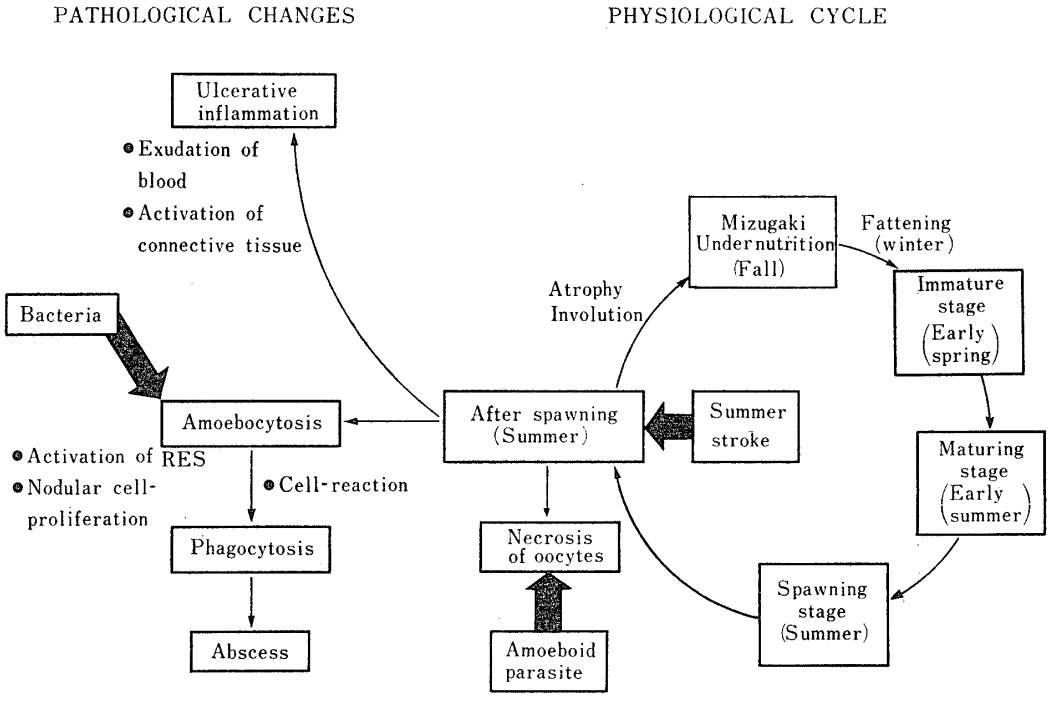

Fig. 16. Possible relation of physiological cycle and pathological changes in oysters cultivated in Matsushima Bay (1965)

pathogenesis of the mass mortality of oysters in Matsushima Bay as is shown in Fig. 16. None of the pathological changes in Fig. 16, however, can be regarded as the direct cause of the mass mortality, because serious pathological changes such as enteritis and multiple abscess occurred rather rarely and the frequency of amoebiasis in the eggs was very rare. We feel that not only further search and investigation of pathogens but also patho-physiological studies are required in order to make a diagram of the pathogenesis in Matsushima oysters a complete one. It must be emphasized that the possible change of non-pathogen into pathogen in connection with the metabolic cycle of oysters should be examined in detail, because serious changes occur only at the stage of the physiological involution due to sexual maturation and spawning of the oyster though bacterial colonies were found regardless of the season.

In conclusion, then, it appears to us that the cause of mass mortality of the oysters in Matsushima Bay is most likely due to the physiological disorder and metabolic disturbance of the animal as a result of heavy gonad formation and spawning under the environmental conditions of high water temperature and rich nutrition, but not due primarily to the bacterial infection or the invasion of the egg by an amoeboid parasite.

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PLATE 1

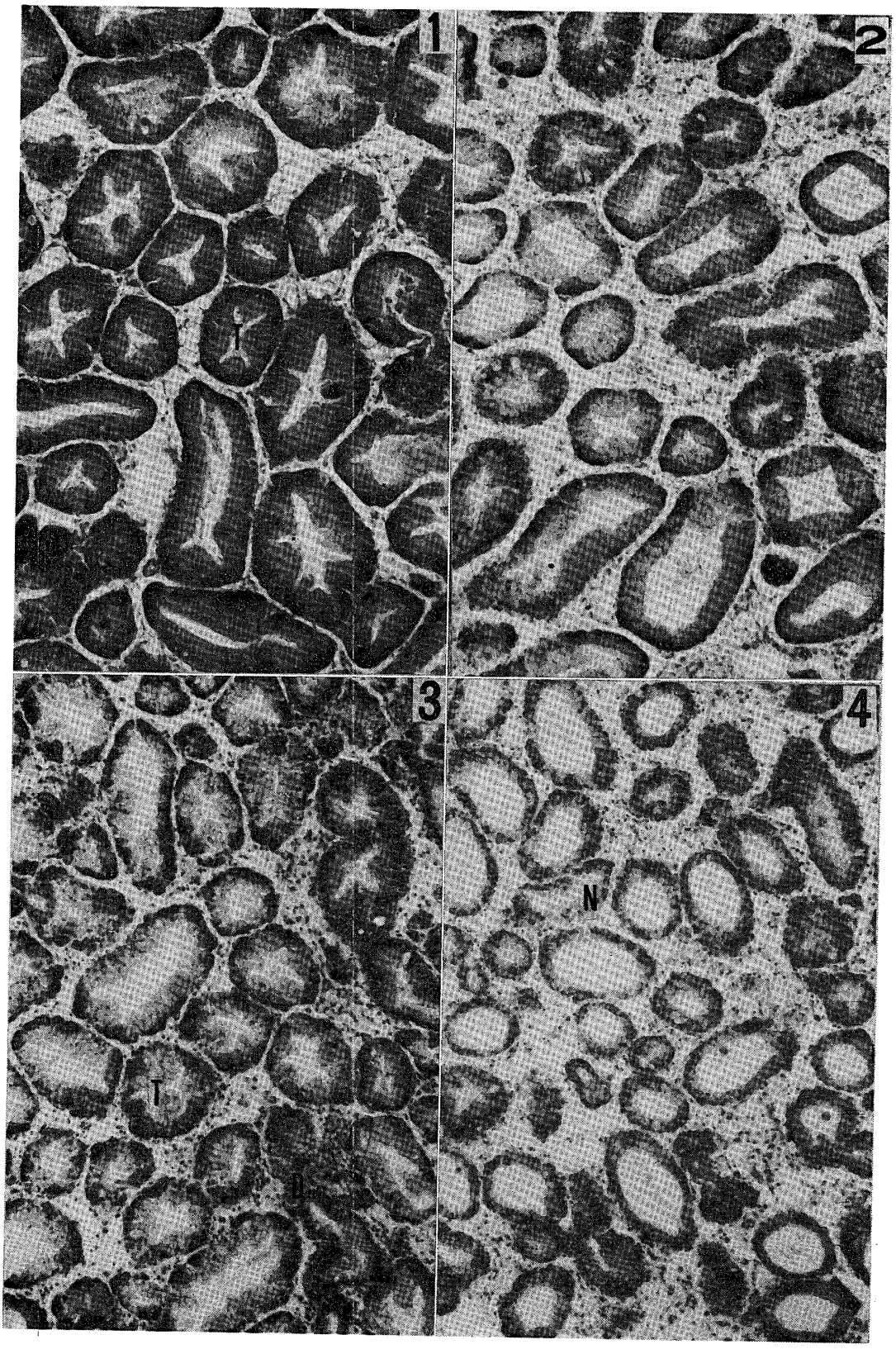
Explanation of the Figures

FIG. 1. Digestive diverticula at the early stage of sexual maturation (May). The tubules (T) are distinctly recognized to consist of two kinds of cells, namely, the dark cells and the light cells. Hematoxylin-eosin stain. × 150.

FIG. 2. Digestive diverticula at the spawning stage (August). The atrophy of epithelia is seen in a good many tubules and the activation of interstitial cells is fairly marked around the tubules. Hematoxylin-eosin stain. × 150.

FIG. 3. Digestive diverticula after spawning (September). The activation of interstitial cells is conspicuous. Vacuoles of various sizes are observed in the epithelia of tubules (T). In a few ducts and tubules, the epithelia show an evagination and the degenerative necrosis (D) is observed in a few cases. Hematoxylin-eosin stain. × 150.

FIG. 4. Digestive diverticula of the Mizugaki, spent oyster, in which the mantle and viscera appear translucent and watery (October). The lumina of the tubules are enlarged and most of the cells in the epithelia become flat. In a few cases, the necrosis (N) is observed in these cells. The marked activation of interstitial cells is seen. Hematoxylin-eosin stain. × 150.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T
2
3
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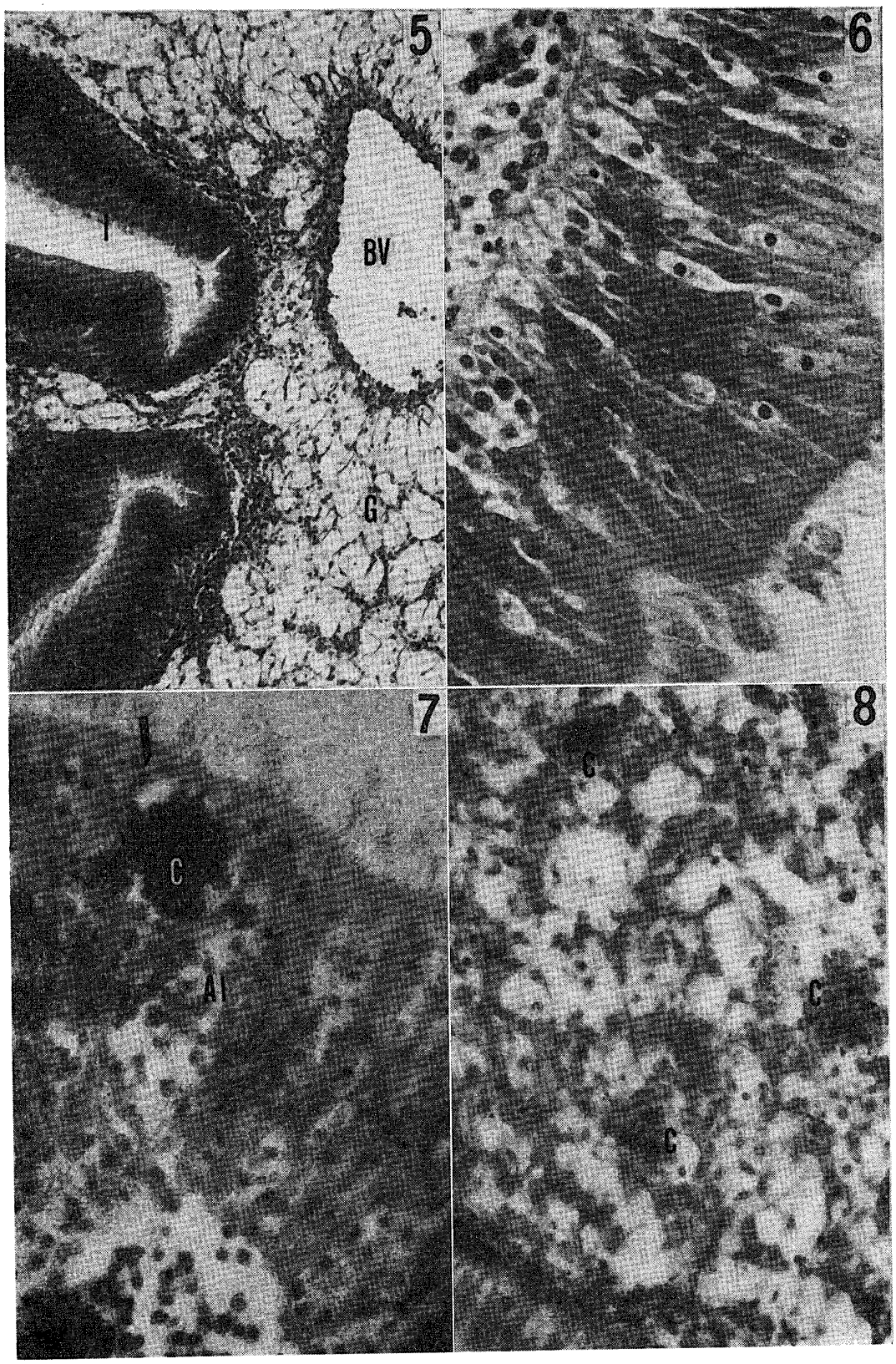

5
I
BV
G
6
7
I
C
AI
8
C
C
C

PLATE 2

Explanation of the Figures

FIG. 5. General view of the intestine (I), blood vessel (BV), and glycogen-bearing tissue (G) of a normal, uninfected oyster, sampled in January. The cilia of the epithelia of intestine are clearly seen. The glycogen-bearing cells in the connective tissue show an enlargement. Aggregations of amoebocytes are found among these cells. Hematoxylin-eosin stain. × 150.

FIG. 6. Enlarged microphotograph of the same intestine as in Fig. 5. No pathological change is observed. Hematoxylin-eosin stain. × 600.

FIG. 7. Epithelia of the stomach in an oyster, infected with bacteria, which was sampled after spawning (September). Bacterial colonies (C) are found among the epithelial cells of the stomach and an intense amoebocytic infiltration (AI) is observed around the colony. A partial falling-off of the cilia is found. A compression atrophy is recognized in the pseudo-stratified epithelia where the colonies of bacteria are found (see the arrow). Gram's stain of Weigert. × 600.

FIG. 8. Connective tissue surrounding the intestine of the same oyster as in Fig. 7. There are colonies of bacilli (C). Nodular amoebocytic infiltration is seen around the colonies, together with a partial exudation. Gram's stain of Weigert. × 600.

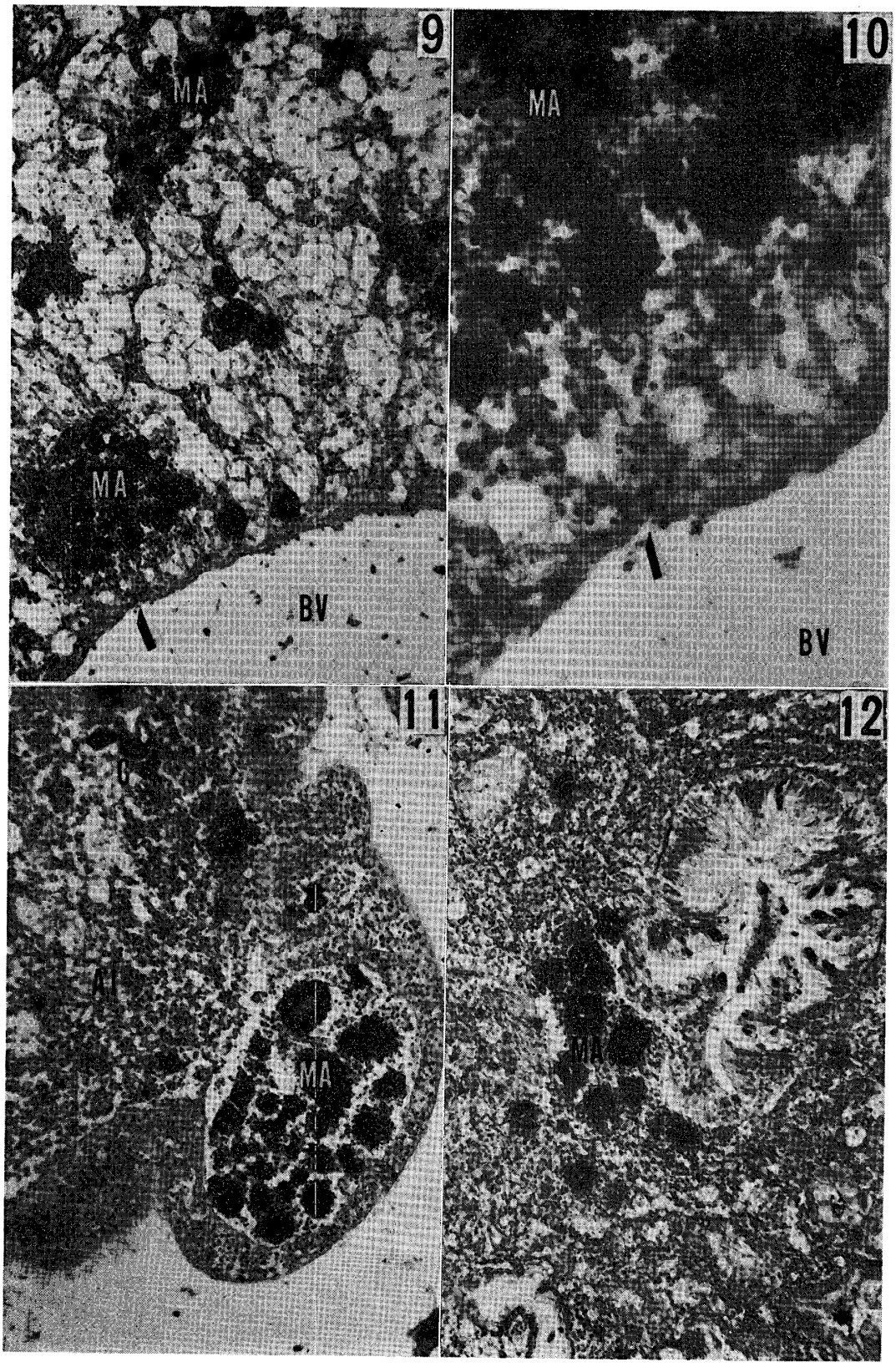
PLATE 3

Explanation of the Figures

FIGS. 9 and 10. Glycogen-bearing connective tissue near the blood vessel (BV) of the same oyster as in Fig. 7. Multiple abscesses (MA) are observed. It is suggested in the picture that the entrance for bacterial invaded is a pore-like space of the wall of blood vessel (see the arrow). Gram's stain of Weigert. Fig. 9, × 150, Fig. 10, × 600.

FIG. 11. Stomach of the same oyster as in Fig. 7. Diffused amoebocytic infiltration (AI) and diffused bacterial colonies (C) are observed in the lamina submucosa, together with the proliferation of amoebocytes around multiple abscesses (MA). Falling-off of the cilia is clearly recognized. Gram's stain of Weigert. × 150.

FIG. 12. Connective tissue around the digestive diverticula of the same oyster as in Fig. 7. Multiple abscesses (MA) are observed, together with the remarkable infiltration of amoebocytes. Gram's stain of Weigert. × 150.


9
MA
MA
BV
10
MA
BV
11
MA
12
MA

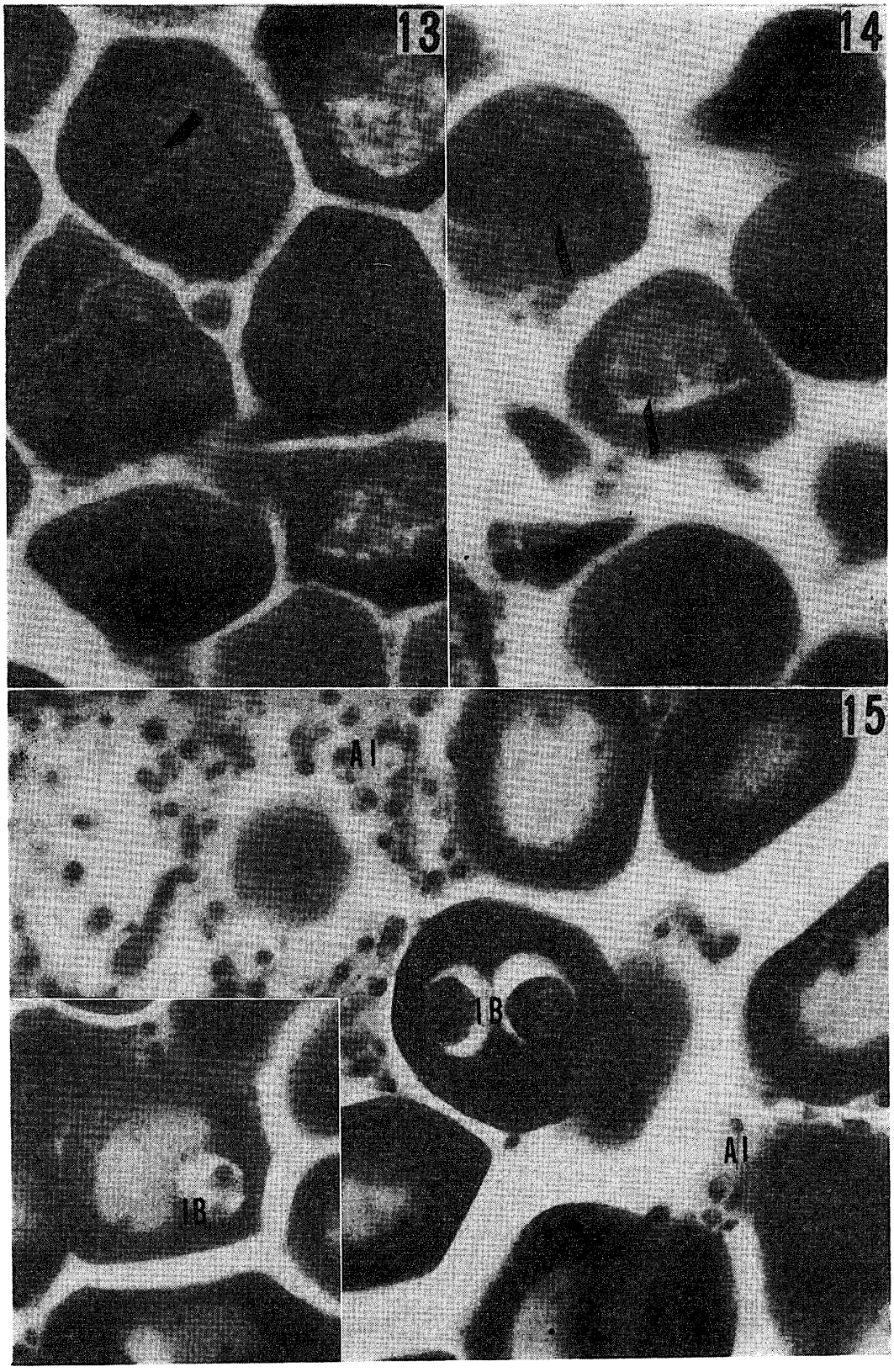

13
14
15
AI
IB
IB
AI

PLATE 4

Explanation of the Figures

FIGS. 13–15. "Amoebocytes invading into the eggs" observed during the spawning and just after that in 1964 (August). They are found as the basophilic aggregates (see the arrow) in the degenerated oocytes without nuclei or as the cytoplasmic inclusion-body (IB) in the cytolytic and vacuolized oocytes. Marked amoebocytic infiltration (AI) is seen around the oocytes. Hematoxylin-eosin stain. × 800.