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Histochemical Study of Dehydrogenases Related to Steroidogenesis in the Tissues of the Fry and Juvenile of Chum Salmon *Oncorhynchus keta*

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Summary

A histochemical study for detecting the dehydrogenases, namely, 3β -hydroxysteroid dehydrogenase (3β -DH), 17β -hydroxysteroid dehydrogenase (17β -DH) and glucose-6-phosphate dehydrogenase (G-6-PDH) was carried out in different organs of the chum salmon (*Oncorhynchus keta*) fry and juveniles. The histochemical distribution of these enzymes was found to correspond with the histological pictures. The activity of 3β -DH was observed only in the interrenal tissues of both the fry and the juveniles. The intensity of this enzyme reaction was found to increase with an increase in the number of steroidogenic cells and also to some extent with an increase in the nuclear diameters of interrenal cells in the head kidneys of the fry and the juvenile. No reaction for 17β -DH was observed in all the tissues examined.

A scattered distribution of G-6-PDH has been observed in most of the tissues of the juvenile salmon examined. In the fry salmon, however, some organs tested did not always show a positive reaction for G-6-PDH.

In the past fifty years a large number of studies on qualitative analysis of steroid hormones in fish have been carried out (1-7). Most of the studies are based on biochemical methods to detect the presence of active steroid hormones such as cortisol, corticosterone, cortisone, 11-ketotestosterone, 17β -estradiol and testosterone in the plasma or tissues. However, the presence of those hormones does not indicate the cellular areas of steroidogenic activities. A method has been recently developed for the histochemical visualization of 3β -hydroxysteroid dehydrogenase (3β -DH), a key enzyme in steroid hormone biosynthesis (20, 21). In the present experiment, this histochemical method has been adopted because of its many merits over other techniques.

Hitherto, there have been very few reports on the histochemical demonstration of 3β -DH, 17β -DH and G-6-PDH in fish.

A histochemical demonstration of 3β -hydroxysteroid dehydrogenase has been carried out in the interrenal tissue of *Anguilla anguilla* and *Conger conger* by

Chieffi Botte (8) and Hanke and Chester Jones (10), in the gonads of *Scomber scomber* and in the adrenocortical tissue of *Fundulus heteroclitus* by Bara (9, 11), in the ovary of *Poecilia reticulata* by Lambert (13), and in *Centropristes striatus* by Reinboth *et al.* (14). Glucose-6-phosphate dehydrogenase has been demonstrated in the ovaries of *Scomber scomber* (12). Recently Mori and Sato (15) have carried out a comprehensive study on the localization of the cellular sites of three kinds of dehydrogenases, 3 β -, 17 β -DH and G-6-PDH related to steroidogenesis in the immature chum salmon, *Oncorhynchus keta*.

The vertebrate steroidogenic organs such as adrenal cortex, gonads and possibly some placentas are able to synthesize steroid hormones. Other tissues, however, may have the ability to carry on only one or two of the ultimate critical steps in the conversion of an inactive steroid precursor into an effective hormone. In view of the paucity of information on the steroid producing glands and target organs in fish, it is of considerable interest to establish the kinds of organs and cells which show evidence of steroidogenesis and sensitivity to the steroid hormones. As far we know, no systematic study has been made regarding the distribution of these enzymes related to steroidogenesis in the different organs at the different stages of a fish. Therefore, we have first attempted to locate the cellular sites of steroid synthesis histochemically in the tissues of *O. keta* during its larval and juvenile stages.

Materials and Methods

Chum salmon fry and juveniles were collected from Tsugaruishigawa Salmon Hatchery, Miyako City, Iwate Prefecture and from Matsushima Aquarium, Matsushima Town, Miyagi Prefecture, Japan. The date of collection, ages and sizes of the fish are shown in Table 1. The albumen embedded blocks of head kidney, body kidney, liver, spleen, heart, gonad and body muscle were prepared in an acetone and isopentane bath at -80°C and sectioned at -20°C by 12 μ in a cryostat microtome. The methods for the visualization of 3 β - and 17 β -DH were the same as those used previously by Mori and Sato (15). However, the method for the localization of G-6-PDH was slightly modified from the previous one. The modification was the only change in the concentration of the substrate. The substrates used in this study are shown in Table 2. They were dissolved in acetone to form a layer at the bottom of the incubating dish. Nitro-BT was used as the final electron acceptor, and NAD was used as cofactor for 3 β - and 17 β -DH and NADP for G-6-PDH. Alternate sections were incubated in a medium which lacked the steroid substrate and were used as a control.

For the histological study, the same kind of tissues as those used for the histochemical study, were fixed in Bouin's or Helly's fluid, embedded in paraffin, and stained routinely with Mayer's hematoxylin and eosin.

TABLE 1. Records of experimental materials, *O. keta*

Stage	Date of sampling	Age of fish after hatching (days)	Habitat	Number of fish	Body	
					Length (cm)	Weight (gm)
Alevin	Mar. 23, 1977	30	F	10	2.8	0.3
Fry	Mar. 23, 1977	45	F	10	3.9	0.5
Fry	Mar. 23, 1977	70	F	10	5.1	1.2
Fry	May 19, 1977	120	F	10	6.6	2.5
Fry	May 19, 1977	120	S	10	8.3	17.5
Juvenile	Dec. 14, 1976	300	S	10	17.5	28.8

F: freshwater pond S: seawater pond

TABLE 2. Substrates and cofactors used for the steroid dehydrogenase reactions

Possible enzyme systems	Substrates* ¹	Cofactors* ²
3 β -Hydroxysteroid dehydrogenase (3 β -DH)	3 β -Hydroxy-5 β -androstan-17-one (I) Dehydroepiandrosterone (DHA) (II) Epiandrosterone (III)	NAD NAD NAD
17 β -Hydroxysteroid dehydrogenase (17 β -DH)	Testosterone (I) Estradiol-17 β (II)	NAD NAD
Glucose-6-phosphate dehydrogenase (G-6-PDH)	D-Glucose-6-phosphate (G-6-P) (monosodium salt)	NADP

*¹ final conc., 5 mM for steroids and 0.5 mM for glucose-6-phosphate

*² final conc., 0.68 mM (NAD) and 0.56 mM (NADP)

Results

General Microanatomy

As in the case of most other teleost fishes (18), the head kidney of the chum salmon fry and juvenile was observed to consist of lymphoidal tissue, interrenal tissue, chromaffin cells, cardinal veins and their branches, a few ganglionic cells, renal tubules, a single large glomerulus and sinusoids (Figs. 1-8). In the fry of chum salmon, the interrenal cells were found as irregular clumps, but in the juvenile salmon, these cells were arranged gradually into one or two layers of cell cords. The nucleus of the cell was small and round, and the ratio of nucleus to cytoplasm was small. Fig. 9 shows the change in the nuclear diameter of the interrenal cell. The diameter exhibited an increase of 4 μ to 6 μ from one month to four months after hatching. However, there was no significant increase in diameter from four months to ten months. On the other hand, the chromaffin cells are few in number in both the fry and the juvenile of the chum salmon. It is of interest to note that small groups of cells (4-5 cells in a group) similar to chromaffin cells were scattered among the hematopoietic tissues (Fig. 7). A ganglion-like structure was found to extend towards the vein (Fig. 8) and have a structural connection with a large number of cells which also took the chromaffin stain. Therefore, it appears that

the chromaffin cells and the nerve ganglion have developed from the same type of cells, and it also seems that the chromaffin cells are stimulated into activity by the ganglionic cell.

The liver is composed of hepatic cells, stroma, blood vessels and sinusoids. The structure of the liver is more or less same throughout the different stages of the chum salmon under one year of age. In the alevin and the two months old fry, the liver cords are spread apart farther than usual.

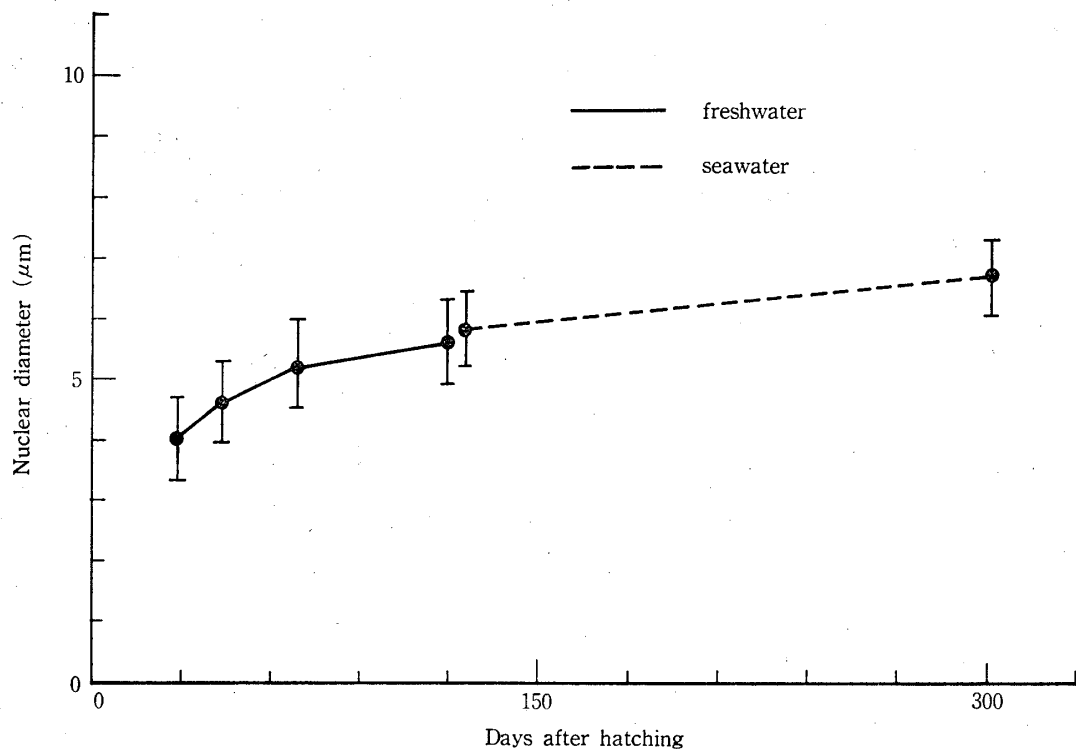


FIG. 9. Variations in the nuclear diameter of the interrenal cells of chum salmon, ages ranging from 30 to 300 days after hatching. The circles are the mean values and the vertical lines are the standard errors.

In general, the kidney contains lymphoid tissue, renal tubules, veins, arteris and their branches, follicle like structures, and glomeruli.

In the spleen, indistinct red and white palps regions are found. Splenic artery, vein and their branches are also present. In the fry, the cells of the red palp are found to be loosely arranged, but in the juvenile fish these cells come close to each other and give the appearance of a compact structure.

The main body of the heart consists of cardiac muscles. These are roughly parallel with one another, but their branching and anastomosing account for the slits present between them. These slits contain loose connective tissue. Moreover, the space between the cardiac muscle fibres is provided with lymphatic capillaries, and it also carries nerve fibers.

The ovary of fish under one year contained the eggs of either perinucleus or perinucleus and chromatin nucleus stages.

Histochemistry

The results obtained in the present study were shown in Tables 3-6.

3 β -DH. Positive reactions were obtained with NAD as a cofactor in the interrenal tissues of the head kidney at the different stages of the fry and the juvenile chum salmon. Reactions of different intensity were obtained with three kinds of 3 β -hydroxysteroids including Δ^5 -3 β -hydroxysteroid, namely, dehydroepiandrosterone. The most intense reaction was found by using a non- Δ^5 -steroid, 3 β -hydroxy-5 β -androstane-17-one as a particular substrate. To some extent the reaction was found to increase in intensity with an increase in the number of interrenal cells (Figs. 10-25) as well as with an increase in the ratio of nucleus to the cytoplasm. No other organs showed positive reactions to 3 β -DH.

17 β -DH. No activity was observed in any of the organs of fry and juvenile fish.

G-6-PDH. As compared to 3 β - and 17 β -DH, this enzyme reaction was quite widely demonstrated in the tissues of the fry and the juvenile. An exceptionally strong reaction was observed in the interrenal cells of all the fish examined, though the fry of one month of age did not show such a strong reaction (Figs. 11, 13, 15 and 17). In the fry, some of the nephrons, liver cells, and gonadal epithelial tissues exhibited a positive reaction. However, no positive reaction was found in the lymphoid tissue of mesonephros, spleen, cardiac muscle and body muscle of fry under four months of age. In the juvenile fish of 10 months of age, the strongest

TABLE 3. *Histochemical reactions of dehydrogenases related to steroidogenesis in different organs of the fry and juvenile chum salmon (incubation: 2 hours for 3 β - and 17 β -hydroxysteroid dehydrogenases and 1 hour for glucose-6-phosphate dehydrogenase at 37°C)*

Age of fish (days)	Key enzymes* and substrates	Interrenal gland	Head kidney	Mesonephros		Liver
				Nephron	Lymphoid tissue	
30 (Alevin)	3 β -DH I	+	-	-	-	-
	II	±~+	-	-	-	-
	III	±~+	-	-	-	-
	17 β -DH I	-	-	-	-	-
	II	-	-	-	-	-
45 (Fry)	G-6-PDH	+	-	+	-	+
	3 β -DH I	+	-	-	-	-
	II	+	-	-	-	-
	III	±~+	-	-	-	-
	17 β -DH I	-	-	-	-	-
70 (Fry)	II	-	-	-	-	-
	G-6-PDH	+	-	+	-	+
	3 β -DH I	##~#	-	-	-	-
	II	+	-	-	-	-
	III	±~+	-	-	-	-
	17 β -DH I	-	-	-	-	-
	II	-	-	-	-	-
	G-6-PDH	##	-	+	-	+

* See Table 2. -: no reaction, ±: slight reaction, ##: maximal reaction.

TABLE 4. Histochemical reactions of dehydrogenases related to steroidogenesis in different organs of the fry and juvenile chum salmon (incubation: 2 hours for 3 β - and 17 β -hydroxysteroid dehydrogenases and 1 hour for glucose-6-phosphate dehydrogenase at 37°C)

Age of fish (days)	Key enzymes* and substrates	Interrenal gland	Head kidney	Mesonephros		Liver
				Nephron	Lymphoid tissue	
120 F (Fry)	3 β -DH I	##~###	-	-	-	-
	II	##	-	-	-	-
	III	##	-	-	-	-
120 S (Fry)	17 β -DH I	-	-	-	-	-
	II	-	-	-	-	-
	G-6-PDH	###	±~+	+	-	##
	3 β -DH I	###	-	-	-	-
	II	##	-	-	-	-
	III	##	-	-	-	-
300 (Juvenile)	17 β -DH I	-	-	-	-	-
	II	-	-	-	-	-
	G-6-PDH	###	±~+	+	-	##
	3 β -DH I	##~###	-	-	-	-
	II	##	-	-	-	-
	III	+	-	-	-	-
	17 β -DH I	-	-	-	-	-
	II	-	-	-	-	-
	G-6-PDH	###	+~##	##	±	###

* See Table 2. F: freshwater S: seawater

TABLE 5. Histochemical reactions of dehydrogenases related to steroidogenesis in different organs of the fry and juvenile chum salmon (incubation: 2 hours for 3 β - and 17 β -hydroxysteroid dehydrogenases and 1 hour for glucose-6-phosphate dehydrogenase at 37°C)

Age of fish (days)	Key enzymes* and substrates	Gonad	Spleen	Muscle	
				Cardiac	Body
30 (Alevin)	3 β -DH I	-	-	-	-
	II	-	-	-	-
	III	-	-	-	-
45 (Fry)	17 β -DH I	-	-	-	-
	II	-	-	-	-
	G-6-PDH	±	-	-	-
	3 β -DH I	-	-	-	-
	II	-	-	-	-
	III	-	-	-	-
70 (Fry)	17 β -DH I	-	-	-	-
	II	-	-	-	-
	G-6-PDH	+	-	-	-
	3 β -DH I	-	-	-	-
	II	-	-	-	-
	III	-	-	-	-
	17 β -DH I	-	-	-	-
	II	-	-	-	-
	G-6-PDH	+	-	-	-

* See Table 2.

TABLE 6. *Histochemical reactions of dehydrogenases related to steroidogenesis in different organs of the fry and juvenile chum salmon (incubation: 2 hours for 3 β - and 17 β -hydroxy-steroid dehydrogenases and 1 hour for glucose-6-phosphate dehydrogenase at 37°C)*

Age of fish (days)	Key enzymes* and substrates	Gonad	Spleen	Muscle	
				Cardiac	Body
120 F (Fry)	3 β -DH I	—	—	—	—
	II	—	—	—	—
	III	—	—	—	—
	17 β -DH I	—	—	—	—
	II	—	—	—	—
	G-6-PDH	+	—	—	—
120 S (Fry)	3 β -DH I	—	—	—	—
	II	—	—	—	—
	III	—	—	—	—
	17 β -DH I	—	—	—	—
	II	—	—	—	—
	G-6-PDH	+	—	—	—
300 (Juvenile)	3 β -DH I	—	—	—	—
	II	—	—	—	—
	III	—	—	—	—
	17 β -DH I	—	—	—	—
	II	—	—	—	—
	G-6-PDH	+	+	+	±

* See Table 2.

F: freshwater S: seawater

reaction was detected in the liver, and a positive reaction was found also in the lymphoid tissues of mesonephros, spleen, cardiac muscle, and body muscle which showed no activity for G-6-PDH in the fry (Figs. 26-31).

Discussion

In the present study, it was observed that the histology of the developing interrenal gland of the immature juvenile of chum salmon was basically similar to that of the fry except for an increase in the number of interrenal cells and chromaffin cells. The chromaffin cells were almost undetectable in the alevin. A gradual increase in the number of both interrenal and chromaffin cells were noticed with the advancement of age. The cells of interrenal tissues were detected as irregular clumps in the hematopoietic tissues of the fry, but the arrangement of these cells in the juvenile gradually changed, and they took the shape of one or two layers of cell cords. These histological changes seem to be correlated with the change in histochemical distribution of 3 β -DH and G-6-PDH and also with the variation in intensity of these enzyme reactions.

To date, there have been no enzyme-histochemical studies done on the fry of chum salmon. However, there has been one report on the histochemical demonstration of 3 β -DH and G-6-PDH in the interrenal tissues of immature chum salmon (15). In this study, the activities of these enzymes (Tables 3 and 4) showed an upward tendency with an increase in number of cells and to some extent

with an increase in interrenal nuclear diameter (Fig. 9). This suggests that some change in the physiological activity is taking place in the interrenal gland. The changes in the histological structure of the interrenal of teleosts which are correlated with the alterations in the level of circulating adrenal steroid during different physiological activities were already reported by Bern (18). The most intense reaction of 3β -DH has been demonstrated by using 3β -hydroxy- 5β -androstane-17-one as a particular steroid substrate (Figs. 10, 12, 14 and 16). The enzyme reaction in the case of using dehydroepiandrosterone was less conspicuous than in the case of above-mentioned substrate (Figs. 18, 20, 22 and 24). No difference in cellular distribution of the formazan crystals was found between the different substrates. This result coincides with the cases of the interrenal gland of *Fundulus heteroclitus* (11) and one-year-old immature chum salmon (15). The enzyme 3β -DH catalyses the conversion of pregnenolone to progesterone and dehydroepiandrosterone to androstenedione, and it is believed to hold a key position in the biosynthesis of the various steroid hormones (16). It is generally accepted that the activity of this enzyme is essential in the early biosynthetic pathways leading to the production of the biologically active steroid hormones (17). This enzyme is specifically concerned with the oxidation of the 3β -hydroxy group. Therefore, the demonstration of 3β -DH in the interrenal tissue suggests that the head kidney of chum salmon is the only steroid hormone producing organ during its fry and immature stages. In our previous study (15), the 17β -DH reaction was not demonstrated in any tissue of the immature chum salmon. In the present study also, no reaction for this enzyme was observed in the tissues of chum salmon during the fry and juvenile stages. These negative reactions might be due to the immaturity of the fish used in these studies.

It has been reported that G-6-PDH is NADP dependent and the most important enzyme, generating NADPH_2 for the hydroxylation mechanism concerned in the corticoid synthesis (19). In the present study, the G-6-PDH showed a positive reaction in most of the tested tissue in the immature fish. The intensity of this enzyme activity varies with not only the tissues but also the developing stages of fish. Also, although strong activity for G-6-PDH was in the interrenal tissue, hepatic cells and the kidney tubules of the fry, this intensity of activity was much lower than the activity obtained in the same kind of organs in one-year-old chum salmon (15). The gradual increase in the activity for G-6-PDH showed a linear relationship to the activity of 3β -DH in the interrenal tissues of both the fry and the juvenile fish. The data of 3β -DH and G-6-PDH suggest the possibility of G-6-PDH to have some significant role in generating NADPH_2 which is necessary for the biosynthetic pathways of steroid hormones.

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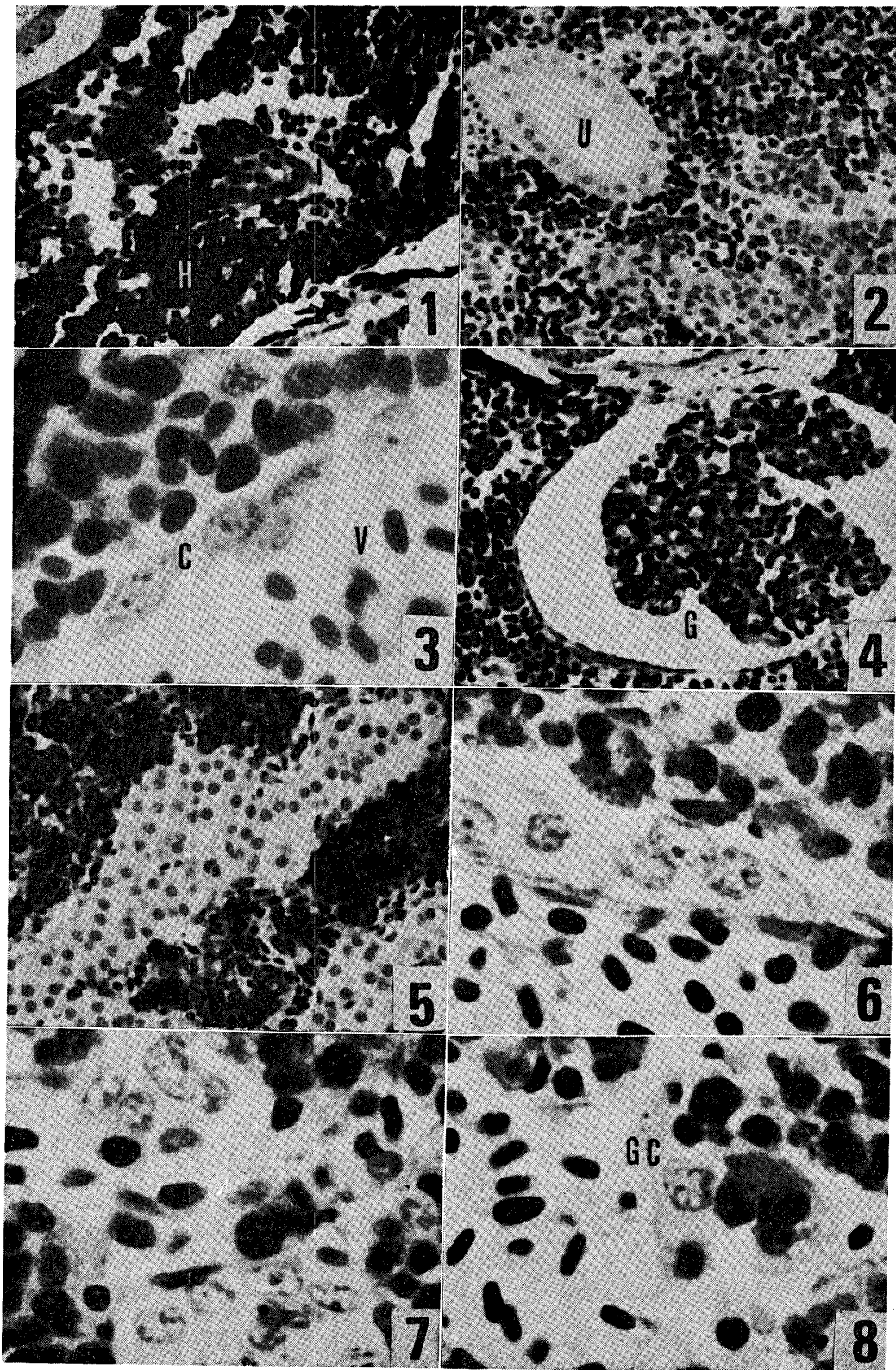
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Explanation of Figures

PLATE 1

- FIG. 1. Head kidney of one-month and fifteen-days-old-fish, showing interrenal gland (I) and hematopoietic tissue (H). Hematoxylin-eosin (H-E) stain. $\times 210$
- FIG. 2. Head kidney of four-months-old-fish (freshwater), showing interrenal tissue and renal tubule (U). H-E stain. $\times 210$
- FIG. 3. Head kidney of one-month and fifteen-days-old-fish, showing chromaffin cells (C) along the cardinal vein (V). H-E stain. $\times 840$
- FIG. 4. Head kidney of one-month and fifteen-days-old-fish, showing a large glomerulus (G). H-E stain. $\times 210$
- FIG. 5. Head kidney of ten-months-old-fish, showing compact clump of interrenal tissue. H-E stain. $\times 210$
- FIG. 6. Head kidney of ten-months-old-fish, showing chromaffin cells along the cardinal vein. H-E stain. $\times 840$
- FIG. 7. Head kidney of four-months-old-fish (freshwater), showing groups of chromaffin cells in the hematopoietic tissue. H-E stain. $\times 840$
- FIG. 8. Head kidney of four-months-old-fish (freshwater), showing a ganglionic structure with a ganglionic cell (GC). H-E stain. $\times 840$



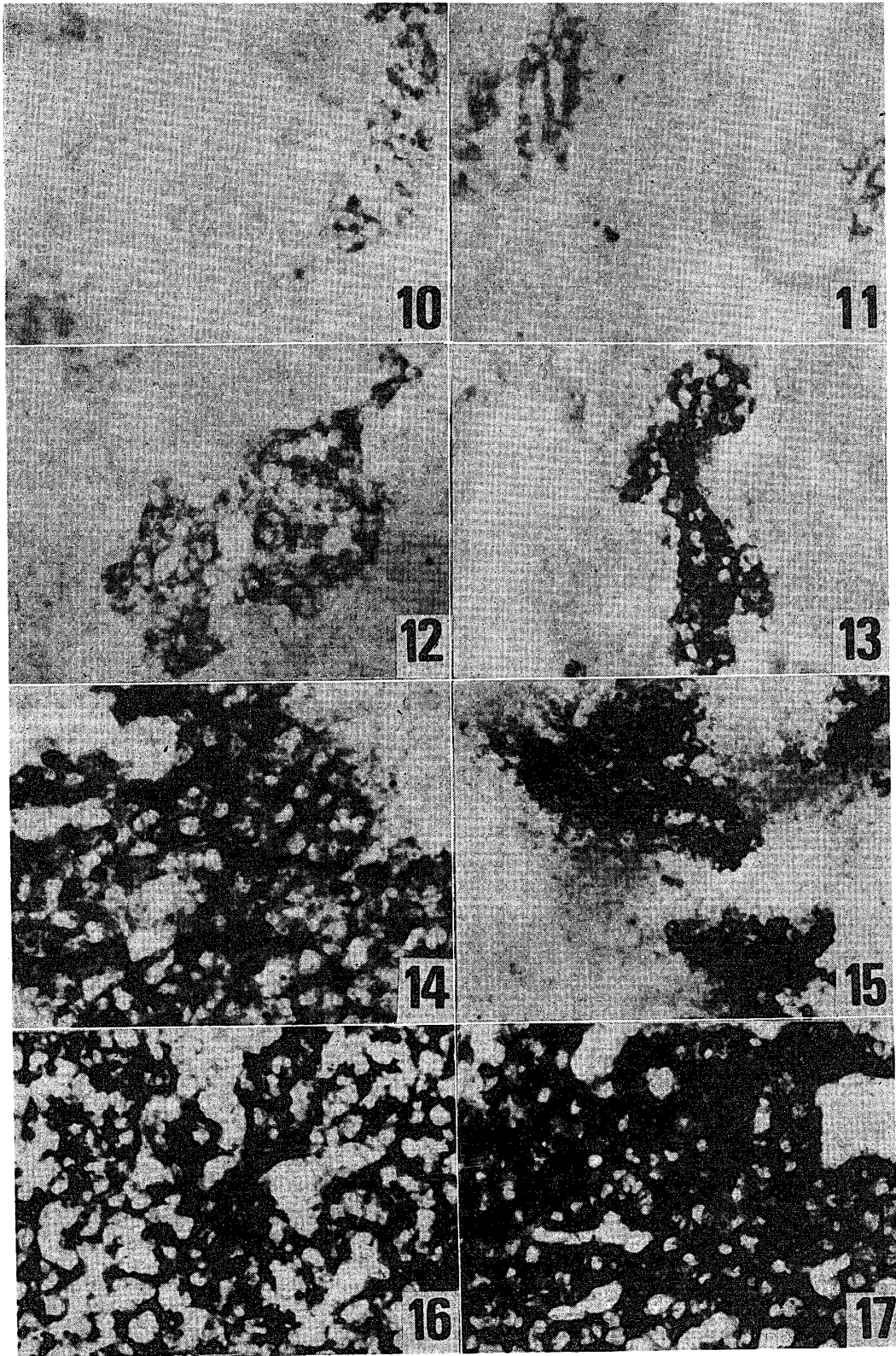
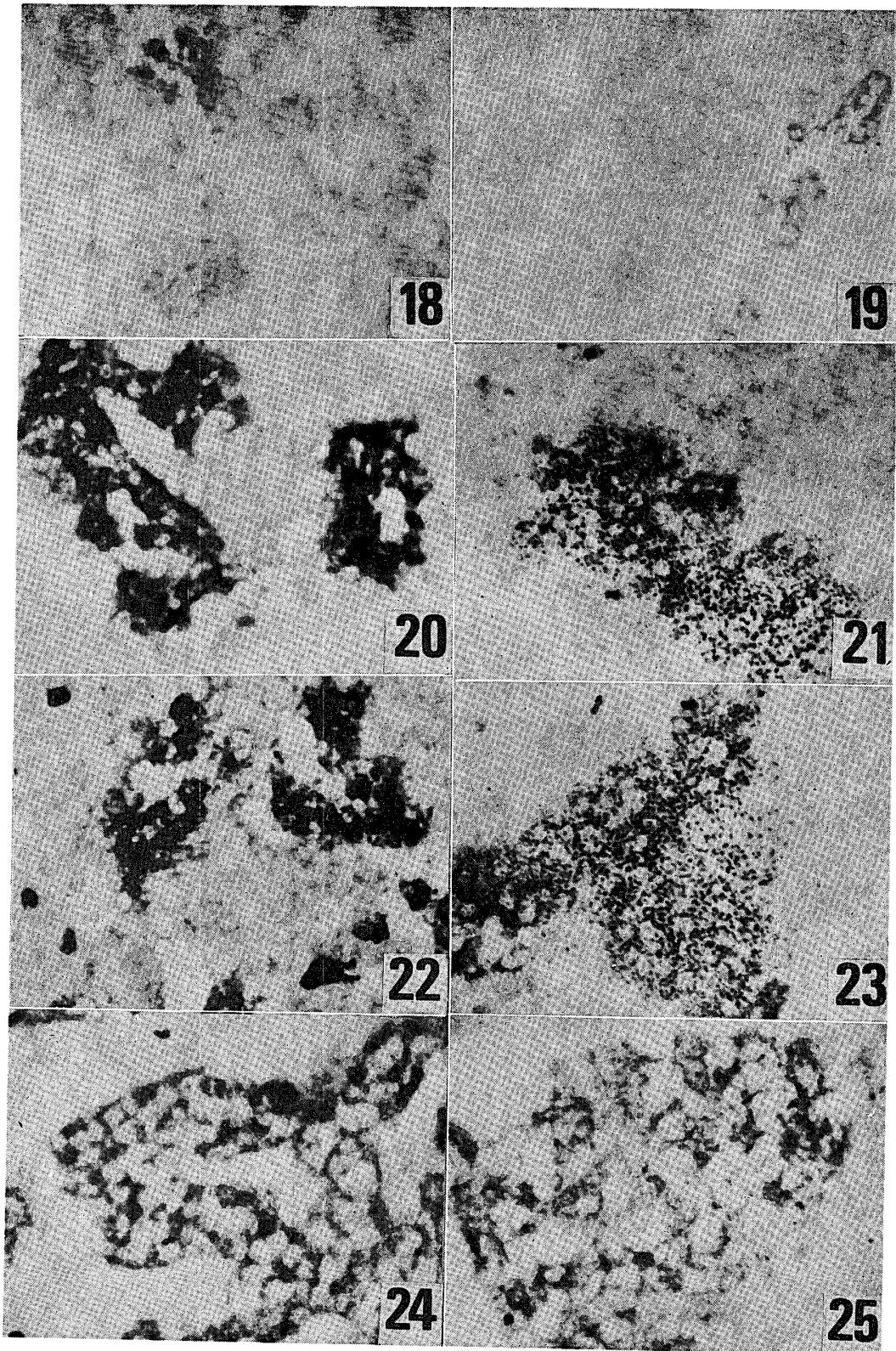


PLATE 2

- FIG. 10. Interrenal tissue of the head kidney of one-month-old-fish, showing reaction with 3β -hydroxy- 5β -androstan-17-one. $\times 210$
- FIG. 11. Interrenal tissue of the head kidney of one-month-old-fish, showing reaction with glucose-6-phosphate. $\times 210$
- FIG. 12. Intrrenal tissue of the head kidney of two-months and ten-days-old-fish, showing reaction with 3β -hydroxy- 5β -androstan-17-one. $\times 210$
- FIG. 13. Interrenal tissue of the head kidney of two-months and ten-days-old-fish, showing reaction with glucose-6-phosphate. $\times 210$
- FIG. 14. Interrenal tissue of the head kidney of four-months-old-fish, showing reaction with 3β -hydroxy- 5β -androstan-17-one. $\times 210$
- FIG. 15. Interrenal tissue of the head kidney of four-months old-fish, showing reaction with glucose-6-phosphate. $\times 210$
- FIG. 16. Interrenal tissue of the head kidney of ten-months-old-fish, showing reaction with 3β -hydroxy- 5β -androstan-17-one. $\times 210$
- FIG. 17. Interrenal tissue of the head kidney of ten-months-old-fish, showing reaction with glucose-6-phosphate. $\times 210$

PLATE 3

- FIG. 18. Interrenal tissue of the head kidney of one-month-old-fish, showing reaction with dehydroepiandrosterone. $\times 210$
- FIG. 19. Interrenal tissue of the head kidney of one-month-old-fish, showing reaction with epiandrosterone. $\times 210$
- FIG. 20. Interrenal tissue of the head kidney of two-months and ten-days-old-fish, showing reaction with dehydroepiandrosterone. $\times 210$
- FIG. 21. Interrenal tissue of the head kidney of two-months and ten-days-old-fish, showing reaction with epiandrosterone. $\times 210$
- FIG. 22. Interrenal tissue of the head kidney of four-months-old-fish, showing reaction with dehydroepiandrosterone. $\times 210$
- FIG. 23. Interrenal tissue of the head kidney of four-months-old-fish, showing reaction with epiandrosterone. $\times 210$
- FIG. 24. Interrenal tissue of the head kidney of ten-months-old-fish, showing reaction with dehydroepiandrosterone. $\times 210$
- FIG. 25. Interrenal tissue of the head kidney of ten-months-old-fish, showing reaction with epiandrosterone. $\times 210$



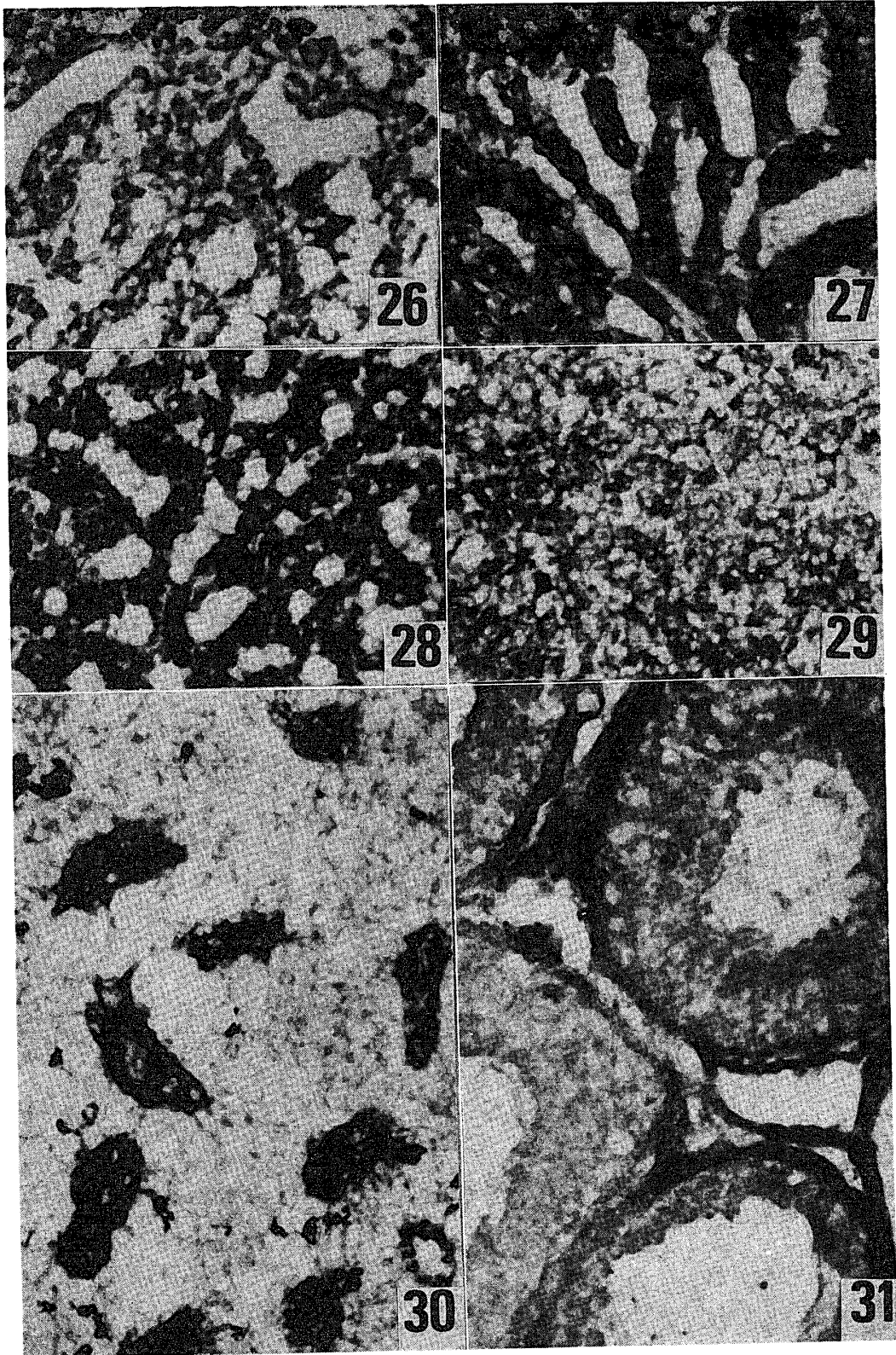


PLATE 4

- FIG. 26. Liver of two-months and ten-days-old-fish, showing reaction with glucose-6-phosphate. $\times 210$
- FIG. 27. Liver of four-months-old-fish, showing reaction with glucose-6-phosphate. $\times 210$
- FIG. 28. Liver of ten-months-old-fish, showing reaction with glucose-6-phosphate. $\times 210$
- FIG. 29. Spleen of ten-months-old-fish, showing reaction with glucose-6-phosphate. $\times 210$
- FIG. 30. Body kidney of ten-months-old-fish, showing reaction, with glucose-6-phosphate. $\times 210$
- FIG. 31. Gonad of ten-months-old-fish, showing reaction with glucose-6-phosphate. $\times 210$