BLOOD CELL CONSTITUENTS IN FISH

I. Peroxidase Staining of the Leucocytes in Rainbow Trout (*Salmo irideus*)

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It is well known that cellular changes in the constitution of the human blood occur in delicate response to any failure of the state of health, and that fluctuation of such values has been utilized universally for the diagnosis of diseases. Dawson has reported that a similar relationship is found also in lower vertebrate animals. According to his studies on lead-poisoning in mud-puppy[1] and catfish[2] much more satisfactory evidence relative to health has been given by its injurious effects on the circulating erythrocytes than by the chemical detection of this metal in both the tissues and body fluids of these animals. Further, he has found in a study on 20 species of marine fishes that a significant correlation exists between the ecological condition of each species and the value of relative proportion of both immature and mature erythrocytes[3].

Thus, it seems that fluctuations of the blood cell constituents are utilizable as a biological indicator in the study of fish physiology. However, that the studies based on this idea have never advanced in this field may be attributed to the fact that the identification of blood cells in this animal is not a little difficult. The difficulty is considered to be partly due to the fact that undifferentiated blood cells are usually found to flow in the blood stream even in the normal condition of fish. It has been reported that such a state resembles that of hemopoiesis at the begining of the third stage of the fetus in the higher vertebrates[4].

In comparison with the cells of the erythrocytic series, the identification for those of the leucocytic series in fish is difficult. Especially, among the cells of the leucocytic series in fish blood, discrimination between the granulocyte and monocyte is considerably difficult, and further, it is much more difficult to distinguish the young cells of both monocyte and granulocyte from the cells of lymphocytic group. Further, as Werzberg[5] and Chûin[4] have observed, the morphological features of the granulocytes in fish are highly multifarious but almost specific according to the species or family. The descriptions of the monocytes in fish which have been reported by previous workers are in disagreement. It seems possible that such confusion is caused by difficulty in the classification of young and transitional cells in the monocytic group. For instance, a large discrepancy has been found among the ratios of the granulocyte, monocyte and lymphocyte in the blood stream of the crucian carp examined in winter[6][7] Such a discrepancy may have been brought about mainly by the following two causes. Namely, one is the personal difference raised by taking a subjective view in the classification of undifferentiated blood cells which are difficult of identification; the other is the ecological

difference such as habitat of the individual fishes to be examined, which brings about variations of the blood cells as reflected in their physiological states.

It is the author's opinion that it is very difficult to settle the cause of such a discrepancy only by application of the Romanowsky staining methods. It is essentially desirable that some staining method by which the blood cells are clearly and easily discernible from one another is employed, if one wishes to utilize changes of the ratio in the leucocytic constituents as a biological indicator in fish physiology. Therefore, on the basis of this idea the present author examined the various tests of both peroxidase and oxidase reactions which have been tried in human blood to distinguish myeloic leucocytes from lymphatic leucocytes. From these tests it was found that a modification of Sato & Sekiya's copper method[8] shows a good result for the purpose stated in the above. By the application of this method to 18 species of fishes, it was clarified that it is not only available on the blood of the rainbow trout, but also on that of these other fishes. Using mainly the blood of the rainbow trout, an establishment of the standardization was made in the method for measuring the constituent ratio of the groups among the leucocytic series.

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MATERIALS AND METHODS

Most of the rainbow trout used in the present experiment were five or six years old, which were reared in a pond of the Hatchery of the Faculty of Fisheries, at Nanaye, near Hakodate.

A fresh, dry blood smear was prepared by a routine method, using blood taken from the caudal vessels of the fish. For preparing the smear, the two methods of Wright-Giemsa's stain[9] and Sato & Sekiya's peroxidase reaction were used. Although several methods were tested to obtain the positive reactions for both peroxidase and oxidase of the blood cells, no satisfactory result was shown by the following methods[9]: Schultze, Pappenheim, Graham, Bloch & Peck, Goodpasture, and Osgood. However, the positive reaction could be obtained clearly by a slight modification of Sato & Sekiya's method described below. Besides this, the Nickel method devised by Mitsui & Ikeda[10] also gave a good result for the reaction concerned.

Sato & Sekiya's method modified in the present investigation is as follows.

Immediately after the fresh, dry blood smear was applied to 1 % solution of copper sulphate, a small quantity of 0.1 % benzidin solution was poured on it. In every use of the last reagent, a few drops of 3 % hydrogen peroxide were added to it to be about 0.04 %, and then, the mixture was warmed to 30° – 40°C. After the smear was applied for 1 minute to the warmed solution, it was washed with water to remove the blue particles precipitated. Next, it was counterstained for 2 minutes with 1 % solution of safranin.

OBSERVATIONS

Peroxidase reaction in the leucocytes

In the peroxidase-stained smear of human blood prepared by Sato & Sekiya's method, cytoplasm of the myeloic leucocyte is stained blue or green producing "positive reaction", while the remaining cells of the blood other than these myeloic leucocytes show "negative reaction", only being coloured red with the safranin of the counterstain. Hollande[11] has stated on the granules stained green or blue by this method that they are to be called "oxybenzidinophilic granules", because peroxidase, an enzyme, which is included within the blood cell, by catalysis oxidizes the reagent used, and then, the so-called peroxidase-granules are stained secondarily by the benzidin blue which is produced by the oxidation.

Similarly as in the case of human blood the peroxidase-positive leucocytes were observed also in the blood of the rainbow trout (Pl. I, Fig. V). As is clearly seen in these figures, there are found the green or blue granules such as above noted within the cytoplasms of these cells. The nuclei are stained by the safranin of the counterstain. In most cases, however, the shape of the nucleus is not clearly observable owing to the presence of the stained granules which are densely distributed within the cytoplasm.

In the peroxidase-negative leucocyte, neither blue nor green granules were found, but the cell was stained red as a whole by safranin and its nucleus was coloured more deeply than the cytoplasm was (Figs. VI–VIII). Although not illustrated in this paper, the cells of the erythrocytic series showed also the negative reaction in the peroxidase-stain; nevertheless, fine granules were often recognized in these cells. However, as the general appearance of the granules in the cells of the erythrocytic series is clearly different from that of the positive leucocyte, it was easy to distinguish them from each other. Further, the granules of the erythrocytic series appear to be quite different in size from those which have been observed in the frog blood.[12]

Peroxidase staining and Wright-Giemsa staining

As stated in the former section, by means of the peroxidase-stained smear the leucocytes of the rainbow trout are classified into the two categories with an exceedingly high contrast, one group showing a positive reaction and the other group a negative

reaction. However, it is a fault in this method that the morphological features of the positive leucocytes are not clear being covered almost wholly by the dense distribution of the stained granules though those features of the negative ones are easily observable. Fortunately, this fault could be remedied by a comparative observation of the leucocytes stained by the peroxidase reaction with those stained by Wright-Giemsa's method. The series of cells in Figs. I-IV show the general features of the leucocytes in the rainbow trout which were stained by the Wright-Giemsa staining. Judging from their morphological characteristics, the cells shown in Figs. I and VI appear to belong to the same group corresponding morphologically to each other. This group is considered to consist of the cells which belong to the lymphocytic group. The groups of cells shown in Figs. II and VII are regarded as spindle cells, both of them corresponding to each other. The morphological appearance of both lymphocytes and spindle cells described above are in general agreement with the descriptions of the previous workers.[2)4)5)] Thus, if the two methods, the peroxidase stained smear and the Wright-Giemsa stained smear, are employed in parallel for staining the leucocytes, a morphological identity will be found between the two groups of the figures obtained. For instance, it becomes clear that the series of cells in Figs. III and IV are identical with those in Fig. V which showed the positive reaction in the peroxidase stained smear. In the series of cells shown in Figs. III and IV, neither granules as stained by the acidic dyes nor by alkaline dyes are found in the cytoplasm. However, the cytoplasm of the cells shown in Fig. III appears blue being relatively deeply stained by alkaline dye, while that of the cells in Fig. IV is stained light blue. In the cells of the former group, the shape of the nucleus is either somewhat rounded or slightly invaginated in part or bilobed though incompletely. The nuclei in the cells of the latter groups are either rounded or rod-like in shape in most of the smaller cells, but those of the larger ones are usually either rod-like or lobated into more than two parts. From these characteristics, it is considered that the two series of cells shown in Figs. III and IV belong respectively to separate groups. Judging from the observations of the previous workers,[4)-7)] the cells shown in Fig. III possibly belong to the monocyte of the leucocytic series. As stated above, there are not found any granules in the cells shown in Fig. IV. However, the nuclei of these cells resemble those of staff cells and segmentocyte which have been found in human blood.[13)14)] In addition to the Wright-Giemsa staining, a few methods belonging to Romanowsky's staining system were tried on such cells as shown in Fig. IV to ascertain whether granules are to be found within them or not. Although all these attempts failed to reveal any granules, the author has assumed those cells to belong to certain group of the granulocyte, considering from their specific morphological features.

Basophilic and eosinophilic granulocytes have been observed in the blood of many fishes similar to those in human blood.[4)-7)] However, Werzberg[5)] has reported the

occurrence of a group of the leucocyte without granules in some fishes, besides the lymphocyte and monocyte which similarly do not possess the granules. The cells shown in Fig. IV are considered to be identical with the type which has been observed by Werzberg. Owing to the further description involved, the author herein has called these cells "specific granulocyte" temporarily.

Fading of the stained granules in peroxidase positive reaction

It seems that the specific granulocytes and monocytes shown in Figs. III and IV are identical with the cells which reacted positively to the peroxidase-stain. Such an assumption will be supported by the fact described below. When the peroxidase-stained smear is left, for instance, for a few weeks as it was mounted with Canada balsam, the blue or green granules lose their characteristic colours and become brownish materials of diphenoquinonediimine. In such a faded smear, the nucleus becomes clearly visible within the cell. Thus, it is clarified that the general appearance of the cells is in agreement with that of cells stained by Wright-Giemsa's method.

Peroxidase-reaction in young leucocytes

The cells with lobated nuclei such as shown in Fig. III, 5,6 and Fig. IV, 6-12 are considered to be mature forms of the monocytic and granulocytic groups. All these cells were figured after it had been made certain that both groups of these cells are identical with each other through a comparative observation of the smear preparations faded from the peroxidase-positive stain to the diphenoquinonediimine as described in the former section. Besides these cells, immature ones belonging to the leucocytes are found in the blood stream of the fish. However, it seems to be very difficult to distinguish these immature cells from those of the other series of the blood elements. For instance, the series of the cells, especially, the apparently immature ones as shown in Fig. VIII, 1 – 3, resemble morphologically the monocytic ones, while those showed the negative reaction to the peroxidase staining. However, although as in the case described above it is very difficult to classify the cells of the leucocytic series, it is very easy to discriminate them from each other according to the peroxidase-positive and -negative reactions. With regard to the questionable cells, however, it seems that further experiments are necessary to establish the author's idea presented in this paper.

Application of the peroxidase-stain to the other fishes

Using the modification of Sato & Sekiya's method, the peroxidase-positive reaction was clearly recognizd in the circulating blood of the following teleost fishes similarly as in the rainbow trout: marine fishes —— atka mackerel, *Pleurogrammus azonus*; starry flounder, *Platichthys stellatus*; 2 species of rockfish, *Sebastes taczanowskii*, *S. thompsoni*; 2 species of puffer, *Fugu vermicularis porphyreus*, *F. pardalis*; wingfish,

Lepidotrigla microptera; dace, *Tribolodon hakonensis hakonensis*; masu, *Oncorhynchus masou*; freshwater fishes —— carp, *Cyprinus carpio*; crucian carp, goldfish, *Carassius auratus*; medaka, *Oryzias latipes*; anadromous form and fry of chum salmon, *Oncorhynchus keta*; dark parr and smolt forms of masu, *O. masou*; smolt form of malma, *Salvelinus leucomaenis*; eel, *Anguilla japonica*.

Kawamoto[15] has reported that the positive leucocytes were not found in eel blood though he tried using the peroxidase reaction of Freisch's method. However, the author could clearly observe them in the eel blood using a modification of Sato & Sekiya's peroxidase staining. In eel blood, there was found a different type of leucocyte containing a few granules stained blue. The occurrence of these two types of leucocytes in the eel appears to be a characteristic for the blood of this fish which is different from that of the other fishes examined.

DISCUSSION

The fact that the morphological features of the granulocytes in fish blood are multifarious but almost specific according to the species or family, has been reported in detail by many workers.[4)-7)] In the rainbow trout, there was not found any leucocyte which contains granules stained with either alkaline or acidic dye, though "specific granulocyte" which has none of the granules was observed. A similar observation has been reported in fish by Werzberg[5)] That is, out of 14 species of the marine and freshwater fishes studied, in 5 species he has found the leucocytes without granules which belong to neither monocyte nor lymphocyte. While Chûin[4)] has reported the occurrence of the granulocytes in all of the 24 species of marine fishes which he studied; he has stated that as the granules of granulocyte in fish are very unstable the fixation of them is difficult, and so, much care in their staining is necessary. The author examined the presence or absence of the granulocyte containing the granules in the blood of 18 species of fishes by Wright-Giemsa's staining, which is the same method as used in the case of the rainbow trout. In consequence of this investigation, it was ascertained that the granulocyte without granules was to be found only in the salmonid fishes studied, viz., in anadromous form and fry of chum salmon, both marine and freshwater forms of masu, malma, and rainbow trout; also it was ascertained that in the blood of all the other fishes studied the granulocyte occurs with granules, which are stained with the dyes acidic and alkaline or either of them. Accordingly, it may be said that the author's observation that the granules were not found in the granulocyte of the rainbow trout is due to neither the staining reagent nor the method used.

Even in the granulocyte in which the granules are easily recognized, colouration of them is not clear in the young cells of this group. Cytoplasm of the young cell has such a common characteristic as to be stained by methylene blue, an alkaline dye.[9)13)14)]

So, the discrimination is difficult between the young granulocyte and young monocyte stained by such an alkaline dye, and it is much more difficult to distinguish the two sorts of young cells from those of the lymphocytic series. A similar relationship occurs also in the case of the specific granulocyte (granulocyte without granules). If the purpose of observation is restricted only to the hemocytological study, perhaps discrimination between them may be possible by means of, for instance, some excellent methods other than Romanowsky's such as vital staining or supravital staining. However, if one would study the fish blood purposing to make its constituents a biological indicator, it is essential that the staining method be simple but sure. As to this point, it was found that the modification of Sato & Sekiya's method used in the present observation is available for use on the 19 species of both marine and freshwater fishes. As the blood smear prepared by this method shows highly contrasted stainings of both the peroxidase-positive and -negative reactions, the relative proportion of the cells reacted to them is easily measurable. In this case, the positive leucocytes are made up of both the monocytic and granulocytic groups which are indistinguishable from each other by this peroxidase-staining. However, so far as the mature cells of these groups are concerned, they can be discriminated from each other by a comparative application of Romanowsky's method and the peroxidase staining.

It may be said that one weak point of the peroxidase-staining is that the classification of young cells is impossible owing to the occurrence of their multifarious types in the blood stream of fish. However, even if it is not possible to discriminate the monocytic group from the granulocytic one by this method, both of them showing the positive reaction are easily distinguishable from the other groups of the leucocytes which show the negative reaction. Consequently, the peroxidase reaction is useful for analysis of the leucocytic constituents as a biological indicator. That is, if the leucocytes which have peroxidase, an enzyme, are considered to be a unit of the cells, fluctuation of their occurrence in fish blood may be utilized to judge any change of physiological states in fish if it has happened.

SUMMARY

1. The peroxidase reaction by which discrimination has been made between myeloic and lymphatic leucocytes in human blood, was applied to the circulating blood of the rainbow trout.

2. To obtain the positive reaction in both the peroxidase and oxidase stainings, the following methods were tried, but none of these gave any satisfactory results in reaction: Schultze, Pappenheim, Graham, Bloch & Peck, Goodpasture, and Osgood.

3. Sato & Sekiya's peroxidase reaction showed a good result in its slight modification as follows. Although the reagent used was the same as that of the original method, the benzidin solution was warmed at about 35°C and the staining was made at the time

when the benzidin had dissolved completely. So, green or blue granules were clearly seen within the cytoplasm of the peroxidase-positive cells.

4. Upon comparison of the result of the positive reaction in this peroxidase staining with results obtained by Romanowsky's staining, it was found that the peroxidase-positive cells included both monocytic and granulocytic groups.

5. So far as the mature cells are concerned, monocyte is distinguishable from granulocyte by Romanowsky's staining. However, identification of the young ones of these cells is difficult, and further, it is much more difficult to discriminate between these young cells and lymphocytic cells. In the peroxidase staining, as both the monocyte and granulocyte show the positive reaction, those groups are easily discernible from the lymphocytic cells and from the other blood elements all of which show the negative reaction.

6. Although by the present peroxidase staining, it is impossible to make distinction between monocytic and granulocytic cells, if both of them are considered to be a unit of the cells showing the positive reaction or containing the same enzyme, peroxidase, fluctuation of the ratio measured between the positive and negative cells seems to be utilizable in fish physiology as a biological indicator.

7. The peroxidase staining was applied to 18 species of fish; pertinent results were obtained similarly to the case of the rainbow trout.

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PLATE I

Explanation of Figures

The figures show the general feature of the leucocytes in the circulating blood of the rainbow trout.

Figs. I, II, III, and IV show the leucocytes stained by Wright-Giemsa's method.

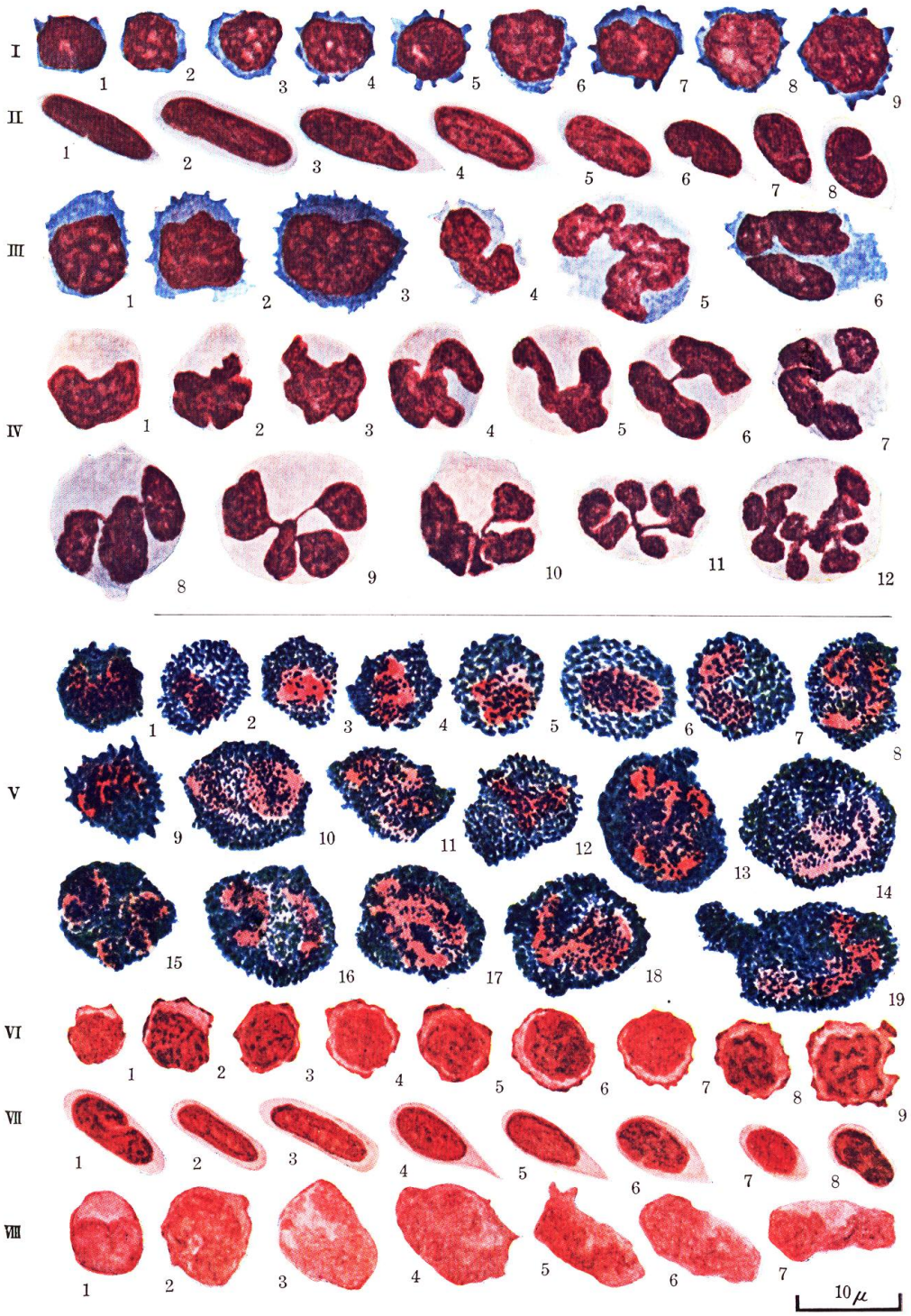
Figs. V, VI, VII and VIII show the leucocytes stained by the modification of Sato & Sekiya's method, V showing the positive reaction and VI – VIII the negative reaction.

The cells shown in Figs. I and VI belong to lymphocytic group, and those of Figs. II and VII to the group of spindle cells.

The cells in Fig. V which showed the positive reaction contain the green or blue granules and correspond to those of Figs. III and IV which were staind by Wright-Giemsa's method. The cells in Fig. III belong to the monocytic group, and those of Fig. IV to the granulocytic group.

Classification of the cells shown in Fig. VIII is not clear at present.

All of these figures were drawn with the aid of an Abbe's transcriber.



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