BLOOD CELL CONSTITUENTS IN FISH—III.
NEW FUNCTIONS OF THE ERYTHROCYTE RESPECTING
COAGULATION IN FISH BLOOD*

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The spindle cells*** in blood of the vertebrates lower than birds have been regarded to have a function in coagulation analogous to that of the platelets in mammals.¹⁻⁴⁾ If a blood smear preparation is made after the fish blood is left purposely as it is until the time just before it begins to coagulate, the following picture is observed; that is, in addition to the usual occurrence of both agglutination and disintegration of the spindle cells, the erythrocytes also are disintegrated. The author considered that such a disintegration of the erythrocytes might be caused by the phenomenon of the cells becoming easy to break down after they have participated in the blood coagulation in the living state of them. If such is the case, it is probable that in the living state also the erythrocytes of fish play an important role in the blood coagulation similar to the function of platelets in mammals. To substantiate this assumption, the following experiments were performed using the blood of adult rainbow trout, *Salmo irideus*, anadromous form of chum salmon, *Oncorhynchus keta* and marine form of pink salmon, *O. gorbuscha*.

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Method and Results

Separation of the erythrocytes: In all cases, the erythrocytes were separated from the defibrinated blood. To remove fibrin, the blood was strained through four layers of washed gauze. The filtrate was centrifuged for 5 minutes at 2000 rpm. The supernatant fluid with the inclusion of as much of the buffy layer as possible was drawn off and replaced by an equal volume of physiological saline of pH 7.4 chilled at 5-7°C. After thorough mixing, the blood was centrifuged similarly as in the former case, and then both the saline supernatant liquid and the layer of the remaining leucocytes were discarded. After repetition of such a procedure 3 times, the suspended erythrocytes were sampled from the middle layer of the residue. Then, these cells were washed

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*** In some papers, these cells have been called also as "thrombocytes" or "kernige Blutplättchen".

several times again with saline solution. Thus, the erythrocytes were completely separated from the other blood elements.

Accelerative function of the fish erythrocytes for fibrin formation: The second clotting factor of platelets in mammalian blood has been well known to catalyze the interaction of thrombin and fibrinogen. To learn whether the erythrocytes of fish have such a function as have the platelets of mammalian blood or not, the following experiments were performed using the blood of rainbow trout and chum salmon. The lyophilized thrombin (Mochida Pharm. Co.) was dissolved into a required unit with physiological saline—(A). Varying numbers of the washed erythrocytes were added to the plasma prepared from the blood containing 10% of anticoagulant of either heparin alone or a mixture of heparin and dual oxalate⁵¹—(B). By adding one volume of (A) to five volumes of (B), the time required for appearance of the first visible evidence of a clot was measured under the temperature 17–18°C. As is seen in Table 1, the time required for fibrin formation in the plasma containing the erythrocytes is found to be clearly shorter when compared with that of the plasma only.

Table 1. Accelerative function of the fish erythrocytes for fibrin formation.

Plasma taken from	Units of thrombin used	Number of erythrocytes suspended in plasma ($\times 10^4/\text{mm}^3$)	First visible evidence of fibrin formation in seconds	Number of determinations
Rainbow trout†	15	0 (plasma only)	44.4 $\pm$ 3.0	5
	15	10.2 $\pm$ 0.4	40.0 $\pm$ 2.0	5
	15	21.3 $\pm$ 0.4	39.0 $\pm$ 2.4	4
	15	38.6 $\pm$ 0.7	31.4 $\pm$ 2.7*	5
Rainbow trout††	20	0 (plasma only)	43.0 $\pm$ 1.0	4
	20	7.9 $\pm$ 2.7	47.0 $\pm$ 0.9	3
	20	18.9 $\pm$ 1.4	22.7 $\pm$ 4.1**	3
	20	19.4 $\pm$ 2.0	21.3 $\pm$ 2.8**	3
Rainbow trout††	20	0 (plasma only)	41.4 $\pm$ 2.4	9
	20	42.7 $\pm$ 1.3	20.6 $\pm$ 2.5**	5
Chum salmon††	20	0 (plasma only)	121.6 $\pm$ 11.3	6
	20	55.2 $\pm$ 0.3	33.3 $\pm$ 3.3**	6
Chum salmon††	25	0 (plasma only)	77.0 $\pm$ 4.5	15
	25	17.5 $\pm$ 0.8	53.5 $\pm$ 3.3**	8
	25	23.1 $\pm$ 0.5	24.4 $\pm$ 3.2**	9
	25	55.2 $\pm$ 0.3	10.0 $\pm$ 0.6**	7

Anticoagulant: † heparin, †† mixture of heparin and dual oxalate.

*, ** Statistically significant more than 95% and 99% of probability, respectively.

In the next experiment, when one volume of the lyophilized thrombin 5 units per ml was added to ten volumes of the solution in which was dissolved 20 mg of "Seegers fibrinogen" purified from bovine (Mochida Pharm. Co.) in 10 ml of physiological saline, the time required for the fibrin formation at 17°C was found to be 24.8 $\pm$ 0.5 seconds, as shown in Table 2. Contrasting with this result, when the erythrocytes of the rainbow trout were suspended in the same fibrinogen solution to the amount of

about 500,000 per 1 mm^3 , the time for fibrin formation was reduced to 18.5 ± 0.9 seconds (Table 2).

Table 2. Accelerative function of the erythrocytes in rainbow trout for "Seegers fibrinogen" solution.

Units of thrombin used	Number of erythrocytes suspended in fibrinogen solution ($\times 10^4/\text{mm}^3$)	First visible evidence of fibrin formation in seconds	Number of determinations
5	0 (fibrinogen solution)	24.8 ± 0.5	10
	9.2 $\pm$ 0.5	23.0 ± 0.9	5
	25.6 $\pm$ 1.1	22.0 ± 1.0	5
	51.2 $\pm$ 0.9	$18.5 \pm 0.9^*$	6

* Statistically significant more than 95% of probability.

It is presumable from these results that the erythrocytes of these fishes have an accelerative factor respecting fibrin formation.

Effect of the fish erythrocytes in the thromboplastin generation test: It has been clearly ascertained that the third clotting factor of platelets in mammalian blood has a function which is closely related to the formation of thromboplastin. Applying the thromboplastin generation test⁽⁶⁾ to blood samples of each the rainbow trout and pink salmon, the author examined whether or not the fish erythrocytes have such a similar function as the platelet factor 3. That is, the erythrocytes suspended in the physiological saline solution was used substituting for platelets which have been used in the generation test for human blood. Both the plasma and the serum of the blood were prepared by the routine method used in human blood,⁽⁶⁾ but the clotting time was measured under the temperature $17\text{--}18^\circ\text{C}$. Curves I, II and III in Fig. 1 graph the

Fig. 1. Effect of the erythrocytes of rainbow trout in the thromboplastin generation test. Number of erythrocytes suspended in saline solution as follows: (I) $52.1 \times 10^4/\text{mm}^3$, (II) $24.7 \times 10^4/\text{mm}^3$, (III) $4.9 \times 10^4/\text{mm}^3$, (IV) 0 (control).

results obtained from the experiments made using the rainbow trout erythrocytes in which varying numbers of erythrocytes were suspended in the saline solution. As is

clear from this figure, the clotting time in each case is distinctly reduced with the lapse of time after incubation. It may be said that this result is almost identical with that of the human blood. Further, it is found that the reduction of the clotting time becomes more distinct, according to the increase of number of erythrocytes suspended, while, in the test of the curve IV to which any erythrocytes were not added, the clotting time was not reduced at all. Similar results were also obtained from the experiment made with the blood of the pink salmon. So, these facts suggest that the erythrocytes of fish possess one of the thromboplastin generating factors in the blood.

Discussion

Although the clotting mechanism of fish blood still remains unexplained, it is presumable that it resembles that of the human blood in many respects. Nevertheless, it is not considered that fish blood has all the factors which have been found in human blood. Recently, GEORGATSOS *et al.*⁷⁾ have reported a new clotting factor which was obtained from the hemolysate of the erythrocytes of the human being and also of various other kinds of mammals. KOBAYASHI *et al.*^{8,9)} also have found the existence of the activity of thromboplastin generation in the hemolysate of human erythrocytes. On the basis of the results reported by these previous workers, it is presumable that in the erythrocyte of the higher vertebrates the clotting function is observed only in the state of hemolysate. However, as described in the present paper, it seems to be highly characteristic that the fish erythrocytes are able to exhibit their clotting ability in the living and intact state. Such functions of the fish erythrocytes may possibly play an important role *in vivo* also, though in fish blood the cells which have the clotting factor have been described to be spindle cells alone.

Summary

This report describes evidence that the erythrocytes of fish, at least, of a few species of the salmonoid have both an accelerative factor for the fibrin formation and a function of the thromboplastin generation.

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