MULTIPLE HEMOGLOBINS IN FISH—II*.

Kanehisa HASHIMOTO and Fumio MATSUURA**

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It was shown previously that blood hemoglobin (Hb) of fish was not always homogeneous electrophoretically¹⁾. BUHLER et al.²⁾ reported also a similar observation on fish Hb's. As for the Hb of chum salmon, the existence of two components was further demonstrated by other means such as solubility or heat coagulation curve, and some fairly distinct differences in chemical and physical properties were pointed out between these two components^{1), 3), 4)}.

The reason why such a heterogeneity exists in fish Hb remains undissolved. Even whether this phenomenon is normal or abnormal (as reported in human) is obscure. According to the results⁵⁾ obtained so far, the proportion of amounts of two Hb's in chum salmon was fairly constant, irrespective of place and season of catching, or sex, which seems to indicate that the duplicity of Hb is not abnormal, or not resulting from secondary changes. Probable physiological significances of the existence of multiple Hb's are enough interesting to be studied, while the phenomenon should be investigated from the genetical point of view, too.

In order to find a clue to such questions, blood Hb's of several species of fishes were analyzed electrophoretically and by heat coagulation method, in succession to the previous investigation¹⁾ (Part 1). The same studies were also performed on a few species of *Salmonidae* fishes, which are nearly related taxonomically each other (Part 2).

Materials and Methods

Materials: Blood was collected either by heart-puncture or by cutting tail from one to scores of live bodies of the following species: black bream, *Mylio macrocephalus*, dolphin fish, *Coryphaena hippurus*, *Inimicus* sp., goby, *Acanthogobius flavimanus*, mullet, *Mugil cephalus*, loach, *Misgurnus anguillicaudatus*, and carp, *Cyprinus carpio*. The bloods were treated as usual and the hemolyzates obtained were analyzed immediately (Part 1).

The blood samples of *Salmonidae* fishes such as chum salmon, *Oncorhynchus keta*, red salmon, *O. nerka* var. *nerka*, pink salmon, *O. gorbuscha*, and king salmon, *O. tshawytscha*, were collected individually by cutting carotid artery of live bodies at the North Pacific Ocean in June, 1959, and kept ice-stored until analyses were undertaken

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** Faculty of Agriculture, The Univ. of Tokyo, Tokyo, Japan. (橋本周久, 松浦文雄: 東大農学部)

after 17 to 22 days, when blood cells were hemolyzing more or less, and so it was obliged to hemolyze completely directly after plasma layers were pipetted off as far as possible. One sample of chum salmon was, however, treated daringly as usual to check the effect of the above-mentioned convenient method, though the most part of the cells were lost during washing with saline. The blood samples of Hime-masu, *O. nerka* var. *adonis* (obtained at the Shikotsu Lake; June, 1959), of rainbow trout, *Salmo gairdnerii irideus*, and brook trout, *Salvelinus fontinalis fontinalis* (both at Nagano Pref.; Feb., 1959) were collected by tail-cutting from live bodies and their hemolyzates were prepared after washing blood cells as usual (Part 2).

Methods of analysis: Electrophoretic analyses were carried out by a HITACHI D-type electrophoresis apparatus in a veronal buffer of pH 8.6 and $I/2=0.1$, on about 2% Hb solutions. No precipitates were discerned in Hb solutions during the dialysis against the buffer. Heat coagulation curves of Hb were measured on both met and cyanmet derivative by the method reported previously⁶⁾.

The analyses were performed on combined samples from several individuals in Part 1, while on individual samples in Part 2.

Results and Discussion

Part 1. The electrophoretic patterns of the Hb's are shown in Fig. 1. For convenience components will be described as I, II, and so on, in the order of decreasing

Fig. 1. Electrophoretic patterns of Hb's of fishes in a veronal buffer of pH 8.6 and $I/2=0.1$. Duration of electrophoresis was 120, 150, 110, 141, 136, 71, and 90 min., respectively.

mobility. Ascending and descending patterns of most Hb's were fairly symmetrical. Only one component was seen in the cases of mullet, goby, and carp, whereas multiple components in the cases of loach, black bream, and *Inimicus* sp. (two components) and dolphin fish (three components). Thus the appearance of multiples Hb's seems to be irrespective of the habitats of the fishes, in accordance with the previous results¹⁾. In Table 1 are shown the electrophoretic mobility and the proportion of amount of each component. Though the carp Hb's (individual samples) behaved either homogeneous or heterogeneous in the previous experiments¹⁾, the pre-

Table 1. Electrophoretic analyses of Hb of several kinds of fishes. For each component, values for the first reading represent mobility $\times 10^6$ cm.² volt⁻¹ sec.⁻¹, and for the second reading (in parenthesis) proportion of amount. Veronal buffer of pH 8.6 and $I/2=0.1$ was used, and calculations were made from measurements of the descending sides only.

Kind of fish	No. of animals	Component*		
		I	II	III
Black bream, <i>Mylio macrocephalus</i>	1	1.6 (55%)	1.3 (45%)	
Dolphin fish, <i>Coryphaena hippurus</i>	several	1.2 (33%)	0.8 (38%)	0.3 (29%)
<i>Inimicus</i> sp.	1	2.9 (62%)	2.1 (38%)	
Goby, <i>Acanthogobius flavimanus</i>	scores	1.6 (100%)		
Mullet, <i>Mugil cephalus</i>	2	1.4 (100%)		
Loach, <i>Misgurnus anguillicaudatus</i>	35	2.3 (55%)	0.9 (45%)	
Carp, <i>Cyprinus carpio</i>	4	1.8 (100%)		

* Nomination of each component is nothing else than for convenience.

sent sample (combined sample from four individuals) was homogeneous. The heterogeneous one might be, therefore, rather exceptional.

The heat coagulation curves of the Hb's are shown in Figs. 2 and 3. All the

Fig. 2. Heat coagulation curves of met-Hb's of fishes. Refer to the previous report⁶⁾ for the method of measuring the curves.

Fig. 3. Heat coagulation curves of cyanmet-Hb's of fishes.

Hb's were shown to be homogeneous by this criterion, though the zone of coagulation temperature is different appreciably species by species. This fact provides a strong contrast between these fishes on one side and *Salmonidae* fishes to be described in Part 2 on the other side.

Part 2. Electrophoretic patterns are shown in Fig. 4. In blood of each species, "Salmon type" fishes:

Fig. 4. Electrophoretic patterns of Hb's of *Salmonidae* fishes. Duration of electrophoresis was 90, 90, 90, 60, 60, 60, and 75 min., respectively.

there were two to four components of Hb. The patterns of both limbs were not so symmetrical as those of the fishes in Part 1, and so the results of calculations are shown in Table 2 for both limbs for comparison. The following points are indicated from these results:

(1) These fishes may be divided into two groups, one group having Hb composed of two components (chum salmon, red salmon, and Hime-masu) and another having Hb composed of three or four components (pink salmon, king salmon, rainbow trout, and brook trout).

Table 2. Electrophoretic analyses of Hb of some species of *Salmonidae* fishes. Refer to Table 1 as to what the numbers mean, and the buffer solution used. Mean values of individual determinations are given.

Name of species**	Limb		Ascending				Descending				Amount of heat-stable component*** (%)
	Component*		I	II	III	IV	I	II	III	IV	
Chum salmon, <i>Oncorhynchus keta</i> (3)			2.7 (58%)	1.5 (42%)			2.6 (46%)	1.7 (54%)			41.7
Red salmon, <i>O. nerka var. nerka</i> (2)			2.8 (56%)	1.7 (44%)			2.7 (39%)	1.9 (61%)			47.3
Hime-masu, <i>O. nerka var. adonis</i> (14)			2.8 (71%)	1.6 (29%)			2.8 (58%)	1.9 (42%)			28.5
Pink salmon, <i>O. gorbuscha</i> (3)			5.5 (15%)	4.2 (4%)	2.9 (38%)	2.0 (43%)	5.3 (13%)	3.8 (6%)	3.1 (23%)	2.1 (58%)	47.5
King salmon, <i>O. tshawytscha</i> (1)			5.2 (7%)	2.6 (53%)	1.4 (40%)		4.9 (8%)	2.5 (35%)	1.8 (57%)		37.9
Rainbow trout, <i>Salmo gairdnerii irideus</i> (3)			2.8 (61%)	1.9 (16%)	1.4 (23%)		2.8 (56%)	2.0 (19%)	1.7 (25%)		37.5
Brook trout, <i>Salvelinus fontinalis fontinalis</i> (1)			2.5 (64%)	2.0 (12%)	1.3 (24%)		2.4 (41%)	2.2 (18%)	1.5 (41%)		32.3

* See foot-note in Table 1.

** Value in parenthesis represents number of individual determinations.

*** Refer to the previous report¹⁾ for the method of estimation.

mon, rainbow trout, and brook trout). For convenience, the former group will be called hereafter as "salmon-type", and the latter as "trout-type".

(2) Though the separation of Hb components of "salmon-type" fishes is comparatively good, the proportion of amounts of two components was different species by species. Two components exist in almost equal amounts in chum or red salmon, while this is not the case in Hime-masu.

(3) Among "trout-type" fishes, pink salmon and king salmon possess four components, whereas rainbow trout and brook trout three components. The degree of separation and the ratio of amounts of components differ species by species. For instance, component II is only a very minor one in pink salmon, but a major one in king salmon. The mobility of component II was closer to that of component III than to component I in rainbow trout, whereas the relation was reverse in brook trout. These four "trout-type" fishes have, however, a thing in common; only one component is separated clearly from the rest.

(4) The electrophoretic mobilities of main components are fairly common over both types, one being $2.5\sim 3.0 \times 10^{-5}$, and another about $1.5 \times 10^{-5} \text{ cm}^2 \text{ volt}^{-1} \text{ sec}^{-1}$.

The heat coagulation curves of these Hb's are shown in Figs. 5 and 6, from

Fig. 5. Heat coagulation curves of met-Hb's of *Salmonidae* fishes.

Fig. 6. Heat coagulation curves of cyanmet-Hb's of *Salmonidae* fishes.

which the following points are noticed:

(1) All of the Hb's of "salmon type" fishes were shown to be composed of two components in accordance with the electrophoretical results, differing from the cases in Part 1. In "trout type" fishes, however, the existence of electrophoretically ob-

served three or four components could not be confirmed by this method, but only two components existed apparently. The half-way shelves of the curves are clearer generally on cyanmet derivative of Hb than on met one.

(2) The Hb of red salmon begins to coagulate at a slightly higher temperature than that of chum salmon does. No difference could be discerned in this respect between red salmon and Hime-masu, although the proportion of amount of corresponding component was not same. The fact seems to support the present theory that Hime-masu is a land-locked form of red salmon, together with the electrophoretic similarity between them.

(3) It was confirmed already that component I and II correspond to the heat-labile one ("component F", as designated by the present authors) and the heat-stable one ("component S"), respectively, in the cases of chum³⁾ and red⁷⁾ salmon. In fact, the proportion of amount of component II on ascending limb is comparable well to the proportion of the heat-stable component in both salmons and moreover in Hime-masu (see Table 2). Such a tendency that the electrophoretically fast component corresponds to the heat-labile one, perhaps holds also in the case of rainbow trout at least among "trout-type" fishes, as shown by the qualitative data on its Hb⁷⁾.

The blood samples were analysed after storing for about three weeks in ice because of technical reasons, and so there was a possibility that some changes of the Hb's might take place during the storage. A storage experiment was carried out, therefore, on fresh blood samples of chum salmon. According to the results, no detectable changes were found to occur during ice-storage of 3 weeks. There was another possibility that plasma proteins might affect on the electrophoretic patterns because the most hemolyzates used were prepared from unwashed red cells. This possibility should be also excluded since no substantial differences were observed between the pattern of hemolyzate prepared without washing blood cells and that of hemolyzate prepared from usually washed cells.

According to the results in the present and previous reports, the electrophoretic properties of fish Hb are specific for each species, but there are some similarities among related species as observed in *Salmonidae* fishes. It was suggested by DESSAUER et al.⁸⁾ that reptiles and amphibians may be classified to a certain degree on the ground of the characteristics of electrophoretic properties of their Hb's, and it may be possible also for fishes. As seen in the case of Hime-masu, the properties of heat coagulation of Hb may be utilized supplementarily for such a purpose.

Summary

(1) Properties of electrophoresis and heat coagulation were investigated on Hb of the fishes (including several species of *Salmonidae*) in sea-, fresh-, and brackish-

water.

(2) The fishes except *Salmonidae* were shown to have one to three components of Hb by electrophoresis, but all proved to have an apparently homogeneous Hb by heat coagulation method.

(3) The Hb of *Salmonidae* fishes was shown electrophoretically to be composed of two to four components, but wholly two by heat coagulation. There are common features in both properties all over *Salmonidae* fishes.

(4) Both properties of fish Hb was more or less species-specific as far as studied, and these may be useful for classifying fishes in general.

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