Multiple Hemoglobins in Fish*

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It had been thought usually that only one type of hemoglobin (Hb) exists in the blood of normal adult animals, until two sheep Hb's, whose solubilities and crystal habits differ from each other, were found in 1952 by Karvonen1). Thereafter, similar phenomena were reported on Hb of normal animals of various species2-9). As for fish Hb, however, there are few articles, except Pedersen's10) in which the electrophoretical heterogeneity of Hb of a fish, Opsanus tau, is described, and the present authors' observation11) that tuna Hb separates into two components in electrophoresis.

Therefore, it seemed interesting to the authors to investigate extensively existence of multiple Hb's in fishes, and the present study was undertaken. Results showed that Hb of most fishes studied here is not homogeneous electrophoretically, but composed of two or three components.

Since the separation of two components of Hb was apparent, especially in the case of salmon, this plurality was examined further by measuring solubility curve, heat coagulation curve, and column chromatographic behavior and the similar heterogeneity as in the electrophoresis was confirmed.

Material and Methods

Material: Blood Hb of the following species was used; yellowtail, Seriola quinqueradiata; swordfish, Makaira mitsukurii; chum salmon, Oncorhynchus keta; rainbow trout, Salmo irideus; and carp, Cyprinus carpio. The blood was obtained by cutting carotid artery or tail from live body in case of the latter three and from dead body (ice-stored for one or two days) in case of the former two. The oxalated blood was then treated as usual; blood cells were separated by centrifugation, washed with saline, hemolysed with water, and the stroma was centrifuged off. The hemolysate thus obtained was analysed immediately.

Electrophoresis: Electrophoretic analyses were carried out by a Tiselius electrophoresis apparatus (Hitachi D-type), in a veronal buffer (pH 8.6, 1/2 0.1) and a phosphate buffer (pH 8.0, 1/2 0.1) at 3~4°C. Most of the analyses were made on fresh hemolysates containing only oxy Hb, but in the case of carp on the hemolysate oxidized with ferricyanide, too.

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Measurement of Solubility Curve: Solubility curve of salmon Hb was determined in ammonium sulfate solution (pH adjusted to 6.9 with ammonium phosphate) at 20°C. Carbonyl form was selected, considering its stability. The procedure adopted was substantially the same as that of ROCHE or ITANO. Concentration of Hb was determined spectrophotometrically on the basis of ε_{540nm}^1M = 11.5 of cyanmet Hb reported by DRABKIN.

Measurement of Heat Coagulation Curve: As details are reported elsewhere, only the outline is given below. Heat coagulation of protein is influenced by many factors, such as temperature, duration of heating, pH, sort and concentration of salt, vessel, and concentration of protein, etc., and moreover by sort of derivative in the case of chromoprotein. When a series of protein solutions is heated at various temperatures, all the other factors being fixed, a S-shaped curve (heat coagulation curve) is obtained by plotting remaining amounts of Hb against temperatures. Each five ml. of Hb solution (final concentration 0.075%) diluted with phosphate buffer (final concentration 0.2 M and final pH 6.7) was pipetted into small test tubes (16×110 mm.). The tubes were stoppered by rubber stoppers, and kept immersed in ice-water. After heating at various temperatures for five minutes, each tube was immersed immediately in ice-water again, and concentration of Hb remaining soluble in supernatant measured spectrophotometrically after centrifugation. Coagulation curves were measured on four derivatives such as met-, cyanmet-, reduced-, and carbonyl-Hb.

Column Chromatography: Columns (15×100 mm.) filled with Amberlite XE-64, were used. As developing agents citrate and phosphate buffers of various pH's and concentrations were used. Hb was either met or carbonyl form, and the operation was carried out at room temperature (10~15°C.), as it took only about one hour for one performance.

Spectrophotometry: Spectrophotometric measurements were carried out with a BECKMAM DU spectrophotometer.

Results and Discussion

Electrophoretic results show that Hb consists of two (one case of carp, and chum salmon) or three components (yellowtail, rainbow trout), whereas Hb of carp (another case) or swordfish is homogeneous (Fig. 1). The percentage composition and electrophoretic mobility of each component are given in Table 1, each component being designated component I, II, and III in decreasing order of mobility.

Number of components, percentage composition and mobility of each component are different among species. In the cases of Hb's of yellowtail and rainbow trout, which are composed of three components, it is noted that component I holds a majority, followed by component III and II, and the electrophoretic mobilities of component II and III differ only slightly from each other. However, it is somewhat questionable whether the presence of three components in trout Hb is intrinsic, because of a pos-
Ascending pattern was also separated into three (yellowtail) or two components (carp), after longer duration of electrophoresis.

** Phosphate buffer of pH 8.0, \( \Gamma/2 \) 0.1.

** Fig. 1. Electrophoretic patterns of Hb's of fish in veronal buffer of pH 8.6, \( \Gamma/2 \) 0.1 (unless otherwise specified). Initial boundaries are shown by dotted lines.

** Table 1. Electrophoretic analyses of Hb's of fish. For each component, values for the first reading give amount in per cent; for the second reading mobility \( \times 10^5 \text{ cm}^2\text{volt}^{-1}\text{sec}^{-1} \)

<table>
<thead>
<tr>
<th>Kind of fish</th>
<th>No. of animals</th>
<th>Component I</th>
<th>Component II</th>
<th>Component III</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>yellowtail *</td>
<td>1</td>
<td>51</td>
<td>14</td>
<td>35</td>
<td>veronal, pH 8.6</td>
</tr>
<tr>
<td><em>Seriola quinqueradiata</em></td>
<td></td>
<td>2.0</td>
<td>1.2</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>swordfish *</td>
<td>a few*</td>
<td>100</td>
<td>1.8</td>
<td></td>
<td>phosphate, pH 8.0</td>
</tr>
<tr>
<td><em>Makaira mitsukurii</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chum salmon *</td>
<td>1</td>
<td>55</td>
<td>45</td>
<td></td>
<td>varonal, pH 8.6</td>
</tr>
<tr>
<td><em>Onchorhynchus keta</em></td>
<td></td>
<td>2.3</td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rainbow trout *</td>
<td>5*</td>
<td>59</td>
<td>17</td>
<td>24</td>
<td>varonal, pH 8.6</td>
</tr>
<tr>
<td><em>Salmo trideus</em></td>
<td></td>
<td>2.6</td>
<td>1.8</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>carp *</td>
<td>1</td>
<td>73</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cyprinus carpio</em></td>
<td></td>
<td>1.8</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>carp *</td>
<td>1</td>
<td>100</td>
<td>0.7</td>
<td></td>
<td>phosphate, pH 8.0</td>
</tr>
<tr>
<td><em>Cyprinus carpio</em>*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Combined.
** Met Hb from another individual.
sibility that state of health or race of each individual might be not completely same. In the cases of carp (oxy form) and chum salmon too, the faster component is dominant. The mobilities of two components of carp Hb are comparatively close, while component I of salmon Hb is twice as faster as component II.

In the cases of carp (met form) and swordfish (both in phosphate buffer), homogeneous Hb was observed. The homogeneity was maintained throughout the electrophoresis (235 min. (carp), and 177 min. (swordfish)). It seems certain that the homogeneity of these Hb's is not due to difference of buffers, in view of the fact that Hb of tuna and horse\(^{11}\) showed multiple components even in the phosphate buffer. However, why carp Hb is separated into two components in oxy form and not in met form, though each sample is not from the same individual, is not clear. There is no reason to believe that met form is not separated into two components at higher or lower pH's.

Thus, as it seemed to be necessary to examine whether electrophoretically distinguishable multiple Hb's show similar differences in the other properties or not, some experiments were carried out on salmon Hb which showed a comparatively nice separation among the fishes tested, and results as expected were obtained. To begin with, as shown in the solubility curve (Fig. 2), a critical zone is located around \(\Gamma/2 = 7.15\), the presence of which is an indication of existence of two components. Heat coagulation curves of some derivatives are shown in Fig. 3. Though heat stability increases in the order of met-, cyanmet-, carbonyl-, and reduced-Hb, each coagulation curve is two-stepped and interpreted to be composed of two curves of two Hb's which differ in heat stability. The relative ratio of two components as estimated by height of the halfway plateaus of the curves is about 55%.

![Fig. 2. Solubility curve of salmon carbonyl Hb in ammonium sulfate solution of pH 6.9 at 20°C.](image)

![Fig. 3. Heat coagulation curves of salmon Hb in 0.2M phosphate buffer of pH 6.7; initial Hb concentration 0.075%.](image)
(heat-unstable component) to 45% (heat-stable), which agrees well to the ratio determined electrophoretically (Table 1).

Separation of carbonyl Hb's of salmon by ion exchanger was then tried using a citrate buffer (pH 5.8, Na+ 0.34 g. ions/l.), by which BOARDMAN\textsuperscript{15}) separated successfully fetal and adult carbonyl Hb of sheep, but it resulted in no separation. By lowering the pH to 5.7, an appreciable separation into two bands occurred. Experiments were performed on met Hb, too, and a fairly good separation was seen when using a citrate buffer (pH 6.5, Na+ 0.17 g. ions/l.) or a phosphate buffer (pH 6.5, 0.1 M) as developer.

All these results coincide with the electrophoretic observation on salmon Hb. Separation and comparison of some physico-chemical properties of two Hb's of salmon will be reported in the following paper\textsuperscript{16}).

Summary

1. It was shown electrophoretically that multiple Hb's occur in blood of some fishes such as yellowtail, salmon, trout, and carp.

2. The heterogeneity of salmon Hb was further confirmed in tests of solubility, heat stability, and chromatographic behavior.

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References

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Addendum, June 10, 1958: Recently, the presence of two Hb's in the blood of a teleost, \textit{Scorpaenichthys marmoratum}, was found by alkali denaturation method (MANWELL, C.: \textit{Sci.} 126, 1175 (1957)). This and the present results make us suppose that heterogeneity of blood Hb is fairly ubiquitous in fishes.