COMPARATIVE STUDIES ON TWO HEMOGLOBINS OF SALMON—IV.
OXYGEN DISSOCIATION CURVE*

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It has been reported previously that chum salmons possess two types of hemoglobin (Hb) (as designated components F and S by the authors) in their blood, whose properties such as electrophoretical behavior, solubility, absorption spectrum, heat coagulability, amino acid composition and molecular shape differ distinctly from each other1-5). In addition, it was found later that component S is more alkali-resistant and more auto-oxidizable than component F is6).

However, in order to clear up probable significances of the existence of the two Hb's it seemed necessary to investigate their properties relating to oxygen dissociation, on which is based the most characteristic function of Hb. The present paper therefore deals with the effects of various factors upon the oxygen dissociation curve of both components.

Experimental

Materials Blood was collected last Dec. from several individuals of chum salmon, Oncorhynchus keta, at a small river (the Takibuchi) of Yamagata Pref., and transported to our laboratory keeping ice-stored overnight.

The oxalated blood was then treated as usual, and the red blood cells obtained were mixed with the glycerol-citrate mixture according to Cushing et al.7) and stored at −20°C. for later experiments. On the day before measurements of oxygen dissociation, the sample was dialysed against ice-water, and the supernatant hemolyzate was electrophoresed overnight on starch block using a veronal buffer of pH 8.6 and 0.05 M8), after the removal of cell debris by centrifugation. Components F and S thus separated were extracted with distilled water, dialysed against ice-water, and then submitted to measurements. Purity of each component was more than 99% as estimated by the heat coagulability test9).

Measurements were carried out not only on each component, but also on hemolyzate itself.

Measurement of oxygen dissociation curve The method for measuring oxygen dissociation curves is based essentially on the Röss's method10). The outline of the method is as follows: Hb solution (4 ml.) is pipetted into a tonometer connected with a special quartz cell, which is a slight modification of the Röss's apparatus, its total

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volume being ca. 500 ml. and the cell depth ca. 0.4 mm. The tonometer is evacuated to 13 mm. Hg usually by water jet pump, into which nitrogen gas is introduced at an atmosphere, and evacuated again. After injecting with a calculated volume of air, the tonometer is rotated slowly (60~80 r.p.m.) horizontally in a water bath at constant temperature (usually 15°C.) to equilibrate the reaction between Hb and oxygen. The degree of the reaction, or the degree of oxygen saturation of Hb, is measured indirectly by tracing the change in absorption spectrum. This operation is repeated with increasing amounts of air, and an oxygen dissociation curve is obtained by plotting the saturation degree of Hb against the oxygen pressure which can be calculated from the volume of air injected and the volume of the tonometer, etc.

All the final concentration of pigment was fixed on ca. 0.3%, because of technical reasons. No concentration effect of the pigment was observed in preliminary experiments, in which the range of concentration for hemolyzate, components F and S were 0.25~2.20%, 0.11~0.73%, and 0.20~0.80%, respectively. In most of the experiments was used the phosphate buffer whose final concentration is 0.1 M and the final pH 7.3~7.4, considering the physiological condition of blood, as far as not specified otherwise.

Results and Discussion

1) pH effect or Bohr effect The effect of pH of the solution upon the oxygen dissociation curve of hemolyzate is shown in Fig. 1. The curve at pH 7.41 is comparable anyhow to that of the Atlantic salmon, Salmo salar, reported by Benditt, et al\textsuperscript{10}. The pH effect is fairly large, and the lower the pH, the more decreases the oxygen affinity of the hemolyzate. There are two noticeable points here: First, oxygen dissociation curve is found to be discontinuous, at 40 mm. Hg at pH 7.20. Secondly, curves remain almost unchanged below pH 7.00.

The pH effect on each component is shown in Figs. 2 and 3. As seen in Fig. 2, the oxygen affinity of component F decreases drastically as pH becomes lower,
especially in the physiological pH range. The pigment could not be oxygen-saturated more than 20% even in the atmosphere at pH 6.8 or below. The "n" value in the HILL's equation was ca. 2.4 between pH 7.76 and 7.15, outside whose region "n" became smaller more or less. On the contrary, component S is affected hardly by pH over the wide range of 5.73~8.48 (Fig. 3). The "n" value was found to be also ca. 2.4.

The discontinuity of the curve in the case of the hemolyzate mentioned above, will be explained well on the basis of the differences of oxygen dissociation property between both components which are present in approximately equal amounts in the hemolyzate\textsuperscript{1).} A similar explanation may be applied unfailingly in the case of 	extit{Opsanus tau}\textsuperscript{11) whose Hb had ever been reported to have also an undulant oxygen dissociation curve.

The log $p_{50}$ (oxygen pressure at half saturation) \textit{vs}. pH curves are shown in Fig. 4, in which the difference of the pH effect for both components is clear at a glance. Though above pH 7.8 the oxygen affinity for component F is superior to that for component S, below the pH the relation is reverse and moreover the curves become almost straight, the slope ($\Delta \log p_{50}/\Delta$ pH) for components F and S being $-1.25$ and $0.0$, respectively. These values were found to remain unchanged essentially at higher concentrations of phosphate (refer to "Salt effect").

In this connection the modes of effect of CO$_2$ and lactic acid, which are regarded as main substances affecting blood pH in live bodies, were studied in other experiments. As far as expressed in the term of pH, they were quite the same as that in the case of phosphate buffer only.

2) \textbf{Salt effect} The effect of concentration of phosphate buffer was studied at pH 7.35. The effect for component F is shown in Fig. 5. As the molarity of phos-
Phosphate buffer increases, the oxygen affinity decreases remarkably, but the curves are inclined to converge gradually. On the other hand, the salt effect on component S is far smaller (Fig. 6). The "n" value for component F was slightly lowered down to 2.0 at high concentrations, while "n" unchanged for component S.

Effects of other salts such as NaCl, KCl, and Na₂SO₄ were also investigated at pH 7.35, when each salt was added to phosphate buffer, the final concentration of the former and the latter being 0.4 M and 0.1 M, respectively. As seen in Fig. 7, the effects of all these salts on component F are very similar among one another and far
smaller compared with the effect of 0.5 M phosphate. Moreover, the dissociation curves shift to the left of that obtained with 0.1 M phosphate only. These are not the case with component S (Fig. 8): It is true that the extent of the effects compares well with the case of component F, but the dissociation curve with Na₂SO₄ overlaps practically that with 0.5 M phosphate only, beyond which the curve shifts somewhat to the right in the cases of the other salts. These results are not agreeable partly with those on human Hb(12) on which the effect of phosphate is most remarkable, followed by Na₂SO₄, and of NaCl or KCl to a far less degree. The "n" values for both components were not altered by the addition of these salts.

3) Temperature effect The effect was measured over the range from 5° to 35°C at pH 7.4. The temperature effect on the hemolyzate is notable and the oxygen affinity decreases acceleratively as temperature rises (Fig. 9). The effect on component

![Fig. 9. The temperature effect on the oxygen dissociation curve of the hemolyzate (0.1 M phosphate buffer, pH 7.4).](image1)

![Fig. 10. The temperature effect on the oxygen dissociation curve of component F (0.1 M phosphate buffer, pH 7.4).](image2)

![Fig. 11. The temperature effect on the oxygen dissociation curve of component S (0.1 M phosphate buffer, pH 7.4).](image3)

![Fig. 12. The log p50 vs. temperature curves for both components and the hemolyzate.](image4)
F is more considerable, but tends to converge at higher temperature (Fig. 10). In passing, at a lower pH of 7.0 only a slight difference was observed between 25° and 35°C. The temperature effect on component S is far minor than on component F, as well as the other effects mentioned above (Fig. 11). The "n" value was unchanged on both components, except a slightly lower value at 35°C. on component F. In Fig. 12, the log $p_{50}$ vs. temperature curves are shown for these three samples. The curve for the hemolysate is comparable with that for rainbow or brown trout. Then the heat of oxygenation calculated from the slopes of these curves by the van't Hoff's equation, is 2.6, 6.4 and 6.3 kcal. (hemolysate), 5.2, 7.2 and 6.3 kcal. (component F), and 1.1, 2.4 and 4.2 kcal. (component S), per mole oxygen for 10°, 20° and 30°C., respectively. These values are smaller more or less than those for mammalian Hb's, especially in the case of component S.

It was reported that the oxygen dissociation curve of Hb solution is different somewhat from that of the suspension of red blood cells. The curve of the cell suspension is, however, not measurable for each component and all the measurements were carried out on Hb solution in the present study.

As mentioned above, component F shows a low oxygen affinity at physiological pH, $p_{50}$ being ca. 30 mm. Hg, the value of which is larger conspicuously compared with Hb's of teleost fishes ever reported which are all half-saturated below 20 mm. Hg. The oxygen affinity of this component is affected strongly by the pH change, the slope ($\Delta \log p_{50}/\Delta \text{pH}$) being -1.25. This value is reminiscent of those of marine teleost fishes such as mackerel (-1.25), sea robin (-1.20) and scup (-1.32). In a striking contrast to component F, component S shows a relatively high oxygen affinity ($p_{50}$=13 mm. Hg) and the affinity is extremely insensitive to pH. Generally, Hb's of fresh-water fishes possess higher oxygen affinities (e.g., carp's $p_{50}$=5 mm. Hg) and is influenced to a less degree by pH than Hb's of marine teleost fishes. From these facts components F and S are comparable roughly with Hb of marine teleost fish and of fresh-water fish, respectively. It should be emphasized, however, that the pH effect-absent Hb such as component S is so unique that there is only one comparable Hb (tadpole) in vertebrate so far as the authors know. In this connection the Bohr effect is known to be absent generally with muscle Hb or myoglobin, although this respiratory pigment is not present in blood. Moreover, the oxygen dissociation curve of component S is affected only slightly by the factors such as temperature and salt concentration. These facts are suggestive of, together with the fact that component S is fairly stable to heat or alkali and autoxidizable tolerably, the corresponding properties of myoglobin of vertebrate including fish. From this point of view component S may be comparable rather to myoglobin. By the way the authors could detect myoglobin in Salmonidae fishes even neither in lateral line muscle, nor in heart muscle, by the methods of absorption spectrum and electrophoresis. Thus it may be guessed that component S might have the similar physiological significance to
myoglobin, though it circulates with component F in the blood of salmon; component S might be rather an oxygen reservoir, providing against the time of oxygen lack such as so-called "Subashiri" (crawling on shallow of river) or probable other emergencies to which salmons may encounter while going up river, leaving component F the role of usual oxygen carrier.

The authors wish to examine by further experiments whether there is any relationship between the ecological specificity of salmon over both sea- and fresh-water and the characteristics of the blood containing two Hb's whose respiratory properties differ appreciably as described above.

It is alleged that experiments on the respiratory function of blood must be performed as quickly as possible, especially in the case of fish, within several hrs. at least after the sampling of the blood. Experiments with fresh blood were, however, impossible due to technical reasons: The place of blood sampling was so remote that it took more than half a day to transport the blood to the laboratory and more one day to isolate both components from the blood by electrophoresis. So there is an apprehension that the samples used in the present study might have undergone some changes. This apprehension seems however to be slight, if any, because results were reproducible enough and because each isolated component gave the almost same oxygen dissociation curve even after one-night-storage in ice as that before the storage, though the dissociation curves became gradually hyperbolic, $p_{50}$ becoming less, after more than two-night-storage. Supplementary experiments will be performed on this point in the near future.

Summary

(1) Effects of pH, salt concentration, and temperature on the oxygen dissociation curve of the two components of chum salmon Hb were investigated.

(2) The effects of pH, phosphate concentration and temperature were much more remarkable on component F than on component S. The mode of effect of the other salts such as NaCl, KCl and Na$_2$SO$_4$ was also different between both components.

(3) Physiological significances of the existence of the two components were discussed.

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