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Some Spectrophotometric Characteristics of Hemoglobin in the Atlantic Hagfish (*Myxine glutinosa* L.)

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By following the light absorption of three purified hemoglobins of the Atlantic hagfish (*Myxine glutinosa* L.) through the spectral region 800–230 mμ for a period of 160 h it could be established that the three hemoglobins found do not differ significantly in their relative rates of autoxidation. The wavelengths of absorption maxima of various hemoglobin derivatives are compared with corresponding data in the literature. The heme group of the three hagfish hemoglobins is of the Protoheme IX type. The differences among the three hemoglobins very probably reside in their globin part.

During our studies on the hemoglobin of the hagfish the question arose, if there is a difference in the prosthetic group and/or the globin part of the three hemoglobins found in the hagfish hemolysate. It also seemed as if a difference in the rate of spontaneous oxidation of the heme iron might exist between these hemoglobins as is observed in the single chains of human adult [1] and foetal [2] hemoglobin.

EXPERIMENTAL

The starting material was prepared from freshly collected blood by washing the erythrocytes twice with 3.3 % saline and once with 17 % sucrose solution at 4°C and hemolysing them in distilled water by freezing and thawing. The samples were stored at −20°C until subjected to isoelectric fractionation according to Svensson,[3-5] and Vesterberg and Svensson.[6] Details of the preparation, the purification, and the separation of the fractions will be published elsewhere.[22] Only the three main oxyhemoglobin fractions obtained by this method were used for further studies.

Spectrophotometric measurements were made with a Cary, Model 14, recording spectrophotometer in matched 1 cm cuvettes from Zeiss or Beckman. The reference solution was either water or buffer solution, except for the difference spectra. The wave-

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lengths of absorption maxima were evaluated from the recorded curves. The error introduced in the determination of wavelength by this method is small (about ± 0.6 mμ) and depends largely on the height of the maximum. Measurements of pH were performed with a pH-meter, model 25 and a combined glass-electrode GK 2021 C from Radiometer, Copenhagen. Sodium dithionite (analytical grade) served as reductant; it was transferred from a fresh bottle to several small, stoppered vessels and kept in the dark until its use. "Matheson" carbonmonoxide, C.P., was used throughout. Pyridine (Stockholms Gasverk; *p.a.*) was used for the preparation of the pyridine hemochromogens. All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

A. The absorption spectra of the three oxyhemoglobins

The hemoglobins were identified according to their isoelectric points of about 5.0, 6.7, and 8.0 as Hb 1, Hb 2, and Hb 3, respectively. This numbering is in accordance with conventional electrophoretic practice, *e.g.* as in numbering lamprey hemoglobins.[7] The oxyhemoglobin solutions were diluted with 0.5 M Trisbuffer, pH 7.0. The samples were kept in stoppered cuvettes at approximately 4°C except for the time of the spectrophotometric measurements (less than 2—3 % of the total time) which were performed at room temperature (20°—23°C). The spectra of the solutions were recorded at daily intervals. Sterile procedures were not used with the solutions, and no preservatives were added. However, no bacterial contamination was observed during the time of the experiment.

The calculation of the oxyhemoglobin concentrations was performed under the assumption that only oxyhemoglobin and methemoglobin were present in the solution as absorbing substances which means

$$[Hb]_{tot} = [HbO_2] + [Hb^+]$$

The following values for the millimolar absorptivity of oxyhemoglobin and methemoglobin at 500 and 577 mμ were used:

$\varepsilon\ mM_{500}\ (HbO_2) = 5.5;^*\ (Hb^+) = 9.5$ (Ref. 8, p. 228)
$\varepsilon\ mM_{577}\ (HbO_2) = 16.6;^*\ (Hb^+) = 3.35\ ^*$

These values were introduced in the equation

$a_{Hb} = \varepsilon_{mM}\ HbO_2 \cdot [HbO_2] + \varepsilon_{mM}Hb^+ \cdot [Hb^+]$ (see Ref. 8, p. 15)

The results are demonstrated in Fig. 1. It can be seen that no significant difference exists in the conversion rate of the three oxyhemoglobins to their methemoglobin derivatives. The differences in content of oxyhemoglobin at time zero among Hb 1 and Hb 2 and 3 is regarded as caused by the pH-difference that arose during the time between the end of the separation experiment and dilution with Tris-buffer, pH 7, at the beginning of the spectrophotometric measurements.

The absorption band at 275 mμ. It has been shown that the absorption band at 275 mμ, of which 70 % is due to the absorption of the heme group

* This figure was calculated from the data given by B. L. Horecker in the appendix of Ref. 8.

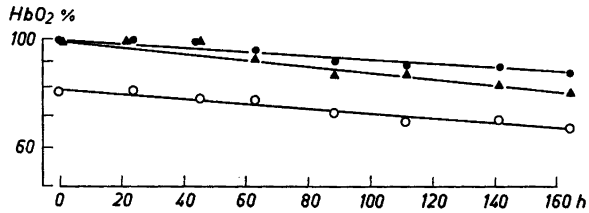

Fig. 1. The change of the oxyhemoglobin content with time; buffered solutions. ○ = Hb 1, 10.5×10^{-6} mole/l; ● = Hb 2, 6.8×10^{-6} mole/l; ▲ = Hb 3, 5.9×10^{-6} mole/l; pH of the samples: 7.0.

and 30 % to the absorption of aromatic amino acids,[9] can provide a valuable clue to the identity of individual hemoglobin chains.[10,11] Small, but reproducible differences have been found in the shape of the absorption bands around 275 mμ of the three oxyhemoglobins (Fig. 2). The "tryptophan notch" is not resolved, only hinted at by a slight inflection at 291 mμ in the curve taken from Hb 2, while a comparable change appears around 285—287 mμ in the curve of Hb 3. In the curve of Hb 1 no such change was observed.

B. The absorption spectrum of deoxygenated hemoglobin

Deoxygenation of diluted oxyhemoglobin solutions of the hagfish by physical methods (evacuation and substitution of air by pure nitrogen) proved to be extremely slow compared to the deoxygenation of mammalian (*e.g.* dog) hemoglobin. Besides that, excessive methemoglobin formation was observed in diluted hemolysates under reduced oxygen partial pressure. For these reasons reduction with sodium dithionite was preferred. The reaction with 0.5—2 mg/ml dithionite was complete within 35 sec as judged by the records of rapid repetitive scanning of the Soret region between 450 and 390 mμ. The spectrum of this reaction mixture was further recorded at intervals for the next 2.5 h, and no change in the absorbance within the Soret region, indicating reaction products of the hemoglobin with peroxides formed by the dithionite,[12,13] could be observed. The pH of these samples was measured

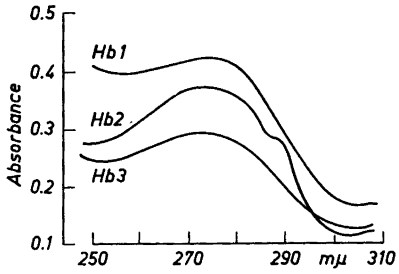

Fig. 2. Comparison of the absorption band at 275 mμ of the three hemoglobins (different concentrations). Note the inflection at 291 mμ (Hb 2) and the shoulder at 285—287 mμ (Hb 3), and the absence of a comparable change in the record from Hb 1.

after the experiments, and was found to lie between 6.75 and 7.0. The spectrum of a fresh hagfish hemolysate, reduced by dithionite, is not different from that of reduced hemolysates of mammals (*e.g.* dog). Values for the wavelength of the absorption maxima are presented in Table 1 together with data for other hemoglobin derivatives.

The absorption spectrum of carbonmonoxyhemoglobin. The absorption spectra of the CO-compounds of the three purified hemoglobins and the CO-treated hemolysate were all alike. Fig. 3 shows the spectrum of CO-treated hemolysate of the hagfish from 700 to 450 mμ, and for comparison the spectrum of the same sample in its oxy-form.

Table 1. Comparison of absorption maxima of hemolysates, separated hemoglobins, and various derivatives of hemoglobins from *Myxine glutinosa*.

	Absorption maxima (mμ)				
	Hemolysate	Hb 1	Hb 2	Hb 3	Literature values
1. Hemoglobin	555.9				555 dog [8], calf [8], human [8]
	431.2				430 human [8]
2. Oxyhemoglobin		577.3±0 3	577.8±0 4	578.0±0 3	576–578 dog [8], calf [8], human [8]
		541.8	542.6	542.9	540–542
		415.3	416.6	416.5	412–415 human, ox [8]
		350	346.9	346.9	350 human [14]
		276.2±0.6	274.6±0 5	274.2±0 5	275 »
3. CO-hemoglobin	570.0				568–572 dog [8], calf [8], human [8]
	538.4				538–540
4. Methemoglobin		636.2	634.6	637.5	630 dog [8], calf [8], human [8]
		501.5	502.2		500
		408.1	408.0		405–407 ox [8]
5. Pyridinehemochromogen, oxid.	575.4	—	574.2	579.4	
	398.1	401.4	398.3	401.3	
6. Pyridinehemochromogen, red.	557.2	558.0	557.1	557.1	557
	541.0[a]	540.3 [a]	539.9 [a]	540.0 [a]	540 [a]
	525.9	526.7	526.1	523.9	526 cow [16]
	479.2	—	479.2	478.9	
	419.3	419.4	418.4	419.3	418.5
7. Difference red.-oxid. Pyridine-hemochromogen	556.7		559.0	557.2	557
	540.0[a]		540.7 [a]	541.3 [a]	541 [a] pig [15]
	526.2		524.9	527.1	526
	419.6		419.5	420.0	

[a] = minimum

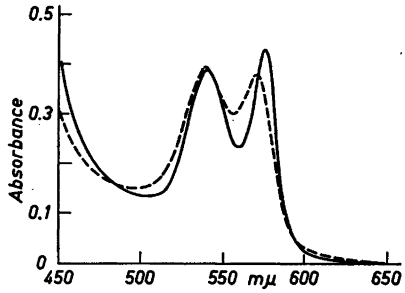

Fig. 3. Absorption spectra of freshly prepared hemolysate (———) and of the CO-saturated hemolysate (— — —).

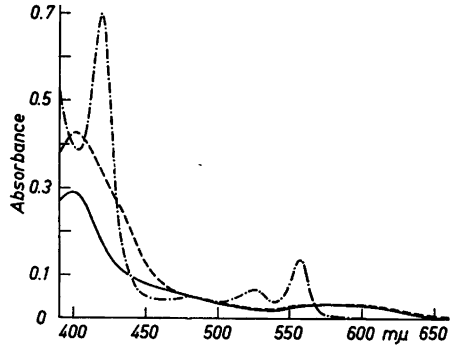

Fig. 4. Absorption spectra of untreated pyridine-hemochromogen (———), pyridine-hemochromogen oxidized with $K_3Fe(CN)_6$ (— —), and dithionite-reduced pyridine-hemochromogen (—·—·) of equal concentrations.

The pyridine-hemochromogen. The reduced pyridine-hemochromogen of the hemoglobins was prepared according to Paul *et al.*[16] Its spectrum was used for the determination of hemoglobin concentration employing the data for the molar absorptivity compiled by Falk.[15] In Fig. 4 the absorption spectra of the untreated, oxidized, and reduced pyridine-hemochromogens are shown. Fig. 5 shows the difference spectrum of reduced minus oxidized pyridine-hemochromogen of the same hemoglobin sample. No difference could be found between the absorption spectra of pyridine-hemochromogens of the three hagfish-hemoglobins and those of hemolysates of mammalian (*e.g.* human, dog) blood. This finding proves that the prosthetic group of hagfish hemoglobin is of the Protoheme IX type [15] (*cf.* Ref. 8, p. 309).

Conclusion. Evidence for the dissimilarity of the globin chains is given by the fact that the three hemoglobins were separated in the same oxidation — and oxygenation — state by isoelectric fractionation (*cf.* Ref. 5, 6, 17—19), which

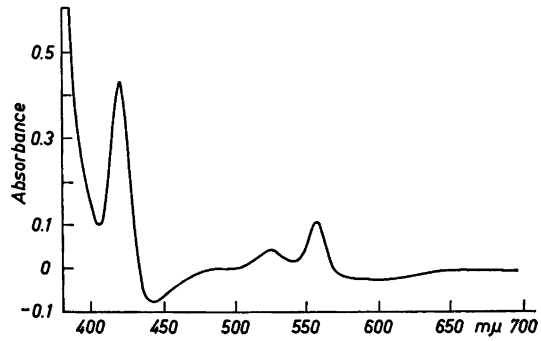

Fig. 5. Difference spectrum, reduced minus oxidized pyridine-hemochromogen; see Fig. 4.

means that they have different isoelectric points. The wavelength-difference of the absorption maximum, around 275 mμ between Hb 1 and the other two hemoglobins and the slight indication of a tryptophan notch in the spectrum of Hb 2 and 3 at different wavelengths may be interpreted as a difference in the amount of tyrosine or tryptophan, or both.[10] Conclusive evidence for either interpretation could not be gained by the method of Bencze and Schmid,[20] because of the high absorption of the heme group in this region.[9,21] It is evident that every determination of the wavelength of absorption maxima of oxyhemoglobin — especially that of the Soret band — is valid only if the presence of even small amounts of methemoglobin can be excluded. This requirement may be met only with great difficulty in cases where a rapid conversion of oxyhemoglobin to methemoglobin occurs as in diluted, slightly acidic samples of Hb 1 of the hagfish.

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