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# Isolation of Five Myoglobins from Seal (*Phoca vitulina*)

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1. Crystalline seal myoglobin contains 5 components. They have been separated by the aid of a cellulose ion-exchanger.
2. All five myoglobins have been crystallized from ammonium sulphate and their crystal shapes were found to be identical under the microscope.
3. The iron content was the same for all five.
4. Mb I is a basic protein, the others have IP values in the pH range 7.6—6.2.
5. Glycine is the terminal group in all five Mb's. Mb IV may contain serine as a second terminal residue.
6. The only differences found amongst the components is the isoelectric point and slight differences in the extinction coefficient of certain bands of the spectrum.

Already in 1934 Theorell [1] found an exceptionally high concentration of myoglobin in the seal, where the muscle press juice contains from 5 to 10 % of myoglobin. This is something like ten times the concentration normally found in mammals. Kendrew, Parrish and others [2] have also found that aquatic mammals, like whales, seals and penguins are particularly rich sources for myoglobin, so that their skeletal muscles are almost black in colour.

In this paper the isolation and some of the properties of seal myoglobin will be described.

## ISOLATION

Seal myoglobin was prepared from the meat of the common seal, *Phoca vitulina*, by the method described for sperm-whale myoglobin by Kendrew and Parrish [3], and kindly given by Dr. Kendrew for this work. The crude preparation, which was sent as a thick paste in phosphate buffer, pH 6.8, was dissolved in a very small amount of water and dialyzed three times against distilled water in the cold, and four or five times against 0.01 M tris-buffer, pH $8.50 \pm 0.02$.

Carboxymethyl cellulose was prepared according to Peterson and Sober [4], and a column was packed with it and charged with tris buffer pH 8.5. The column dimensions were $1.9 \times 23$ cm. The load of the protein was 119 mg in a volume of 2.5 ml. The temperature was 4°C; no difference was noticed if the chromatograms were run at room temperature.

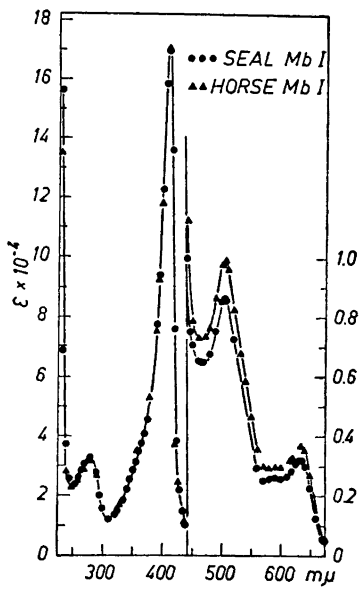


*Fig. 1.* Light absorption curves of seal ferri-Mb I and horse ferri-Mb I.

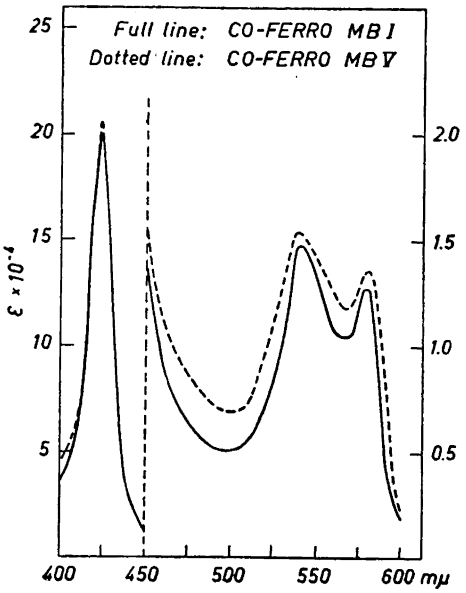


*Fig. 2.* Light absorption curves of seal CO-myoglobins I and V.

The chromatogram was eluated with the same buffer. On the column five distinct bands separated, moving with different speeds. The fastest component was named Mb V, and the slowest, which contained about 90 % of the total amount, was named Mb I. The distances between the bands were: Mb I 3 cm from the top, the distance between Mb I and II was 3.7 cm, between II and III 4.5 cm, between III and IV 2.5 cm, and Mb V came rather close to Mb IV. The yield of Mb I was about 90 % of the total; Mb II 2.5; Mb III 1.7; Mb IV 1.7 and Mb V 3.4 %. The high content of Mb I may be explained by the repeated crystallization from phosphate and ammonium sulphate, which followed after the extraction from the meat.

The five components were dialyzed over night in the cold against distilled water, and each component was put separately on a column, and eluated with tris buffer. In this way it was possible to concentrate the solutions, and at the same time to complete purification.

## PROPERTIES

The *homogeneity* of the five myoglobins was demonstrated by moving boundary electrophoresis in phosphate buffer $\mu = 0.1$, at various pH in the range 6.1—9.0. The isoelectric point, determined by boundary electrophoresis, was found to be near pH 8.05 for myoglobin I and for myoglobin II at pH 7.6. The isoelectric points for Mb III, IV and V were not determined, because of lack of protein.

The *sedimentation constant* of Mb I determined in the Spinco centrifuge, was found to be $S_{20} = 1.95\ S$, a value which is similar to those obtained for horse myoglobin I by Theorell and Åkeson [5].

The *minimum molecular weight* deduced from the iron content (0.30 % for Mb I, 0.29 % for Mb II, 0.30 % for Mb III, 0.29 % for Mb IV, and 0.30 % for Mb V) is 18 600 for all five myoglobins. These values are comparable with those found by Theorell [5] for pure and homogeneous horse myoglobin.

The *spectrophotometric analysis* of the seal myoglobins showed that the five myoglobins have light adsorption maxima and minima exactly at the same wave lenghts as pure horse myoglobin I, but they differ in extinction coefficients. Seal myoglobin I and horse Mb I have the same molar light absorption at 280, 315 and 409 mμ, but they differ at the wave lenghts 465, 505, and 635 mμ, which can be seen from Fig. 1. The results for all five myoglobins are given in the Tables 1 and 2.

The *prosthetic group* of the seal myoglobin I is identical with that of horse Mb I; this was demostrated by resynthesis from seal apo-protein and protohematin. Resynthesized Mb I was crystallized and the crystals were identical with those of the native seal myoglobin. The yield of Mb I after resynthesis from apo-protein and protohematin was 93.5 %, determined from the optical density of the solution at 409 mμ. Myoglobin I was crystallized from ammonium sulfate (80 %) at pH 8. For Mb II the pH was adjusted to pH 7.5, and for the other components to 7. All five myoglobins crystallized in the same form.

*Free amino group of the five myoglobins.* For the determination of the N-terminal group of the seal myoglobins Sanger's method [6], slightly modified, was used.

*Table 1.* Ferrimyolglobins, $\varepsilon \times 10^{-4}$.

| mμ | 250 | 280 | 315 | 409 | 465 | 505 | 590 | 635 |
|---|---|---|---|---|---|---|---|---|
| Seal Mb I | 2.31 | 3.31 | 1.22 | 17.1 | 0.657 | 0.868 | 0.260 | 0.323 |
| » » II | 1.93 | 2.96 | 0.99 | 17.2 | 0.591 | 1.02 | 0.284 | 0.366 |
| » » III | 2.58 | 3.66 | 1.44 | 17.2 | 0.755 | 1.01 | 0.310 | 0.374 |
| » » IV | 2.45 | 3.35 | 1.39 | 17.1 | 0.721 | 0.95 | 0.280 | 0.355 |
| » » V | 2.76 | 3.64 | 1.39 | 17.2 | 0.781 | 1.05 | 0.329 | 0.383 |

*Table 2.* CO-ferromyoglobins, $\varepsilon \times 10^{-4}$.

| mμ | 580 | 560 | 540 | 500 | 424 |
|---|---|---|---|---|---|
| Mb I | 1.28 | 1.05 | 1.45 | 0.502 | 20.55 |
| Mb II | 1.24 | 1.00 | 1.43 | 0.505 | 20.50 |
| Mb III | 1.32 | 1.06 | 1.52 | 0.565 | 20.60 |
| Mb IV | 1.30 | 1.06 | 1.48 | 0.522 | 19.40 |
| Mb V | 1.36 | 1.19 | 1.54 | 0.694 | 20.45 |

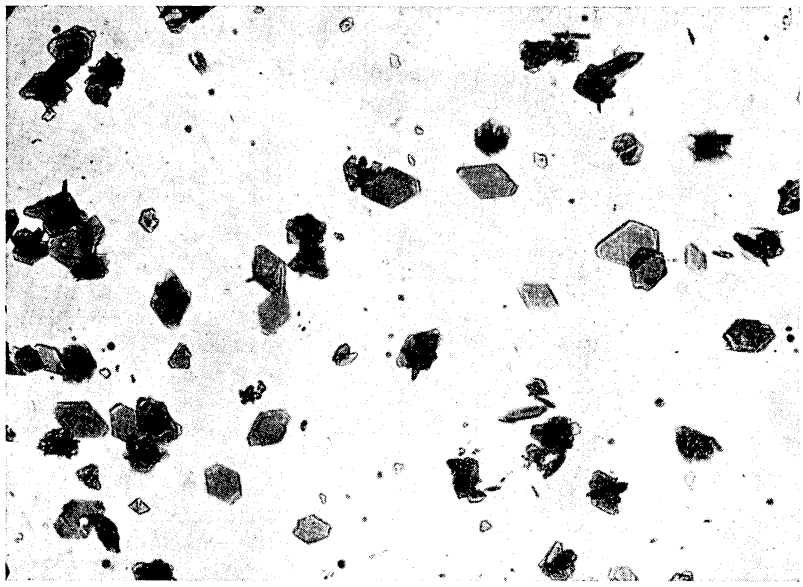

*Fig. 3.* Crystals of native seal Mb I from ammonium sulfate ( × 83).

In each experiment 3 mg (0.25 ml) of pure salt-free myoglobin, denatured with 0.75 ml ethanol was used, and 0.5 ml of a 10 % solution of $NaHCO_3$ and 0.005 ml liquid 1,2,4-fluorodinitrobenzene were added. The mixture was then vigorously stirred for 2 h at room temperature. The precipitate formed was centrifuged off, washed with water, ethanol and finally with ether. The precipitate was hydrolyzed with 6 N HCl at 110°C for 4.6 and 8 h. After hydrolysis, the solution was cooled and evaporated to dryness, water added and dried. After dissolving in a small volume of water, the solution was extracted twice with ethyl acetate. The extracts were combined, evaporated under vacuum to a small volume, applied to a corner of a Whatman No. 1 paper and run by the ascending technique with butanol-ammonia [7] (*n*-butanol was equilibrated with the same volume of 5 % ammonia and the top phase used). After 24 h the paper was dried at 38°C for 4 h. The paper was then run in the second dimension using the descending technique and 1.5 M aqueous phosphate buffer, pH 6. The paper was dried and the $R_F$ values were compared with those of known DNP-amino acids. In myoglobins I, II, III, and V only one spot was observed, which was found to be DNP-glycine. In Mb IV two spots were observed, with $R_F$ values corresponding to $\varepsilon$-DNP-lysine or DNP-serine, and DNP-glycine.

To identify them, the DNP-amino acids were eluated from the paper after chromatographic separation and heated with concentrated ammonia [8] in a sealed capillary for 2 h at 108°C. The DNP-derivatives had been split, and the free amino acids could be run by one-dimensional paper chromatography, using phenol as solvent [9]. On the paper lysine could be identified, but whether serine

was present or not was difficult to see, because of the tailing of the spots. The experiment could not be repeated because of the shortage of fraction IV. The N-terminal group of unpurified seal myoglobin has been determined by Orláns [2], who found glycine as the terminal residue.

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