THE REVIEW OF PHYSICAL CHEMISTRY OF JAPAN, VOL. 29, No. 2, MAR. 1960

DENATURATION OF HEMOGLOBIN UNDER HIGH PRESSURE, I

BY KEIZO SUZUKI AND KIYOSHI KITAMURA

(*Received February 15, 1960*)

The behaviors of hemoglobin under high pressure of 0~9000 kg/cm² were examined mainly on oxyhemoglobin at temperature of 20~40°C. Experiments were also performed on four kinds of hemoglobin: oxyhemoglobin, carbonylhemoglobin, reduced hemoglobin and methemoglobin to study the influences owing to the difference of the prosthetic group.

From the experimental results, it is supposed that the denaturation mechanism of hemoglobin is the same in itself as that of ovalbumin[3], and that the secondary structure responsible to denaturation under pressure may be affected in a certain extent by the state of heme and largely by the valency of iron ion in the center of protoheme.

The effect of high pressure on the denaturation of albumin was investigated previously[1-3]. It seems to be necessary to find whether the characteristics observed in ovalbumin, especially that the rate of denaturation has the negative temperature coefficient, are generally applicable for other globular proteins or not.

Though it has been already reported that carbonylhemoglobin coagulates at a pressure of 9000 atm[4], and that the oxygen equilibrium of hemoglobin is not altered by pressure at 680 atm[5], more detailed informations should be required to suffice the above purpose. From this point of view, the behavior of oxyhemoglobin of bovine was investigated with a special regard to the influences of temperature and magnitude of pressure on the rate of denaturation.

In addition, hemoglobin is a conjugated protein constituted of protein, globin and pigment, protoheme, and the behavior of denaturation of globin may be influenced by the prosthetic group, heme. Hence, it seems also to be interesting to investigate the problem with regard to the interaction between heme and globin, which enables this protein to combine with oxygen reversibly without undergoing oxydation.

Experiments for this purpose were carried out using four kinds of hemoglobins with different heme states: oxyhemoglobin, carbonylhemoglobin, reduced hemoglobin and methemoglobin under the same condition, and the results were compared with one another.

---

1) K. Suzuki, *Memoirs Res. Inst. Sci. and Eng., Ritumeikan Univ.*, 2, 19 (1957)
2) K. Suzuki, K. Kitamura, S. Kagawa and K. Tamura, *ibid.*, 3, 1 (1958)
3) K. Suzuki, *This Journal*, 28, 24 (1958)
4) P. W. Bridgman and J. B. Conant, *Proc. Natl. Acad. Sci.*, 15, 680 (1929)
5) F. H. Johnson and F. McK. Schlegel, *J. Cell. Comp. Physiol.*, 31, 421 (1948)

Experimentals

Preparations of hemoglobins Oxyhemoglobin: fresh bovine red cells well airated and repeatedly washed with isotonic saline solution, were hemolyzed by addition of distilled water, and then the stroma was removed by centrifuging. The solution thus obtained was stored as a stock solution in ice box of 0°C and used within 12 hours*.

Reduced hemoglobin; small amount of powdered hydrosulphite was added to oxyhemoglobin solution.

Carbonylhemoglobin; carbon monoxide gas was bubbled through oxyhemoglobin solution to saturation.

Methemoglobin; sodium nitrite was added to oxyhemoglobin solution and then the solution was dialyzed against running water over night.

These stocks were diluted with water and phosphate buffer of pH 6.8 composed of sodium phosphate dibasic and potassium phosphate monobasic to a given protein concentration and a buffer concentration of M/40.

High pressure apparatus and procedures The compressing apparatus was the same as in the previous paper[1]. About 3 ml of hemoglobin sealed in polyvinyl chloride sack was exposed to hydrostatic pressure. Then the solution was filtered to remove the coagulated hemoglobin as soon as possible, because of the reversibility described below, and the hemoglobin in the filtrate was determined colorimetrically[6].

Results

Oxyhemoglobin denatures and coagulates in the salt containing solution of pH 6.8 when

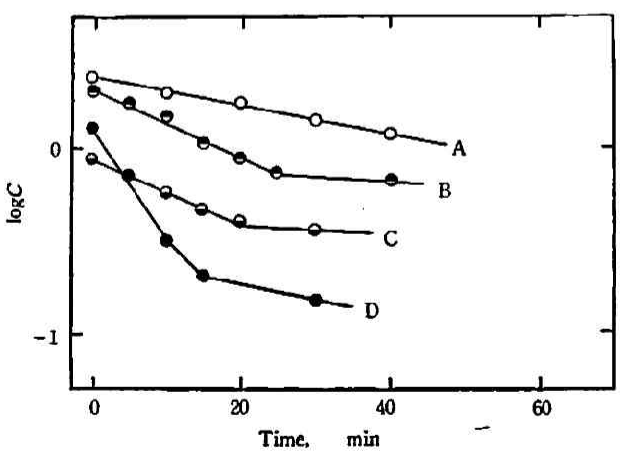

Fig. 1 Relations between logarithm of protein concentration in filtrate *C* and pressing duartion

protein: oxyhemoglobin
buffer: M/40 phosphate buffer, pH 6.8

	temperature °C	pressure kg/cm²
A:	24	6000
B:	20	6000
C:	20	6000
D:	20	7000

* Similar results were obtained by using the pure sample recrystallized many times from ethanol solution[7], but most of the experiment were carried out by using the preparation above described, since the freshness seemed to be rather important than the purity.

6) D. L. Drabkin and J. H. Austin, *J. Biol. Chem.*, **112**, 51, 86 (1935~36); D. L. Drabkin, *ibid.*, **146**, 605 (1942)

7) M. Suzuki, A. Kajita and C. Hanaoka, *J. Biochem.*, **41**, 401 (1954)

The Review of Physical Chemistry of Japan Vol. 29 No. 2 (1959)

several thousands kg/cm² of hydrostatic pressure is applied. The process does not follow the simple first order kinetics as shown in Fig. 1, and the coagulation does not proceed completely until the protein concentration in supernatant becomes zero, even if very high pressure is applied for a long duration (*cf.* Figs. 2 and 5).

The color of the filtrate is turned to brownish from deep red of the initial solution, as the fraction of the coagulated protein increases. The differences of these spectra are shown in Fig. 2.

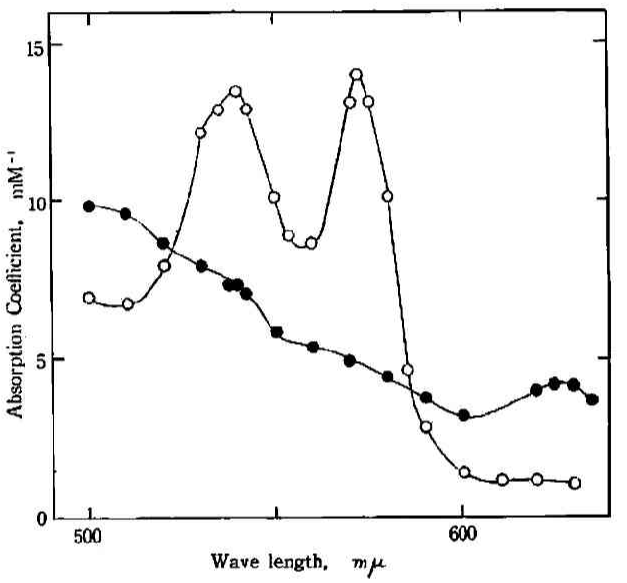

Fig. 2 Change of the color of filtrate after compression of oxyhemoglobin

○ : absorption coefficient before compression

● : absorption coefficient after compression

(9000 kg/cm² of pressure is applied for 30 min at 20°C; hemoglobin concentration of the filtrate is about 1/20 of the beginning after compression, in M/40 phosphate buffer, pH 6.8)

This result will be presumably due to auto-oxydation of heme to hemine. In addition, coagulation seems to be reversible, since it is found that a part of coagulated hemoglobin redissolves again in the solution as shown in Table 1.

Table 1 Reversibility of coagulation

t: time between releasing pressure and filtration

C: concentration of hemoglobin in filtrate

t	C, mM
2.5 min	0.058
1 hour	0.108
8 hours	0.146
1 day	0.144
2 days	0.144
7 days	0.154

initial concentration: 0.456 mM (ca. 0.3%) oxyhemoglobin in M/40 phosphate buffer, pH 6.8
compression: at 20°C, under 8000 kg/cm², for 5 min
stood: at the room temperature for 1 hour and then kept in 0°C ice box before filtration

t	C, mM
2.5 min	0.032
10	0.035
15	0.037
30	0.039
40	0.040
60	0.041

initial concentration: 0.070 mM (ca. 0.05%) oxyhemoglobin in M/40 phosphate buffer, pH 6.8
compression: at 20°C, under 7000 kg/cm², for 5 min
stood: at 20°C before filtration

The behaviors of oxyhemoglobin under pressure are much complicated in such ways, but the inclinations of the influences of temperature and pressure are closely similar to those of ovalbumin. The results around room temperature show similar patterns for three kinds of temperature as given in Fig. 3; above a certain pressure, further increase in pressure results in a rapid increase of the precipitation up to the point above which further increase in pressure causes little change. And it is also found that the temperature coefficient is negative, that is, the increase in temperature lessens the precipitation under the same pressure. But the relation is reverse above a certain temperature; above which further increase of temperature increases the quantities of precipitation as shown in Fig. 4, namely, there appears a minimum of rate between 30 and 40°C.

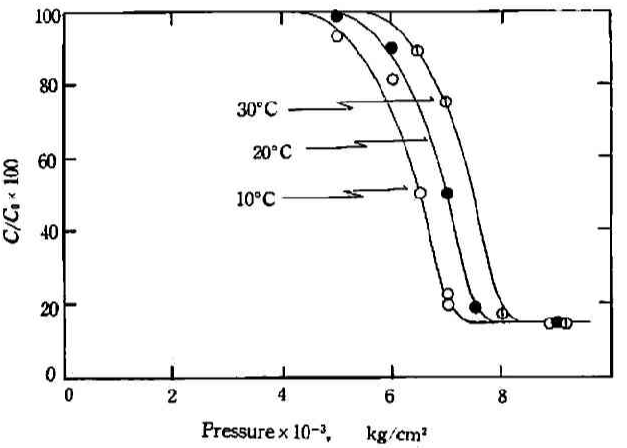

Fig. 3 Relations between $C/C_0 \times 100$ and pressure on oxyhemoglobin (C_0 is the initial protein concentration and C is the concentration of remaining protein in filtrate after compression.)

buffer: M/40 phosphate buffer, pH 6.8
duration: 5 min
C_0: ca. 0.5%

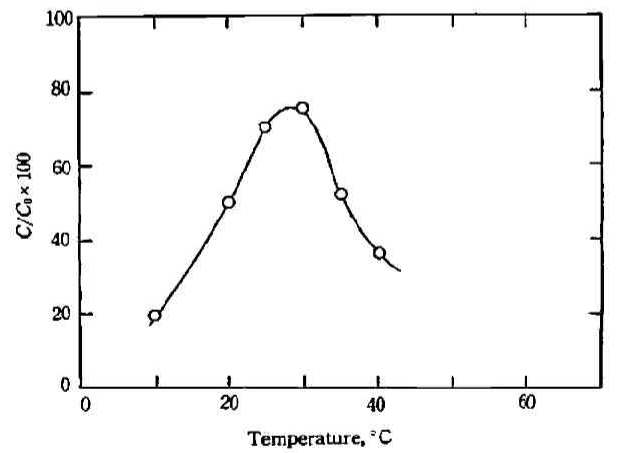

Fig. 4 Influense of temperature on the denaturation of oxyhemoglobin under high pressure

buffer: M/40 phosphate buffer, pH 6.8
duration: 5 min
pressure: 6500 kg/cm²
C_0: ca. 0.5%

The results of the pressure dependence on the process of coagulation are compared between four kinds of hemoglobin in the different states of heme at the same temperature, 20°C as shown in Fig. 5. The plots show the similar patterns, though the magnitude of pressure causing the same amounts of precipitation is different for each state of heme, and higher in the order of oxyhemoglobin, carbonylhemoglobin, reduced hemoglobin and methemoglobin. And it is to be

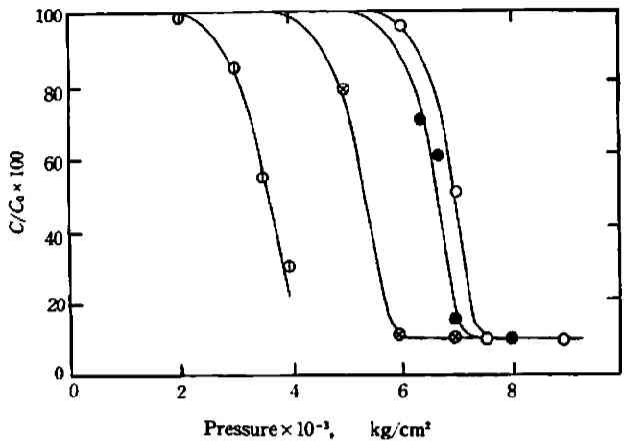

Fig. 5 Influence of pressure on oxyhemoglobin, carbonylhemoglobin, reduced hemoglobin and methemoglobin

buffer: M/40 phosphate buffer, pH 6.8
temperature: 20°C
duration: 5 min
Co: ca. 0.3~1.0%
○: oxyhemoglobin
●: carbonylhemoglobin
⊗: reduced hemoglobin
Ⓘ: methemoglobin

noted that methemoglobin is especially far apart from the others.

Considerations

From the experimental results mentioned above, it is supposed that the denaturation mechanism or the way of destruction of the subsidiary structure of hemoglobin is the same in itself as that of ovalbumin, and then may be generally applicable for other proteins, though there are considerable differences in the rate of denaturation between hemoglobins with ferrous ion and methemoglobin with ferric ion which is the most susceptible to pressure.

It is supposed that the subsidiary structure responsible to denaturation under pressure may be affected in a certain extent by the state of heme which combines the substituent such as oxygen, carbon monoxide and so on and largely by the valency of iron ion whether it is ferrous or ferric at the center of protoheme.

In the case of oxyhemoglobin, the final product of denaturation being of hemichrome type, it is doubted whether oxidation from ferrous to ferric is preceded or followed by the denaturation in globin part. But, from the fact that the resistance against pressure of oxyhemoglobin is similar to that of carbonylhemoglobin, while the denaturation product of the latter is hemochrome, we suppose that oxydation occurs in succession to denaturation in globin part. Similar step is assumed in denaturation of oxyhemoglobin by cationic detergents[8].

The authors have great pleasure in expressing their sincere thanks to Prof. Wasaburo Jono, Dr. Jiro Osugi and the late Prof. Ryo Kiyama for their valuable guidance and encouragement throughout the course of this work.

Laboratory of Physical Chemistry
Faculty of Science and Engineering
Ritumeikan University
Kyoto, Japan

8) T. Saito, *J. Biochem.*, 28, 56 (1956)