Hemolysis Under High Hydrostatic Pressure[1] (35679)

WALLACE O. FENN AND VIRGINIA P. BOSCHEN

Department of Physiology, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14620

At pressures up to 2000 atm, Ebbecke (1) found no spontaneous hemolysis of erythrocytes, but the cells lost their biconcave shape and became spherical. Haubrich (2) confirmed this but found that, subsequently, the treated cells were more easily hemolyzed. At pressures of 3500 atm or more, Dow (3) and Dow and Matthews (4) found disintegration of the cells and denaturation of the hemoglobin. No one seems to have tried the reaction of cells with a hemolytic agent under pressure except Bernardini and Pryor (5), who reported a slight inhibition of hypotonic hemolysis at a pressure of 2–3 atm and an acceleration of hemolysis by the same solutions at simulated high altitudes. Our studies were undertaken to cover intermediate pressures between these and 2000 atm.

I. Immediate Hemolytic Effects of Pressure up to 41 atm. Method. The apparatus used for this study is shown in Fig. 1. It consisted essentially of a 20-ml syringe containing 5 ml of blood cells into which 5 ml of water could be injected from a 5-ml syringe attached to it by a short rubber tubing. The water was driven into the cells by a strong spring which could be released from outside the pressure chamber by activating a solenoid. The opacity of the cell suspension was monitored by a beam of light passing through the cell suspension and falling on a photoelectric cell, the output of which was recorded on an Esterline Angus ink writer. A deflection of 5–7 cm was obtained when the water was injected into the cell suspension. The dilution effect was recorded in about 1 sec and hemolysis was 80% complete in 5–9 sec, and 100% complete in 1–3 min or less. In all cases before use the blood cells were washed three times in 0.9% NaCl and made up to 40 times the original blood volume with saline. The whole apparatus was heat-insulated with sponge rubber and cotton and was enclosed in a pressure chamber rated for 80 atm and used safely (after testing) at 40 atm. It was pressurized with air to about 3 atm and then with nitrogen or helium to the desired pressure. Closing the chamber and pressurization required 5–8 min, and the spring was released usually 10 min after installing the syringes. Due to the insulation, the temperature in the blood sample rose much more slowly during pressurization than that of the ambient gas and after the usual interval of 10 min, it had usually risen 2–3° at 20 atm and proportionately less at lower pressures. The temperature was recorded by a thermistor inserted into the lumen of the syringe through a tiny hole bored in the bottom of the plunger. A few tests at 1 atm of pressure before and after warming the syringes about 5° showed no significant change in the percentage of hemolysis obtained when the blood was diluted with an equal volume of water. After the galvanometer deflection had reached its maximum, the pressure was reduced and the percentage hemolysis in the diluted sample was measured in a spectrophotometer. For this purpose 3-ml samples taken before and after centrifuging were diluted with a further 5 ml of H_2O and the hemoglobin was estimated at 540 mμ.

The galvanometer record was started always immediately after the syringes were installed and before the pressure was raised. In all cases there was a gradual deflection in the direction of increased light transmission because of the slow sedimentation of the cells. If left a half hour or more, the injection of the

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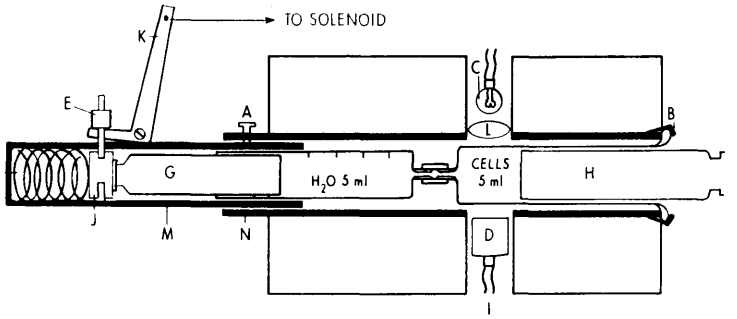

FIG. 1. Diagram of apparatus for measuring hemolysis under pressure. E is a pin pulled by K to release J so that the spring F can push the plunger of the 5-ml syringe G. C is a light bulb, L a lens, and D a photoelectric cell. The brass tube M is set in the brass tube N by the set screw A. B is a spring to hold the 20-ml syringe H in place. The syringe G is drawn too large relative to H. Both syringe plungers are open to avoid an implosion under pressure. The tube N is surrounded by heat-insulating material as indicated.

H_2O into the blood gave a backward deflection due to the stirring of the solution. The deflection recorded, however, was always the difference between the initial and the final reading. The injection of the H_2O was very rapid and effectively stirred the whole solution very thoroughly according to visual observation. In any event it averaged out as equal in both the pressurized and control experiments. Usually only about 10% of the total deflection occurred prior to triggering the injection of H_2O. Both control and pressurized samples were triggered after the same initial delay.

Four different series of experiments were performed which are presented as parts 1–4. Different samples of blood were used for these different series as follows. Part 1: pooled human blood samples from the clinic anticoagulated with EDTA. Parts 2 and 3: sheep's blood with oxalate; Part 4: blood from senior author with 3 vol of Alsevier's glucose–citrate solution. Only in the experiments of part 4 was the temperature of the blood measured with a thermistor and the percentage of hemolysis measured after each experiment. In parts 1–3 the temperature was estimated by a thermistor in a model syringe similarly exposed and the percentage of hemolysis was monitored roughly by single preliminary experiments without pressure.

Results. A sample of the galvanometer response resulting from the injection of 5 ml of water and 5 ml of saline is shown in Fig. 2. The much more rapid response due to dilution with saline is plainly visible. We measured only the maximal deflection obtained under pressure for comparison with a similar experiment without pressure. The results are expressed as the difference between these two curves in percentage of the control deflection.

Figure 3 shows the way in which the galvanometer deflection increased when increasing amounts of water were injected into 5 ml of cell suspension at atmospheric pressure. The lower curve represents similar experiments with saline instead of water and represents the simple dilution effect of the saline. Controls at atmospheric pressure are indicated by dots and samples at 13.6 atm are shown by open circles. There is no significant difference between the pressurized and the control samples either in water or saline. The shaded area between the two

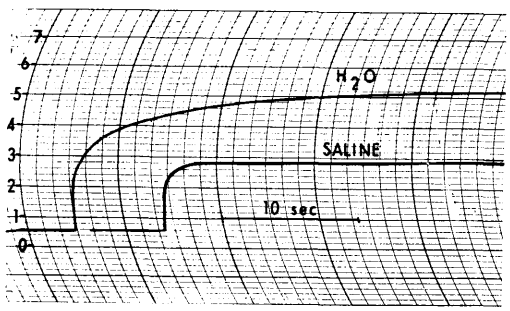

FIG. 2. Galvanometer deflections obtained when saline or water is added to an equal volume of washed cells. The water curve is higher because of the decreased opacity due to hemolysis.

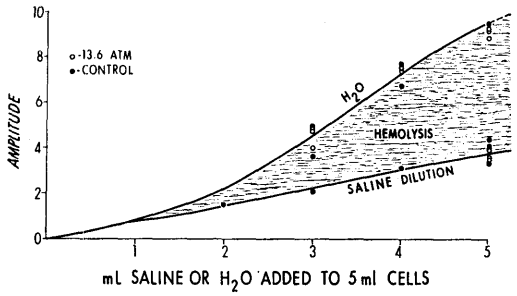

FIG. 3. Record of one experiment in which increasing volumes of 0.9% NaCl or H_2O (abscissae) were added to 5 ml of washed cells at 1 and 13.6 atm pressure. Ordinates are galvanometer deflections obtained. No constant difference due to pressure is seen.

curves indicates the effect of hemolysis which increased naturally as the dilution increased.

The experimental results with this technique are conveniently presented in four parts. Part 1 includes 42 experiments completed before we realized the importance of equalizing the time interval between the first insertion of the syringe and the triggering of the dilution. Using pressures varying from 3 to 14 atm, the average apparent percentage of inhibition of hemolysis in these early experiments was 9.5 ± 1.9. We believe that this result was due to the stirring up of red cells which had settled out prior to injecting the H_2O.

In part 2 the time delay was equalized and the average result in 19 experiments was 0.8 ± 2.6% at pressures of 14–20 atm. Part 3 was similar except for some additional minor improvements in technique. The results of 39 such experiments are given in Table I and show an average inhibition of 0.5 ± 0.8%.

TABLE I. Hemolysis Under Pressure with No Significant Inhibition.[a]

Pressure (atm)	No. of expt.	Pressure effect (%)
3	11	−1.9 ± 1.1
14	6	−3.7 ± 2.1
14	5	−2.1 ± 3.3
20	6	+2.0 ± 4.2
20	6	+2.1 ± 1.3
41	5	−1.0 ± 2.7
	39	0.5 ± 0.8

[a] In all cases 5 ml H_2O is added to 5 ml of cells. Percentage of inhibition is the pressure hemolysis deflection minus the control hemolysis deflection in percentage of the control deflection with standard error of the mean added $[\Sigma d^2/n(n-1)]^{1/2}$. The pressure is gauge pressure, the control being at atmospheric pressure. All these experiments were done on washed cells from oxalated sheep blood.

There seemed to be no effect either at 3 atm or 41 atm.

The 35 experiments of Part 4 are summarized in Table II. The percentage change in the hemolysis and in the deflection are recorded together with the standard error of the mean. It is evident that none of the changes are more than twice the calculated error and it must be concluded that no statistical effect of pressure has been demonstrated. The percentage of hemolysis measured spectrophotometrically might have been influenced by further hemolysis occurring after the reduction of the pressure but the deflection of the galvanometer measured while the sample was still under pressure showed no inhibition or acceleration.

II. Acceleration of Spontaneous Hemolysis

TABLE II. Effect of Pressure on Hemolysis.[a]

Pressure (atm)	Control values: Hemolysis (%)	Control values: Defl. (cm)	No. of expt.	Change due to pressure: Hemolysis (%)	Change due to pressure: Defl. (cm)
3	34	5.5	17	− 0.25 ± 2.1	−0.07 ± 0.16
6.8	31	4.5	3	+12.3 ± 10	+4.5 ± 4.0
13.6	41	5.6	10	+ 4.8 ± 3.4	−3.5 ± 1.9
20.5	58	6.7	7	+ 7.4 ± 5.2	+2.5 ± 4.0

[a] The last two columns represent pressure–control values ($E-C$) in percentage of control together with standard error of mean, *i.e.*, $100(E-C)/C$.

at Pressures up to 540 atm. Method. The above experiments have shown that pressure up to 41 atm has no immediate effect on hemolysis. The same method could not be used for longer periods of exposure to pressure because of the sedimentation of the cells in the syringe. For this reason another series of experiments was undertaken in which samples of cells washed three times in physiological salt solution were exposed in a 10-ml syringe to pressure for periods up to 17 hr with a control sample under 1 atm of pressure. Some slight spontaneous hemolysis always occurred during this period. The samples were then centrifuged and the hemoglobin in the supernatant fraction was determined by a spectrophotometer at a wavelength of 540 mμ. The absorbances E and C, of the experimental (pressurized) and control samples, respectively, were recorded.

Results. In general it was found that pressure tends to increase the small amount of hemolysis which occurred, but the effect was much greater at 6° than at 25°. No effect was observed in whole blood but only in washed cells. Averaging all the experiments with pressures 200–540 atm at both 5 and 16 hr the average percentage increase in hemolysis due to pressure, 100 $(E—C)/C$, was 149 ± 28 in 30 experiments at 6° and 19 ± 7 in 22 experiments at 25°.

To show the effect of different pressures, the values of E/C found in the 16-hr exposures at each of these temperatures are plotted as logarithms against pressure in Fig. 4. The number of experiments averaged is indicated beside each point, and the standard error is indicated by the vertical bars. The enhanced effect at the lower temperature is evident.

These results were confirmed by some 25 similar preliminary experiments using washed cells from pooled human blood samples from the clinical laboratory. There were some minor variations in technique, but the results were in no way essentially different from these already described and they need not be reported here.

Discussion. The effect of pressure on the rate of any chemical reaction can be expressed by the equation given by Johnson, Eyring, and Polissar (6).

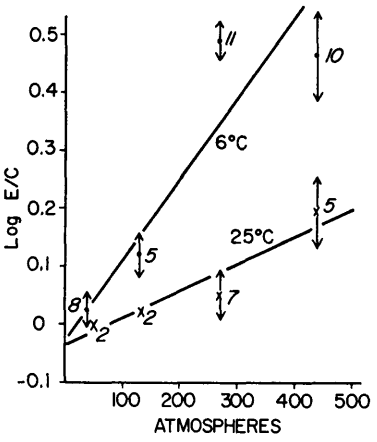

FIG. 4. Spontaneous hemolysis of red cells in isotonic saline at 6° and 25° as a function of hydrostatic pressure in atmospheres for 16 hr. The ratio E/C is the ratio of the hemolysis under pressure to that at 1 atm. The slopes of these lines give the value of the activation volume which is —69 ml/mole at 6° and —22 ml/mole at 25°. Pressures varied for each of the points plotted being, respectively, for the four pressures plotted, 21–50, 160–200, 200–350, and 350–540 atm. There were also 24 experiments for periods of 5–6 hr, but there were no significant effects except perhaps for four experiments at 6° with an average value of E/C of 1.38 ± 0.12, and eight experiments at 25° with an average value of 1.13 ± 0.05, both at the highest pressure levels of 350–540 atm.

$$k_p = k_0 e^{-p\Delta V\ddagger/RT},$$

where k_p and k_0 are the reaction rates with and without additional pressures, p, in atm, $\Delta V\ddagger$ is the volume of activation of the limiting reaction in milliliters per mole, R the gas constant, and T the absolute temperature.

In using this equation we have taken our values of E and C as proportional to the reaction rates because the percentage of hemolysis is so small that the changes in the concentrations of reactants and end products could hardly have been large enough to influence the rates of the reaction. They might also be regarded, however, as representing the equilibrium value for the condition of the blood at the time. In that case the same values for ΔV can be obtained by essentially the same equation (6), but then it represents an overall measurable volume change and not merely a calculated volume of activation.

According to this equation, pressure should have a greater effect on k_p at a lower temperature and log k_p/k_0 = log E/C should be a linear function of the pressure, p. While the points plotted do not strictly confirm a linear relation, straight lines have been drawn for convenience. From the slopes of these lines (Fig. 4) average values of $\Delta V^{\ddagger}$ can be calculated as −69 ml/mole for 6° and −22 ml/mole for 25°. From these differing values it seems likely that the limiting reaction for hemolysis at 6° is not the same as at 25°.

Since our experiments for prolonged exposures to high pressures predict a decrease in volume due to hemolysis, it should be mentioned that we have measured the volume change in hundreds of experiments (unpublished) and have found regularly decreases in volume in hypotonic hemolysis of the order of magnitude of 0.01% or less. This agreement may, of course, be only a coincidence if our pressure experiments predict only the volume of activation of some reaction rather than a true equilibrium volume change. Moreover, we have found a similar decrease in volume using laked cells so the result may be due to conformational changes due to dilution of the protein.

It is impossible to identify the actual reaction involved in the hemolysis observed and it does not seem likely that it concerns hemoglobin directly but rather some constituent of the cell membrane. It is, perhaps, instructive, however, to calculate the volume change to be expected if hemoglobin is the important molecule. With 15% hemoglobin and a mol wt of 66,000 the value at 25° would be 22 ml/mole × 15/66,000 (moles %) = 0.005%. This is certainly of the right order of magnitude since many of our observations were distinctly less than 0.01%. The same calculation might be applied to other cell constituents such as, for example, ATP, but in that case the predicted volume change would be much less because the molar concentration of ATP is lower than that of hemoglobin. The importance of ATP as a factor of importance in hemolysis is well illustrated by the paper of Weed, LaCelle, and Merrill (7).

These experiments have provided no evidence of the effect on hemolysis of pressures up to 41 atm for short periods. Our results do do not directly contradict the experiments of Bernardini and Pryor (5) who reported a slight inhibition of hemolysis due to relatively low pressure (2–3 atm) because the methods were so totally different. In longer exposures to high pressures up to 540 atm we have found a slight acceleration of the spontaneous hemolysis which occurs especially at low temperatures in red cells washed in isotonic saline. While the actual percentage of hemolysis observed was very small, this is presumably the initial effect of the more pronounced effect reported by Dow and Matthews (4) at pressures of 3500 atm.

All of our results apply to cells washed free of plasma in saline thus providing a condition in which they are probably particularly sensitive to hypotonic hemolysis. Since we found no immediate effect of pressure up to 41 atm with these cells and very little effect with longer exposures to even higher pressures, we were not encouraged to continue further.

Summary. Hydrostatic pressures up to 41 atm had no significant immediate effect on the percentage of hemolysis of washed red cells when they were diluted with an equal volume of water. After prolonged exposures to pressures up to 540 atm there was a significant increase in hemolysis due to pressure which was 2–3 times greater at 6° than at 25°.

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