THE EFFECT OF HYDROGEN ION CONCENTRATION ON SAPONIN HEMOLYSIS.

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The purpose of the present study has been to determine the change in the resistance of corpuscles to saponin with changes in pH, the acids employed being the common inorganic acids. Ponder (1) has shown that the rate of saponin hemolysis is increased in the presence of certain acids and has applied the equation

\[ C_1 = R (C_2 + kR) \]

to systems in which an acid is the accelerating agent.

Details of technique in preparing the red blood cell suspensions and measuring the rate of hemolysis will be found elsewhere (2, 3), but to explain the significance of the terms in the equation given above, it is necessary to refer to certain features of the procedure which Ponder has adopted in his studies of hemolysis and which have been followed in a portion of this investigation. In determining the effect of an agent which either accelerates or retards the action of saponin, a time-dilution curve is first constructed which shows the relation of the time taken for complete hemolysis of a standard cell suspension to the dilution of saponin, in the absence of inhibiting or accelerating agents, the determinations being made at a constant temperature. The dilutions of saponin are usually 1 part in 10,000, 20,000, 30,000, etc. The concentrations corresponding to these dilutions \( s_1 \) are expressed in the above equation by \( C_1 \), the value of which in these experiments is the number of mg. of saponin contained in the tube to which the standard red blood cell suspension is added.

Upon introducing into the simple cell-saponin system an agent which hastens hemolysis, a different time-dilution curve is obtained. For a given dilution of saponin complete hemolysis of a
standard cell suspension now occurs in a shorter time. Accord-
ingly, by referring to the original time-dilution curve, the dilution
of lysin ($\delta_2$) may be determined, which by acting alone would

![Graph](http://www.jbc.org) Fig. 1. Curve A represents the relation of the resistance, $R$, of the red blood corpuscle to the initial pH of the saponin solution. Dilution of saponin, $\delta = 30,000$. The clear circles, squares, triangles, and rhombs represent the data for dog corpuscles, with hydrochloric, nitric, sulfuric, and phosphoric acids, respectively. The solid circles, squares, etc., represent the data for human corpuscles, with the same acids. Curve B represents the relation of the resistance of the corpuscles to the pH of the cell-
saponin-acid system when hemolysis is complete.

have produced complete hemolysis in the same time taken in the presence of the accelerating substance. The concentration cor-
responding to this dilution is $C_2$. If the values for $C_1$ are plotted against the corresponding values for $C_2$, a straight line is
obtained which makes an intercept on the $C_2$ axis. This intercept is represented by $k_R$. If the straight line passes through the origin, $k_R = 0$ and $R = \frac{C_1}{C_2}$. Ordinarily, the value of $k_R$ is small, being treated by Ponder as an empirical constant, probably an expression of the fact that the real relation between $C_1$ and $C_2$ is not linear, but given by a flat curve.

$R$ is the resistance constant of corpuscles by means of which the effects of two or more hemolytic systems may be compared. For example, referring to Curve A in Fig. 1, it will be seen that corpuscles suspended in a solution of saponin in saline ($\delta = 30,000$), acidified with an inorganic acid to an initial reaction of $pH = 3.0$ have 0.25 the resistance which they exhibit at a $pH$ of about 4.5, or in the control experiment, in which saponin is dissolved in neutral saline.

Curve A, in Fig. 1, represents the data obtained with human and dog corpuscles, in each case the four inorganic acids, hydrochloric, nitric, sulfuric, and phosphoric, being employed, the dilution of saponin (Merck) being 30,000. Complete studies have also been made with $\delta = 10,000$ and 20,000. With the dilution of 10,000, in the presence of acids, hemolysis may be so rapid as to introduce a significant experimental error. More consistent results are obtained with $\delta = 20,000$, and these have been found to approximate closely the data obtained with $\delta = 30,000$, as may be seen by examining the curves in Fig. 5. When the saponin concentration is relatively low, $\delta > 40,000$, the end-point of complete lysis is not very sharp and this may likewise introduce an appreciable experimental error. Accordingly, while valuable data may be obtained over a wide range of dilutions of saponin, the most reliable results, with the methods employed in the present work, are observed with $\delta = 20,000$ and $\delta = 30,000$. Dog and human corpuscles have about the same resistance to saponin over the range of hydrogen ion concentrations represented by Curve A in Fig. 1.

This curve, which is based upon the $pH$ values at the beginning of hemolysis, shows that the acceleration of saponin hemolysis depends upon the hydrogen ion concentration and is approximately the same for the four inorganic acids studied. The shape of the curve, moreover, suggests a resemblance to a titration curve.
It is obvious, however, that any relationship that may exist between the hydrogen ion concentration and the resistance of corpuscles is likely to be a relationship involving, not the initial hydrogen ion concentration, but some concentration intermediate between the initial concentration and that at the end of hemolysis, since, as has been previously shown (3), acids, even in very dilute solutions, react rapidly with the constituents of the red blood corpuscle. In the more concentrated solutions, the neutralization of the acid produces relatively little effect on the hydrogen ion concentration, whereas in the dilute solutions, the neutralizing effect of the cell buffers causes much greater shifts in pH.

The time required for complete hemolysis being determined by Ponder's method (2), parallel determinations were made in which neutralization of the acid in the saponin solutions was measured by means of a quinhydrone electrode. In each of these determinations, 4 cc. of the red blood cell suspension were added to 16 cc. of the acid-saponin solution. Quinhydrone did not seem to affect appreciably the rate of saponin hemolysis and it was assumed that the hemoglobin liberated from the 0.1 cc. of corpuscles, contained in the 4 cc. of suspension, did not influence the determinations materially because of the effect of the hemoglobin in altering the ratio of oxidant to reductant. Readings of the E. M. F. were taken before adding the cells and at intervals of 10 to 30 seconds during hemolysis, until equilibrium was attained. From the results obtained in this way, it was possible to determine the pH shift from the initial value which occurred in the interval required for complete hemolysis, as determined in the parallel determinations by Ponder's method. This shift may be represented by $\Delta p$H. The value of $p$H + $\Delta p$H, in any given case, is therefore the pH at the end of hemolysis.

Curve B, Fig. 1, is based on the values of $p$H + $\Delta p$H. The actual relation between $p$H and the resistance of cells to saponin would probably be represented by a curve plotted between Curves A and B. Such a curve, except for the portion representing the lower values of $R$, would also bear some resemblance to a titration curve. (Compare, for example, these curves with the titration curves for various proteins given by Cohn (4).)

The value of $R$, at any given $p$H, being approximately proportional to the amount of lysin needed to produce a given effect, as
compared with the amount needed in the absence of acid, the preceding results indicate that the acceleration of saponin hemolysis by acids may depend either (a) on the formation of a dissociable acid-saponin compound, or (b) on a similar combination of hydrogen ion with one or more constituents of the red blood corpuscle.

Experiments with “Acid” and “Basic” Saponins.—With few exceptions, the saponins are either neutral or weakly acid. The product used in this work, Merck’s pure saponin, was slightly acid. In a dilution of 1:30,000, in saline, the pH was 6.00; in a solution containing 1 part in 2500, the pH was 5.76. Saponin reacts both with acids and bases, and accordingly, small amounts of “acid” and “basic” saponins were prepared. 2 gm. quantities of saponin were dissolved in 50 cc. of 0.1 N HCl and, after being cooled in the refrigerator, the acid-saponin was precipitated with acetone. The precipitate was redissolved in a small volume of warm alcohol, reprecipitated with acetone, filtered, and dried. Alkali-saponin was prepared by dissolving 2 gm. portions of saponin in 50 cc. of 0.1 N NaOH, cooling, and precipitating with alcohol or, preferably, an alcohol-ether mixture. The precipitate was redissolved in water and reprecipitated with alcohol-ether, filtered, and dried. Portions of the commercial saponin were further purified by being dissolved in water and precipitated with alcohol-ether and alcohol-ether-acetone mixtures.

The preparation of “acid” and “basic” saponins did not result in the hydrolysis of any of the saponin, as shown by the absence of reducing sugars at any stage in the preparation and in the final products.

The acid-saponin, dissolved in physiological salt solution, gave the following values: δ = 2,500, pH 4.50; δ = 5,000, pH 5.42; δ = 10,000, pH 5.85; δ = 20,000, pH 5.96. The alkali-saponin, dissolved in physiological salt solution gave the following values: δ = 2,500, pH 7.28; δ = 5,000, pH 6.90; δ = 10,000, pH 6.77; δ = 20,000, pH 6.64. The original saponin, similarly dissolved, gave the values: δ = 2,500, pH 5.76; δ = 5,000, pH 5.89; δ = 10,000, pH 5.91; δ = 20,000, pH 5.96; δ = 30,000, pH 6.00. The repurified saponin was somewhat less acid, giving the following values: δ = 2,500, pH 6.05; δ = 5,000, pH 6.10; δ = 10,000, pH 6.13; δ = 20,000, pH 6.17; δ = 30,000, pH 6.24.

The results of the experiments with the “acid” and “basic”
Saponin Hemolysis

Saponins are represented by the curves in Fig. 2. These show that the formation of a dissociable acid-saponin compound is not the cause of the acceleration of saponin hemolysis by acids. In fact, acid-saponin was much less effective as a hemolytic agent than the original saponin. In a dilution of 20,000, hemolysis occurred in 1 minute when the original saponin was used, but, with acid-saponin, hemolysis was not complete even at the end of 30 minutes. Alkali-saponin was likewise less hemolytic than either the untreated or repurified preparations.

**FIG. 2.** Time-dilution curves of various saponin preparations.

*Experiments with Red Blood Corpuscles, Treated with Acid and Alkali.* When erythrocytes are treated with dilute acid, their resistance to hemolysis by saponin is diminished. Ponder (1) has observed this effect in the case of glutamic and aspartic acids. On the other hand, when cells are treated with arginine, which inhibits hemolysis, their resistance to saponin increases.

Dog corpuscles, centrifuged from 50 cc. of a standard suspension, were treated with 50 cc. of 0.001 N HCl (the HCl was made up in salt solution, the concentration of the salt being adjusted so that
the acid solution would be isotonic with the cells). After 5 minutes, the acid-cell suspension was centrifuged; the cells were again suspended in 50 cc. of acid saline, centrifuged and washed three times with physiological salt solution. Another 50 cc. portion was
similarly treated with \(0.001\, n\) \(\text{NaOH}\) in saline, the cells being suspended in the alkaline saline but once, in view of the rapid hemolysis which occurs on repeated treatment with alkali.

The behavior of saponin toward the acid- and alkali-treated cells is shown by the curves in Fig. 3. From these it is clear that

![Graph](image)

**Fig. 5.** The relation of the resistance, \(R\), to the initial pH of saponin solutions. Dilutions, \(\delta = 20,000\) and \(\delta = 30,000\).

the effect of the acid in accelerating hemolysis and of alkali in inhibiting it must depend largely on the action of these on the corpuscle. At the beginning of hemolysis, the saponin solution, \(\delta = 30,000\) had a pH value of 5.96 in all cases. On addition of the washed, untreated cells, the pH shifted to an equilibrium value of 7.01 when hemolysis was complete, the shift being due to the
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liberation of the cell buffers. On the other hand, the acid-treated cells caused a shift in the opposite direction, from pH 5.96 to 5.88. As compared with these observations were those noted with the alkali-treated cells which produced a shift from pH 5.96 to pH 7.40.

A comparison was also made of the action of "acid," "basic," and approximately neutral saponins on the acid-treated cells. The results, represented by the curves in Fig. 4, show that each of the saponin preparations hemolyzed the acid-treated cells more readily than the untreated cells (compare with Fig. 2). Here, also, the "acid" and "basic" saponins were much less hemolytic than either the untreated or repurified preparations. For example, in a dilution of saponin, $\delta = 20,000$, hemolysis of the acid-treated cells, with the original saponin, occurred in 0.52 minute, whereas with the "basic" saponin, hemolysis occurred in 13 minutes, and with the "acid" saponin, 17 minutes were required.

Near the neutral point, the resistance of the red blood corpuscles increases slightly with increasing pH, to approximately pH 8.5, after which the resistance increases more rapidly, reaching a maximum at about pH 10. The effect of further increase of alkalinity on saponin hemolysis cannot be studied satisfactorily, owing to the independent hemolytic effect of the alkali. In the experiments upon which the curves in Fig. 5 are based, the saponin solutions were adjusted to a given pH by the addition of either HCl or NaOH, the pH being determined colorimetrically.

DISCUSSION.

The effect of hydrogen ion concentration on saponin hemolysis is apparently twofold. The reaction between acid and saponin yields an ionizable acid-saponin compound which retards hemolysis, an effect which manifests itself over the entire range of acidity and which probably accounts for the higher resistance of corpuscles observed in a saponin solution, acidified to pH 4.5, or higher, than is noted in a saponin solution prepared in neutral saline. The second and predominant effect, and the one responsible for the acceleration of hemolysis, is due to the reaction of the acid with the cell constituents.

Coulter (5), in studying the cataphoresis of red blood cells, determined that their movement in an electric field was a function
of the hydrogen ion concentration. For normal sheep corpuscles he obtained the value pH 4.6 for the isoelectric point, that is, the point at which no movement of the cells occurred. On the alkaline side of the isoelectric point the cells are negatively charged, the charge increasing with the alkalinity, whereas on the acid side, the charge is positive and increases with the acidity. The behavior of corpuscles toward acids and bases corresponds with that found for protein. On the acid side of the isoelectric point, the red blood corpuscles combine chemically with H\(^+\) and Cl\(^-\) ions, whereas on the alkaline side, combination occurs with cations.

A variety of phenomena, such as agglutination and stability of blood cell suspensions, seem to be linked in some way to the electric charge of the red corpuscles. Coulter (5) has observed that the optimum for agglutination of normal cells is at pH 4.75, a point at which "the cells exist most nearly pure, or least combined with anion and cation." From their studies of the influence of electrolytes on the stability of red blood corpuscle suspensions, Oliver and Barnard (6) are led to conclude that a red blood cell when suspended in fluid reacts in regard to its electrophoretic properties as if it were possessed of a surface of globulin.

Judging from the curves in Figs. 1 and 5, the behavior of saponin is likewise modified by the electric charge of the corpuscles. The greater the positive charge, the less the resistance to saponin, the point of inflection of the curves appearing approximately at the isoelectric point of the corpuscles. On the alkaline side of the isoelectric point, as the cells become negatively charged, their resistance to saponin increases somewhat, but not markedly so until after a pH of about 8.5 is attained. The second inflection in the curve (Fig. 5) occurs approximately at the point which marks the reversal of permeability of the corpuscle to anions, as has been recently shown by Mond (7). This investigator observed that erythrocytes treated with alkali become impermeable to anions but permeable to cations, the reversal occurring between pH 8.0 to 8.3. This value corresponds closely to the isoelectric point of globin (from the erythrocytes of cattle), determined by Osato (8) to be pH 8.1 ± 0.1. Accordingly, Mond concludes that the membrane of the red blood corpuscle contains protein phases consisting of globin. While the present writer is inclined to accept the view of Mond with regard to the possible importance of this protein in red
blood cell permeability, he is unaware of any direct evidence that globin, as such, is a constituent of the red blood corpuscle.

SUMMARY.

The relationship of pH to the resistance of dog and human erythrocytes to saponin has been studied and the results represented by means of curves.

The effect of inorganic acids and bases on saponin hemolysis is believed to depend principally on their chemical combination with globulin and possibly other proteins of the cell membrane, such as globin.

On the acid side of the isoelectric point of the red blood corpuscle (pH about 4.6), as the positive charge increases, the resistance of the cell to saponin diminishes. On the alkaline side of the isoelectric point, as the cells become more and more negatively charged, their resistance to saponin increases gradually to about pH 8.5, after which the resistance increases more rapidly, reaching a maximum at about pH 10.

BIBLIOGRAPHY

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