THE AGGLUTINATION OF RED BLOOD CELLS IN THE PRESENCE OF BLOOD SERA.

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(Received for publication, January 6, 1922.)

The optimum for the agglutination of normal sheep cells in isotonic saccharose solution has been given as pH 4.75.[1] To correct a possible error in the colorimetric measurements originally employed electrometric determinations have been made in a similar series of experiments in which graduated amounts of N/10 to N/40 HCl have been added to suspensions of red blood cells in saccharose solution and measurements made of the reaction of the supernatant fluid from which the cells have been removed 15 to 30 minutes after the addition of acid. The average pH 4.76 of the following values thus found corresponds closely with the result of the colorimetric method: pH 4.55, 4.57, 4.79, 4.90, and 5.03.

Cells sensitized with approximately 10 units of immune rabbit serum at pH 5.3, the optimum for combination of the cells with the immune sensitizer[2] and washed with pure saccharose solution at the same reaction agglutinate most promptly at the following reactions in a series of experiments: pH 5.22 to 5.45, 5.26, and 5.06 to 5.30. The average is pH 5.26. The colorimetric method had given from a larger series the value pH 5.3. If the cells be not washed after the addition of immune serum which was present in a concentration of 0.5 per cent by volume, the optimum occurs at a slightly higher figure, pH 5.5 approximately.

If a similar small volume of active normal rabbit serum be added to the cells in place of the immune serum, the optimum for agglutination occurs at the same point, pH 5.5.

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[1] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.

[2] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 513.

The euglobulin precipitates most promptly and completely from rabbit serum diluted 1:20 with distilled water at the same reaction, pH 5.5, and it is apparent that the agglutination of the cells is intimately related to the precipitation of the serum euglobulin.

The same relation is observed in the agglutination of sheep cells to which a like small amount of their own active serum has been added, as shown by the following values for the optimal point for agglutination in the presence of homologous serum: pH 5.58, 5.44, and 5.38. The euglobulin itself precipitates best from sheep serum diluted 1:20 with distilled water at approximately pH 5.5.

Guggenheimer[3] has made an observation which corresponds closely with this, that if defibrinated sheep blood be washed directly with isotonic saccharose solution the euglobulin of the serum is carried down with the cells and will serve as the mid-piece fraction of complement to persensitize the cells on the subsequent addition of sensitizer.

The relation mentioned is noted again when sensitized sheep cells in saccharose solution are persensitized by the addition of active normal guinea pig serum. If such serum be added in the amount of 8 per cent of the total volume to an emulsion of sensitized cells of such concentration that one unit of complement is present, the optimal point for agglutination has been found at the following electrometric values: pH 6.19, 6.35, and 6.15. Five other experiments in which the estimation was made colorimetrically gave values between pH 5.9 and 6.3. The euglobulin has been found to precipitate best from guinea pig serum diluted 1:20 with distilled water between pH 6.2 and 6.4 (electrometric).[4]

If the cells were persensitized at pH 6.2 and washed by allowing them to settle spontaneously from pure saccharose solution of pH 6.0 the optimal point of agglutination was noted at the following reactions (electrometric): pH 5.71, 5.79, 5.76 to 6.18, 5.38 to 5.80, 5.78, and 5.69 to 5.77. This shift toward a more acid zone runs parallel with that observed in the precipitation of guinea pig globulin which has been washed as precipitate and redissolved by bringing to pH 7.4 with NaOH. Precipitation then has its optimum between pH 5.1 and 5.7.[4]

[3] Guggenheimer, H., *Z. Immunitätsforsch. Orig.*, viii, 1910–11, 295.
[4] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 771.

A specific reaction in the immunological sense may be supposed to take place between the sheep cells and the immune or native sensitizers of rabbit or guinea pig serum. A reaction of the same nature can hardly be thought of as occurring between sheep cells and their own serum, and yet these sera act alike as protective colloids to sheep cells since in their presence agglutination of the cells is not observed at pH 4.7; and further, they sensitize the cells to agglutination at the characteristic reactions of the serum euglobulins.

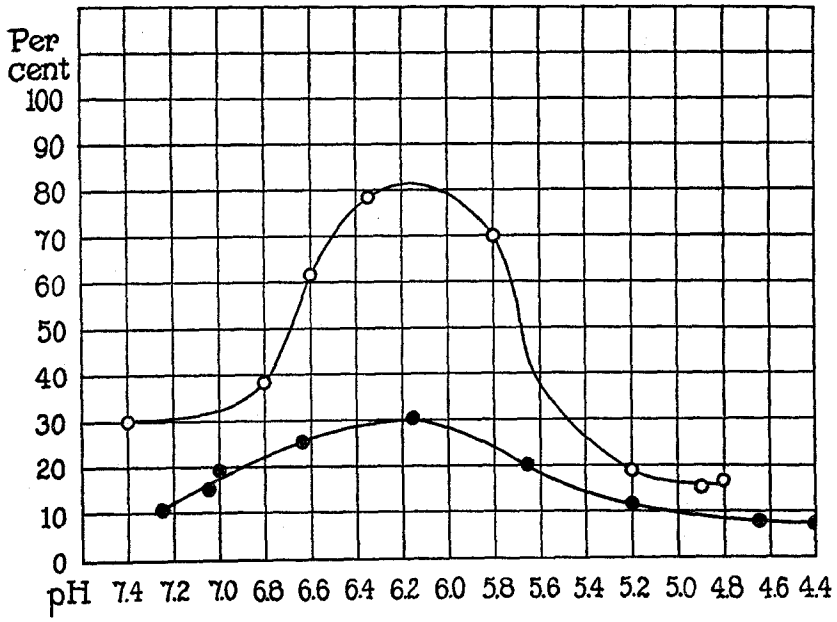

FIG. 1. Each curve represents a single experiment and gives as ordinates the percentage of the total amount of complement added which has been absorbed by sensitized cells at 4°C., with varying H ion concentrations as abscissæ.

If the cells from the tubes to which guinea pig serum has been added be sedimented and reemulsified in isotonic saline solution, it is found that they are not only persensitized, but within a certain range of reaction have combined apparently with the end-piece fraction of complement as well, since hemolysis occurs without further addition of end-piece. This is shown in the experiments recorded in the curves (Fig. 1). The phenomenon is observed regardless of whether the

cells have first been sensitized with rabbit serum or not; in the latter case the normal sensitizer of guinea pig serum, too small in amount to be recognized by the usual means, is probably united with the cells. In the experiments recorded cells were sensitized with approximately 8 units of sensitizer and added to tubes along with guinea pig serum in such amounts as to give a concentration of one unit of complement. N/10 NaOH and HCl were immediately added and the tubes kept at 4°C. for 45 minutes. The cells were then sedimented, the supernatant fluids pipetted off as completely as possible, and their pH measured electrometrically. The cell sediment in each tube was reemulsified in a constant amount of isotonic saline solution and the tubes were incubated at 37°C. The curves show the percentage of the total complement present which has been absorbed by or removed along with the cells. This percentage was plotted from the titration curve of the complement alone according to the method described by Brooks[5] and employed with slight modification[4] by the author. Five other experiments have given the same result; namely, that at the point of optimal agglutination of the persensitized cells the greatest amount of whole complement has been bound by the cells.

Guggenheimer[3] found that sensitized sheep cells in saccharose solution carry down with them in sedimentation the mid-piece fraction of guinea pig serum, and that the amount of mid-piece removed increases with the degree of sensitization of the cells, so that a true binding probably occurs. He could not detect, however, an absorption of the whole of complement by sensitized cells under these circumstances, even when the cells had been sensitized with 100 units. In the experiments described above in which such an absorption appears to have taken place a small amount of end-piece must have been present in the liquid phase of the sedimented cell mass, which could not have been removed by washing without disturbing the equilibrium relations between the cells and the sensitizer[2] and complement. According to the experience of Zinsser,[6] the trace of end-piece retained by the globulin sediment in the partition of complement by dialysis is sufficient to give complementary power to the globulin

[5] Brooks, S. C., *J. Med. Research*, 1919–20, xli, 399.

[6] Zinnser, H., Infection and resistance, New York, 1914, 180.

sediment alone. However, in the experiment recorded in the upper curve of Fig. 1, an hemolysis corresponding to an activity of 78 per cent of the total amount of complement originally present points distinctly to a true binding of end-piece by the persensitized cells.

It is generally believed that the protective and sensitizing effects of one colloid upon another with reference to precipitation by a third substance are due to a combination of some sort between the two colloids. There is no evidence available on which to base a judgement as to the nature of the combination between the cells and the serum euglobulins considered here, whether physical or chemical. However, the fact that the presence of the sera displaces the optimum for agglutination of the cells quite sharply to the reaction characteristic of the flocculation of the euglobulin added, or of that added last and in largest amount as in the case of the guinea pig serum, suggests the occurrence of a surface combination or condensation of the serum protein upon the surface of the red cell. The conclusion is supported by the observation of Porges[7] that bacteria which have been treated with such an excess of immune serum that agglutination does not appear, show the salt precipitation limits of the serum euglobulin and not those of the native bacteria.

The cells in the experiments reported here were not permeable to hemoglobin since hemolysis did not appear, and as will be mentioned later were very little permeable to inorganic ions, so that a penetration of the euglobulin into the interior of the cell does not seem possible. It would be difficult to explain furthermore how the small amount of serum protein relative to the mass of cells could give its own point of optimum flocculation to a mixture of cells and serum in any other way than as a surface deposition.

The euglobulin appears to be the active protein of the serum in combining with the cells.

A phenomenon based, as far as one may judge, upon precisely the same mechanism in the combination of a protein with a surface has been observed by Loeb.[8] This author has found that collodion membranes always acquire the characteristics of the protein with which

[7] Porges, O., *Centr. Bakt., Orig.* 1906, xl, 133.
[8] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 577.

they have been brought into contact, and that if such a membrane be treated with a solution of gelatin or oxyhemoglobin, for example, after the surplus protein has been washed away, the isoelectric point of the membrane is now that of the protein with which it has been treated.

This observation in connection with those reported here indicates the importance of factors which are non-specific in the serological sense in the mechanism of agglutination.

This view of the importance of the surface conditions in agglutination receives further support if the H ion concentration of the interior of the red cells be examined in relation to that of the fluid in which they are suspended This can be done by sedimenting the cells, removing the supernatant fluid and dissolving the cell sediment in a small amount of distilled water. Experiments which are shortly to be reported in full have shown that on the acid side of pH 7.4 at least, the reaction of the interior of fresh cells in a medium of low electrolyte content is maintained at a more alkaline level than that of the fluid outside. The following figures give the reactions of the outside fluid and of the dissolved cells in an experiment in which agglutination occurred at the two most acid reactions, although not promptly:

pH outside.	pH inside.
5.92	7.28
5.83	7.26
5.61	7.18
5.44	7.03
4.08	6.97

In numerous other experiments the suspending fluid has been brought very near pH 4.8 with the appearance of immediate agglutination, and the reaction of the dissolved cells found to lie between pH 7.20 and 6.8 if the reaction be measured within 15 minutes after the addition of acid.

The ionic state of the hemoglobin must be a large factor in the electric charge carried by the cell as a whole; the value pH 4.6, in the suspending fluid, as the isoelectric point of red blood cells[1] appears thus to correspond under the conditions of the determination with a

reaction within the cell of pH 6.8, which is the isoelectric point of hemoglobin.[9]

With older cells the reaction within the cell may be at pH 6.8 when that of the outside fluid is at the same point without the appearance of a trace of agglutination. The phenomenon of agglutination appears then to be related closely to an optimal reaction in the suspending fluid and probably of the cell membrane and not to a definite reaction in the interior of the cell.

CONCLUSIONS.

1. The addition of blood serum displaces the optimum for agglutination of red blood cells in a salt-free medium to the reaction characteristic of flocculation of the serum euglobulin.

2. This effect is not due merely to a mechanical entanglement of the cells by the precipitating euglobulin, since at reactions at which the latter is soluble it protects the cells from the agglutination which occurs in its absence.

3. A combination of some sort appears therefore to take place between sheep cells and sheep, rabbit, and guinea pig serum euglobulin, and involves a condensation of the serum protein upon the surface of the red cell.

4. At the optimal point for agglutination of persensitized cells both mid- and end-piece of complement combine with the cells.

5. Agglutination is closely related to an optimal H ion concentration in the suspending fluid, and probably of the cell membrane, and not to a definite reaction in the interior of the cell.

[9] Michaelis, L., and Takahashi, O., *Biochem. Z.*, 1910, xxix, 439. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xli, 102. Michaelis, L., and Airila, Y., *Biochem. Z.*, 1921, cxviii, 144.