

THE EQUILIBRIUM BETWEEN HEMOLYTIC SENSITIZER  
AND RED BLOOD CELLS IN RELATION TO  
THE HYDROGEN ION CONCENTRATION.

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INTRODUCTION.

The reversible nature of the union between various kinds of antigen and antibody in the usual physiological saline solution has been demonstrated many times.<sup>1-12</sup> Landsteiner and Jagić<sup>3</sup> have interpreted the reaction between antigen and antibody as a reversible reaction which is essentially similar to that taking place between simpler and definitely known chemical substances and in which an equilibrium is reached depending upon the concentration of the reacting substances and the temperature. Further evidence that suggests such an equilibrium state is given by Bail<sup>9</sup> and Matsui<sup>11</sup> in experiments on the splitting off of anticholera sensitizer. However, this point cannot be regarded as definitely proved by experiment.

That the chemical reaction, or the acidity or alkalinity, is a factor in the combination of antibody and antigen appears in any event

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<sup>1</sup> Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 1905.

<sup>2</sup> Morgenroth, J., *Münch. med. Woch.*, 1903, l, 61.

<sup>3</sup> Landsteiner, K., and Jagić, N., *Münch. med. Woch.*, 1903, l, 764.

<sup>4</sup> Landsteiner, K., and Jagić, N., *Münch. med. Woch.*, 1904, li, 1185.

<sup>5</sup> Landsteiner, K., and Reich, M., *Centr. Bakt., 1te Abt., Orig.*, 1905, xxxix, 83, 712.

<sup>6</sup> Bail, O., and Tsuda, K., *Z. Immunitätsforsch., Orig.*, 1908-09, i, 546.

<sup>7</sup> Tsuda, K., *Z. Immunitätsforsch., Orig.*, 1909, ii, 225.

<sup>8</sup> Spät, W., *Z. Immunitätsforsch., Orig.*, 1910, vii, 712.

<sup>9</sup> Bail, O., *Z. Immunitätsforsch., Orig.*, 1914, xxi, 202.

<sup>10</sup> Bail, O., and Rotky, K., *Z. Immunitätsforsch., Orig.*, 1913, xvii, 566.

<sup>11</sup> Matsui, I., *Z. Immunitätsforsch., Orig.*, 1915, xxiii, 233.

<sup>12</sup> von Liebermann, L., *Biochem Z.*, 1907, iv, 25; *Arch. Hyg.*, 1907, lxii, 277.

from a number of investigations. Von Liebermann<sup>12</sup> found that small amounts of alkali inhibit the action of hemolytic sera, while acid in small amount increases this action and in larger amount inhibits it. Hecker,<sup>13</sup> Sachs and Altmann,<sup>14</sup> and von Eisler<sup>15</sup> have confirmed this effect of alkali. Rondoni<sup>16</sup> has carried the analysis of this phenomenon farther and has shown the action of alkali in inhibiting the union of hemolytic sensitizer and red cells, and a similar but less marked effect of acid, which did not depend apparently upon destruction of the immune substance but rather upon a reversible modification of it.

The influence of reaction has been shown likewise on the dissociation or splitting off of antibody from combination with its antigen. Hahn and Trommsdorf<sup>17</sup> were able to separate agglutinin from sensitized bacteria by digestion with  $N/100$  NaOH and almost as well with  $N/100$  H<sub>2</sub>SO<sub>4</sub>, while physiological saline solution was found ineffective. Von Liebermann and von Fenyvessy<sup>18</sup> effected the separation of hemolytic sensitizer from sensitized cells by dilute H<sub>2</sub>SO<sub>4</sub>. Rondoni<sup>16</sup> found that digestion of sensitized cells with alkali or acid yielded a larger amount of free sensitizer than did a like volume of physiological saline solution. The separation in alkali was more complete than in acid; in several experiments with alkali approximately 60 per cent of the total sensitizer in combination was obtained free.

The presence of electrolytes appears also to be concerned in this combination. Although Ferrata<sup>19</sup> and Sachs and Teruuchi<sup>20</sup> have shown that sensitizer and cells will combine in a salt-free medium, von Eisler<sup>21</sup> found that the combination is less rapid and complete in salt-free than in salt-containing media. Kosakai<sup>22</sup> has found

<sup>13</sup> Hecker, R., *Arb. Inst. Exp. Therap. Frankfort*, 1907, iii, 39.

<sup>14</sup> Sachs, H., and Altmann, K., *Berl. klin. Woch.*, 1908, xlv, 699.

<sup>15</sup> von Eisler, M., *Centr. Bakt., 1te Abt., Orig.*, 1908, xlvi, 353.

<sup>16</sup> Rondoni, P., *Z. Immunitätsforsch., Orig.*, 1910, vii, 515.

<sup>17</sup> Hahn, M., and Trommsdorf, R., *Münch. med. Woch.*, 1900, xlvii, 413.

<sup>18</sup> von Liebermann, L., and von Fenyvessy, B., *Centr. Bakt., 1te Abt., Orig.*, 1908, xlvii, 274.

<sup>19</sup> Ferrata, A., *Berl. klin. Woch.*, 1907, xlv, 366.

<sup>20</sup> Sachs, H., and Teruuchi, Y., *Berl. klin. Woch.*, 1907, xlv, 467, 520, 602.

<sup>21</sup> von Eisler, M., *Z. Immunitätsforsch., Orig.*, 1909, ii, 159.

<sup>22</sup> Kosakai, M., *J. Immunol.*, 1918, iii, 109.

extraction with isotonic saccharose solution an effective means for separating sensitizer from cells.

Michaelis and Davidsohn<sup>23</sup> have endeavored to relate the phenomenon of specific agglutination to the electrical properties of the reacting substances, but state that typhoid bacilli and immune serum combine readily when the particles of both are negatively charged, so that their union cannot depend upon an affinity due to opposite electrical charge. They conclude further that specific typhoid agglutination and precipitation are independent of the hydrogen ion concentration—a conclusion which if applied to the general reaction between antigen and antibody would seem at variance with the facts related above.

We have shown in an earlier paper<sup>24</sup> that the agglutination of sensitized sheep cells has an optimum at pH 5.3 at which point the occurrence of agglutination is independent of the presence of electrolyte. This point does not coincide with the isoelectric point of the cells which was found for both the normal and sensitized cells to be about pH 4.65, and the suggestion was made that the optimum for agglutination is related to the isoelectric point of the immune serum. The present work is an outcome of the earlier investigation and is concerned with the relation of the hydrogen ion concentration to the union of hemolytic sensitizer and cells.

#### EXPERIMENTAL.

The methods employed were similar to those already described. To investigate the combination of sensitizer with cells in the absence, as far as possible, of electrolyte, sheep cells were washed in four changes of isotonic saccharose solution after washing in saline solution. The cells were made up to 10 per cent by volume of the concentrated sediment in saccharose solution. A series of eight to ten tubes was prepared, each containing 5 cc. of isotonic saccharose solution (9.2 per cent) and varying amounts by drop addition of N/10 NaOH or N/10 HCl. To each tube were then added precisely 0.1 cc. of undiluted immune rabbit serum and immediately afterward

<sup>23</sup> Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.

<sup>24</sup> Coulter, C. B., *J. Gen. Physiol.*, 1920-21, iii, 309.

5 cc. of 10 per cent cell suspension. The tubes were stoppered with paraffined corks, gently agitated, and kept in the water bath at 38°C. for 35 minutes, with gentle agitation every 5 minutes. The tubes were then centrifugated and the supernatant fluid was drawn off into two equal portions. To the first portion indicator was added and the pH determined colorimetrically by comparison with a standard series. The second portion served as a color screen for the standard tube. After the determination of the pH value, the first tube was titrated with  $N/100$  HCl or  $N/100$  NaOH to a pH between 6.5 and 6.0 and the amount of acid or alkali so required added to the second portion of test fluid. This portion was then diluted with saline solution and its content of sensitizer determined by titration in the usual way, with physiological saline solution as the medium. 0.04 cc. of guinea pig serum was used as complement. The cells used were 0.5 cc. of a 3 per cent suspension of the same sheep cells used in the first part of the experiment. The total volume of each tube was 2.0 cc. In consequence of the adjustment of the reaction of the test fluid and the buffer action of the complement added the hydrogen ion concentration of the tubes in this titration was sensibly constant. For each experiment the immune serum itself was titrated directly, using the same saline solution, complement, and cells. The hemolytic value was determined by interpolation, as, for instance, where with a 1:10 dilution of the supernatant test fluid the readings were 0.4 cc. complete, 0.35 cc. almost complete, 0.3 cc.  $\pm$ ; the value chosen was 0.375 cc. By this means and by the use of a 3 per cent cell suspension the error is within 5 per cent of the true value for any given reading. In the calculations the alteration in volume of the test fluid by the two additions of acid or alkali was taken into consideration.

The effect of electrolyte was determined by adding 1 or 2 cc. of physiological saline solution together with 4 or 3 cc. of saccharose solution to a series of tubes and then adding acid or alkali, sensitizer, and cells as before.

The dissociation of sensitizer from cells was investigated as follows: 5 cc. of concentrated cell sediment from saccharose were sensitized with approximately 50 units of sensitizer per unit of cells in a volume of 50 cc. Saccharose solution was used as the medium so that the

only electrolyte present was that added with the immune rabbit serum. The mixture of cells and serum was left standing for 30 minutes in the water bath at 38°C. and 1 hour in the refrigerator at about 8°C., then centrifugated, and the supernatant fluid drawn off. The pH of this was usually about 6.5. The cells were then washed once or twice in saccharose solution and made up to 10 per cent strength. From this point the procedure was the same as for the combination of sensitizer except that the addition of 0.1 cc. of sensitizer was omitted. The supernatant fluids from the sensitization and

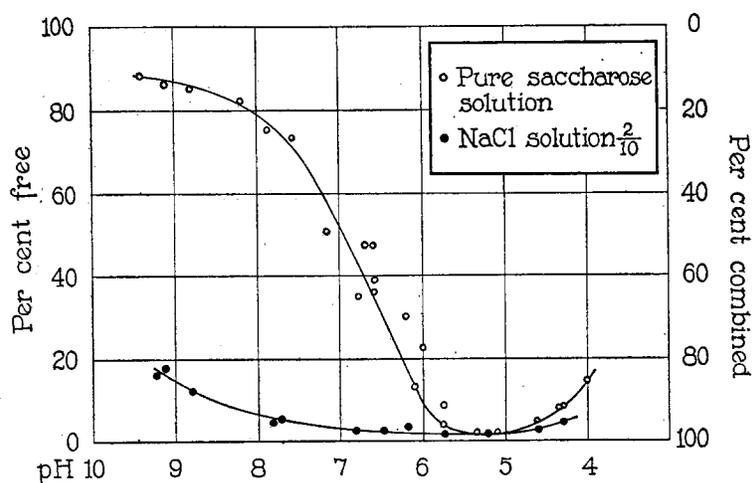


FIG. 1. Curve showing the proportion of the total sensitizer present either free or combined with cells, when the two combine *de novo*.

from the washing of the sensitized cells were titrated for their content of antibody and the sum of these values subtracted from the value of the immune serum itself. The remainder gives the amount of sensitizer actually in combination with the cells.

The results of both series of experiments are given in the form of curves, Fig. 1 for combination and Fig. 2 for dissociation. The abscissæ represent pH values, the ordinates the percentage of the total amount of sensitizer present either free or combined with cells. The curves show that near pH 5.3 the amount of sensitizer free in the supernatant fluid is at a minimum and the amount combined with the

cells is at a maximum. On the alkaline side of this point in the absence of electrolyte the percentage of sensitizer uncombined increases with the alkalinity and reaches a maximum of nearly 100 per cent at about pH 10. On the acid side of pH 5.3 the percentage of sensitizer uncombined increases with the acidity but somewhat less rapidly than for a corresponding increase in alkalinity.

It is impossible to carry the observations to reactions more acid than pH 4 on account of hemolysis. The fragility of heavily sensitized cells is well known; both normal and sensitized sheep cells

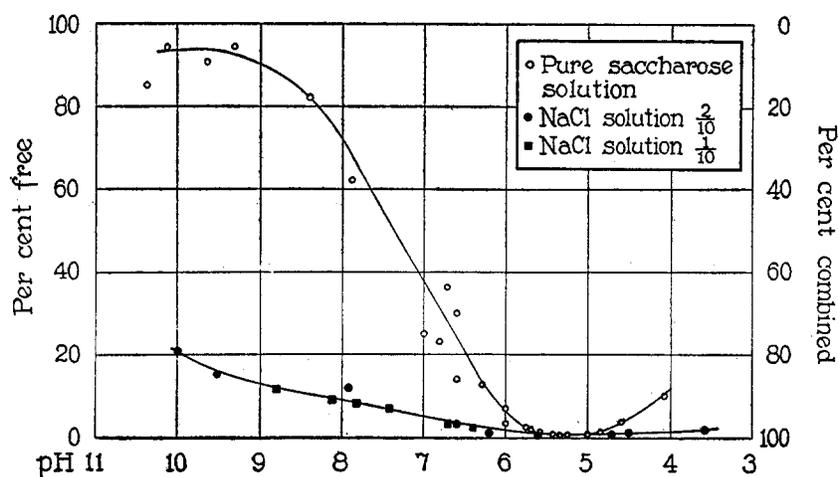


FIG. 2. Curve showing the proportion of the total sensitizer present either free or combined with cells, when the sensitizer dissociates from combination.

can endure, in the absence of electrolyte, a reaction of pH 4.5 without a trace of hemolysis provided they are not agitated. At this and more acid reactions a considerable degree of hemolysis can be caused by shaking or even gentle agitation. The agitation to which the cells were subjected while in the water bath caused a trace of hemolysis at all reactions; this hemolysis was apparently no greater at pH 10 than at pH 7.4, the normal reaction of the blood. Numerous observations were made on the acid side of pH 5 which are not recorded because of the difficulty in satisfactory colorimetric determination

of the pH value in consequence of hemolysis. All agree, however, in showing an increased dissociation with increased acidity.<sup>25</sup>

The presence of electrolyte as NaCl greatly increases the proportion of sensitizer combined with cells at all reactions except those near pH 5.3. At this point the combination of sensitizer with cells is independent of the presence of electrolyte. This recalls the observation, to which reference has already been made, that the agglutination of sensitized cells is independent of electrolyte at pH 5.3; it occurs as readily in the presence as in the absence of salt.

From a comparison of the two curves (Figs. 1 and 2) it is seen that they are almost identical. Since the volumes in the experiments are practically constant it is evident that, under the conditions both of combination *de novo* and of dissociation from combination, an equilibrium is established in a given volume between the amount of sensitizer free and that combined with cells, for any given hydrogen ion concentration.

The isoelectric point of serum globulin in which fraction the immune bodies are believed to be carried has been given by Rona and Michaelis<sup>26</sup> as about pH 5.4, and the isoelectric point of typhoid agglutinin has been found by Michaelis and Davidsohn<sup>23</sup> to lie near pH 5.2. It is probable that the hemolytic sensitizer used here has the same value. The point of maximal combination of sensitizer and cells coincides therefore with the isoelectric point of the sensitizer.

The amphoteric electrolytes, with which the immune bodies must be classed on the basis of their behavior in the electric field (Michaelis and Davidsohn;<sup>23</sup> Landsteiner and Pauli<sup>27</sup>), owe their electrical charge

<sup>25</sup> It was found that no destruction or irreversible modification of the sensitizer is brought about by the degrees of acidity or alkalinity reached in the experiments. A series of tubes, each containing 0.1 cc. of sensitizer in a volume of 10 cc. of saccharose, was brought to various reactions corresponding to those in the experiments with cells, and kept at 38°C. for 35 minutes. After centrifugation the supernatant fluids were adjusted in reaction and titrated for antibody content. No significant differences were found between any of the tubes; the differences were within the experimental error.

<sup>26</sup> Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193.

<sup>27</sup> Landsteiner, K., and Pauli, W., *25th Kong. Inn. Med.*, cited by Landsteiner, K., *Kolloide und Lipide in der Immunitätslehre*, in Kollo, W., and Wassermann, A., *Handbuch der Pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, ii, 1241.

to ionization. On the alkaline side of the isoelectric point they ionize as acids, on the acid side as bases; at the isoelectric point the ionization is at a minimum. It is evident that the combination of sensitizer and cells is related intimately to the ionization of the immune body. The curves showing the fraction of sensitizer free in solution in a salt-free medium follow closely the curves given by Sörenson,<sup>28</sup> after Michaelis,<sup>29</sup> to represent the degree of ionization of an amphoteric electrolyte. The ionized fraction of the sensitizer, both as anion and as cation, corresponds with that fraction which is uncombined with cells, so that we may conclude that the cells combine only with the undissociated molecules of sensitizer.

The ionization of the cells appears not to be a factor in their combination with sensitizer. There is no inflection in the curves at pH 4.6, the isoelectric point of the cells. At pH 5.3, the reaction at which the maximal amount of sensitizer is combined, the cells are considerably ionized as is demonstrated in the curve showing their rate of movement in the electric field.<sup>24</sup>

On the alkaline side of the isoelectric point proteins combine only with cations.<sup>30</sup> In the presence of NaCl, a Na salt could be formed therefore with the immune body at reactions more alkaline than pH 5.3. If this salt had a small dissociation constant, so that only a small concentration of ampholyte anion could exist in the presence of Na without combining to form undissociated salt, the degree of ionization of the ampholyte would be represented by such a curve (Michaelis<sup>31</sup>) as that showing the proportion of sensitizer uncombined in the presence of NaCl. While we possess no information as to the degree of dissociation of such a Na sensitizer salt, the effect of NaCl is at least suggestive of a depression in the ionization of the sensitizer with combination between the cells and all undissociated molecules of sensitizer, either pure or united with cation to form a salt.

<sup>28</sup> Sörenson, S. P. L., *Ergebn. Physiol.*, 1912, xii, 393.

<sup>29</sup> Michaelis, L., *Biochem. Z.*, 1911, xxxiii, 182.

<sup>30</sup> Loeb, J., *J. Gen. Physiol.*, 1918-19, i, 39, 237, 363, 483, 559.

<sup>31</sup> Michaelis, L., *Biochem. Z.*, 1920, ciii, 225.

## CONCLUSIONS.

1. In a salt-free medium the proportion of the total amount of hemolytic sensitizer present, combined with the homologous cells, reaches a maximum of almost 100 per cent at pH 5.3. On the alkaline side of this point the proportion combined diminishes with the alkalinity and reaches a minimum of approximately 5 per cent at pH 10. On the acid side of pH 5.3 the proportion combined diminishes with the acidity but somewhat less rapidly than for a corresponding increase in alkalinity.

2. The presence of NaCl greatly increases the proportion of sensitizer combined with cells at all reactions except those in the neighborhood of pH 5.3. At this point the combination of sensitizer with cells is independent of the presence of electrolyte.

3. The curves representing the proportion of sensitizer combined or free run almost exactly parallel, both when the sensitizer combines *de novo* and when it dissociates from combination; therefore, in constant volume, at a given hydrogen ion concentration, and at a given temperature, an equilibrium exists between the amount of sensitizer free and that combined with cells.

4. The combination of sensitizer and cells is related fundamentally to the isoelectric point of the sensitizer.

5. The dissociated ions of the sensitizer, formed either by its acid or its basic dissociation, do not unite with cells. Combination takes place only between the cells and the undissociated molecules of the sensitizer.