HEMOLYSINS IN ACQUIRED HEMOLYTIC ANEMIA

Effect of pH on the Activity in Vitro of a Serum Hemolysin

By J. V. Dacie, M.B., M.R.C.P. London

Hydrogen ion concentration has a controlling effect upon many hemolytic systems, both simple and complex. Osborn in 1934 reviewed the early literature on the effect of pH on the hemolysis by complement of corpuscles sensitized by hemolytic immune body. He found that the optimum reaction for the hemolysis of sheep corpuscles by guinea pig serum was about pH 7.5 with inhibition below pH 5.5 and above pH 9.7. More recently, Seifter et al. have reported unimpaired activity of human complement between pH 6.1 and 8.4 and irreversible and rapid destruction below pH 4.2 and above pH 10.1.

The effect of pH or carbon dioxide concentration on the activity in vitro of hemolytic antibodies of human origin has seldom been considered except in the case of chronic hemolytic anemia with nocturnal hemoglobinuria (Ham, Dacie and Richardson), in cold hemoglobinuria where the adjuvant effect of carbon dioxide on hemolysis has been sometimes referred to (Van den Bergh, Hannema and Rytma, Wagley, Zinkham and Siebens) and in a case of acute hemolytic anemia in infancy reported by David and Minot.

In the present communication are reported observations on the activity in vitro of an abnormal hemolysin in the serum of a patient with idiopathic acquired hemolytic anemia, and the effect of pH on its action. It was found that although little or no hemolysis resulted when normal Group O corpuscles were suspended in unacidified patient’s serum (pH 8.0), hemolysis readily took place if the pH of the serum-corpuscle suspension was adjusted to an optimum (pH 6.8 to 7.0) by the addition of suitable volumes of acid. If graded amounts of acid were added to serum it could be shown that the range of pH within which hemolysis could be observed corresponded quite closely to that found in chronic hemolytic anemia with nocturnal hemoglobinuria (Dacie and Richardson). In the final section of this paper, these observations are contrasted with the pH ranges for the hemolysis by complement of erythrocytes sensitized by anti-A or anti-B iso-hemolysin and of group O erythrocytes sensitized by a “cold” hemolysin present in the serum of a patient with cold hemoglobinuria.

General Technical Methods*

Serum was obtained by defibrinating blood around a roughened glass rod in a conical flask. The pH of the serum was approximately 8.0. Serum intended as a source of complement was used within three hours of collection and stored frozen until utilized.

The pH of the serum was modified by the addition of 10 per cent by volume of N/5, N/10 or N/20 NaOH or N/20, N/10, N/5, N/4, N/3.5, N/3 or N/2.5 HCl.

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* Other technical details are given in footnotes to table 1.
Erythrocyte suspensions were prepared from packed saline-washed corpuscles and used at final concentrations of 2 per cent or 5 per cent by adding to the (acidified) serum 10 per cent by volume of a 10 per cent or 50 per cent suspension.

Serum-corpuscle suspensions were generally incubated in a water bath at 37°C for 30 minutes. Hemolysis was measured photoelectrically after diluting in N/100 NaOH volumes of the supernatants obtained after centrifuging the corpuscle-serum suspensions.

The pH of the corpuscle-serum suspensions was measured by a glass electrode at the end of the period of incubation and after resuspending the corpuscles, or, when only small volumes of serum were available, by the use of indicators (phenol red, bromthymol blue or methyl red).

Summary of Clinical History and Routine Laboratory Findings

Miss B., aged 18. Idiopathic acquired hemolytic anemia

Splenectomy had been performed for hemolytic anemia approximately five years before the present series of observations was made. The cause of the original hemolytic attack was uncertain; the family history did not suggest a familial incidence.

The patient was admitted into a hospital in London in December 1946, severely ill with signs of intense hemolysis. Repeated blood examinations then revealed a severe macrocytic anemia; the erythrocyte count was 1.0 to 1.5 million per cu. mm., with 3.5 to 5.5 Gm. hemoglobin; there was a high reticulocytosis (25 to 50 per cent) and a raised mean corpuscular volume (up to 160 cu. micra). In films of peripheral blood there were occasional normoblasts, much polychromasia and postsplenectomy basophilic stippling, and rarely instances of erythrophagocytosis by mononuclear cells. The Coombs test was positive and cold autohemagglutinins were present to a titer of 1:512 at 2°C; there was just perceptible autohemagglutination at 37°C. The plasma bilirubin level was continuously raised (up to 4 mg. per 100 ml.) and there was a slight increase in plasma globulin (albumin 4.0 Gm.; globulin 3.3 Gm. per 100 ml.).

Hemolysis continued at an extremely rapid rate with only minor fluctuations, and blood transfusions were only of transient benefit. Hemoglobinuria was generally absent, but was observed on several occasions after transfusions. Data obtained by the differential agglutination technic (Dr. J. F. Loutit) confirmed that the transfused blood was very rapidly eliminated. The patient died in April 1947.

Nature of the Serum Hemolysin

Samples of the patient's blood were investigated on several occasions between January and March 1947.* It was repeatedly found that normal group O erythrocytes and the patient's own corpuscles underwent hemolysis in vitro in the patient's serum. The hemolytic antibody seemed to be distinct from the cold hemagglutinin antibody and was absorbed on to corpuscles better at 37°C than at lower temperatures. The amount of hemolysis was largely determined by the pH of the cor-

* I am indebted to Dr. J. F. Loutit for blood from this patient and to Dr. J. F. Hawkesley for details of the clinical history.
Hemolysins in Acquired Hemolytic Anemia

Table I
For Procedures and Remarks, see below

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Serum</th>
<th>Corpuscles</th>
<th>Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a.</td>
<td>Patient*</td>
<td>Patient</td>
<td>Nil.</td>
</tr>
<tr>
<td>b.</td>
<td>Patient*</td>
<td>Normal (O)</td>
<td>5</td>
</tr>
<tr>
<td>c.</td>
<td>Acidified patient's serum</td>
<td>Patient</td>
<td>3</td>
</tr>
<tr>
<td>d.</td>
<td>Acidified patient's serum</td>
<td>Normal (O)</td>
<td>60</td>
</tr>
<tr>
<td>2a.</td>
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<td>Normal (O)</td>
<td>10</td>
</tr>
<tr>
<td>b.</td>
<td>Inactivated acidified patient's serum</td>
<td>Normal (O)</td>
<td>6</td>
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<td>3</td>
</tr>
<tr>
<td>3.</td>
<td>Inactivated acidified patient's serum</td>
<td>Normal</td>
<td>(a) 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(b) 70</td>
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<tr>
<td>4a.</td>
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<td>Normal</td>
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</tr>
<tr>
<td>b.</td>
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<td>Normal</td>
<td>2</td>
</tr>
<tr>
<td>c.</td>
<td>Fresh acidified patient's serum</td>
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<td>d.</td>
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<td>50</td>
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<tr>
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<td>Patient</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(2) Nil.</td>
</tr>
<tr>
<td>b.</td>
<td>Inactivated acidified patient's serum</td>
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<tr>
<td></td>
<td></td>
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<td>(2) 25</td>
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<tr>
<td>c.</td>
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<td>Patient</td>
<td>(1) 10</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>(2) 10</td>
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<tr>
<td>d.</td>
<td>Inactivated acidified patient's serum</td>
<td>Normal</td>
<td>(1) 55</td>
</tr>
<tr>
<td></td>
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<td>(2) Nil.</td>
</tr>
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</table>

1 a, b, c and d: Procedure: The corpuscle-serum suspensions were centrifuged after 30 minutes at 37 C. Remarks: Demonstrates the effect of pH on the hemolysis of the patient's corpuscles and of normal group O erythrocytes. The patient's own corpuscles are less sensitive than are the normal erythrocytes.

2: Procedure: (a): The corpuscles were sensitized in the patient's serum for 30 minutes at 37 C. The suspension was then centrifuged; the deposited corpuscles were washed in warm saline and re-suspended in fresh normal acidified serum and incubated at 37 C for a further 30 minutes. (b): Same as (a), but the corpuscles were sensitized in the patient's serum at 16 C. (c): Same as (a), but the corpuscles were sensitized in the patient's serum at 2 C. Remarks: Demonstrates that the hemolysin is less readily absorbed at temperatures below 37 C.

3: Procedure: The corpuscle-serum suspension was incubated for 30 minutes at 37 C, then centrifuged, and the corpuscles washed once in warm saline. The sensitized corpuscles were then divided into two equal portions, (a) and (b). To (a) was added a volume of heated normal serum at its natural pH (8.0); to (b) was added heated acidified normal serum (pH 7.0). Both tubes were held at 37 C for 30 minutes; then centrifuged and fresh acidified normal serum added to the deposited corpuscles (pH 7.0 approx.), and the suspensions incubated at 37 C for a further 30 minutes. Remarks: Shows that the hemolysin is absorbed best at a relatively acid reaction and may be liberated from the corpuscles into serum of a more alkaline reaction (a).

4: Procedure: (a): The corpuscle-serum suspension was incubated at 37 C for 30 minutes, then centrifuged. To the deposit was added absorbed guinea pig serum. The tube was incubated at 37 C for one hour. (b) Unsensitized normal corpuscles were suspended in absorbed guinea pig serum and incubated at 37 C for one hour (control for a). (c) Incubated at 37 C for one hour. (d) Same as (c), but with the addition of absorbed guinea pig serum. Remarks: (a and b): Sensitized normal corpuscles are hemolyzed by fresh guinea pig serum complement. (c and d): Hemolysis is increased in the presence of additional guinea pig serum complement.

5: Procedure: (a): (i) The suspension was incubated at 37 C for 30 minutes, then centrifuged. Acidified fresh normal serum was added to the deposit and the tube incubated at 37 C for one hour.
puscle-serum suspension; hemolysis was maximal at about pH 6.8 to 7.0 and was inhibited below pH 6 and above pH 8, and there was but a trace of hemolysis in unacidified serum. This restricted pH-hemolysis range seemed due to the hemolysin being poorly absorbed at the alkaline side of neutrality, and it was demonstrated that hemolysin absorbed at the optimum pH was liberated again if the sensitized corpuscles were suspended in a more alkaline serum. The antibody was found to be thermostable and withstood heating to 56 C. for thirty minutes. Complement was required for hemolysis and either fresh human serum or guinea pig serum was satisfactory. The titer of the hemolysin (determined against normal corpuscles under what was thought to be optimum conditions) was 1:8 (final serum dilution).

Absorption experiments showed that normal corpuscles absorbed hemolysin active against patient's corpuscles and vice versa, and there seemed to be no difference in sensitivity to the hemolysin between the patient's immature corpuscles (reticulocytes) and her mature erythrocytes. Repeatedly, the patient's corpuscles were shown to be less sensitive to hemolysis than were normal erythrocytes.

Some of the data on which the above description is based are recorded in table 1.

**DISCUSSION**

Although the cause was obscure there can be little doubt as to the nature of the disorder from which the subject of this report was suffering; the negative family history, the severe anemia and high reticulocytosis, the presence of cold hemagglutinins, the positive Coombs test, the relapse after splenectomy and the transient benefit of blood transfusions due to a rapid elimination of the transfused corpuscles, and the presence of an abnormal auto- and isohemolysin in the serum all indicate a severe idiopathic acquired hemolytic anemia.

The presence in the patient's serum of an abnormal hemolysin is the most unusual feature and has seldom been observed. It is probably only in the most severe forms of hemolytic anemia when autoantibodies are being formed in large quantities that hemolysin of the abnormal type is seen. Further patient's corpuscles were added to supernatant. The suspension was centrifuged after 30 minutes; fresh acidified normal serum was added to the deposited corpuscles and the tube incubated at 37 C for one hour. (b) Same as (a) except that normal corpuscles were used throughout. (c): Same as (a), except that normal corpuscles were used in the second stage of the experiment to test for the absorption of the hemolysin by the patient's corpuscles. (d): Same as (a), except that patient's corpuscles were used in the second stage of the experiment to test for the absorption of the hemolysin by the normal corpuscles. Remarks: Demonstrates the cross absorption of hemolysin between patient's and normal corpuscles, and the relative insensitivity of patient's corpuscles compared with the normal.

* Serum not acidified (pH approximately 8.0).
† Serum acidified by the addition of 10 per cent by volume of N/4 HCl. The pH after the addition of the corpuscles and incubation at 37 C. for 30 minutes was approximately 7.0.
‡ Serum inactivated by heating to 56 C. for 30 minutes; acidified with 10 per cent by volume of N/4 HCl after inactivation.

Fresh guinea pig serum was absorbed with equal volumes of washed normal human corpuscles for 30 minutes at 2 C. The serum was used at a final dilution of 1 in 5.
amounts that there is sufficient for detention in the serum over and above that absorbed on to the patient's own corpuscles; this probability, and the fact that adjustment of pH to an optimum for hemolysis is important in the demonstration of hemolysins of the type now described, perhaps accounts for the fact that observations similar to the present have seldom been reported.

In France, however, about forty years ago, the role of hemolysins in acute hemolytic anemia was well recognized (Chauffard and Troisier, Chauffard and Vincent), and these early papers and some others are referred to by Dameshek and Schwartz in their review of acute hemolytic anemia. Following these early papers, however, the association of hemolysins and acute hemolytic anemia seems to have been forgotten until in 1938 Dameshek and Schwartz published 3 cases of their own. More recent reports are those of Farrar, Burnett and Steigman, David and Minot, Neber and Dameshek, and of Ellis, Wollerman and Stetson. Only in the report of David and Minot has the effect of pH on the demonstration of hemolytic activity been investigated. These authors observed a substantial increase in hemolysis when the corpuscles were suspended in serum acidified with 5 per cent N/3 HCl instead of in unacidified serum, in one instance an increase from 47 to 111 mg. in the concentration of liberated hemoglobin.

As has already been mentioned, the effects of pH on the action of guinea pig

Fig. 1.—On the left, A (continuous line), a pH-hemolysis curve for the hemolysis of normal corpuscles by the patient's (Miss B's) serum and B (interrupted line) the effect of pH on the absorption of the hemolysin; there was only a trace of hemolysis in unacidified serum, indicated by the black arrow, due to absorption being inhibited by increasing alkalinity.

On the right, C (interrupted line), the effect of pH on the absorption of a "cold" hemolysin present in the serum of a patient suffering from "cold" hemoglobinuria, and D (interrupted line), the absence of any effect of pH on the absorption of anti-A (or anti-B) isohemolysins. Curve B is reproduced for comparison.
and human serum complement are well recognized. In the present instance, there was evidence that the absorption of antibody was also controlled by pH, and that it was this effect which was responsible for the comparatively restricted pH range between which hemolysis could be demonstrated.

It was of interest to contrast the behavior of this patient's hemolysin with two other types of antibody, the anti-A and anti-B isoheomolysins and a "cold" hemolysin from a patient suffering from "cold" hemoglobinuria. The effect of pH on the absorption of these three types of hemolytic antibodies is indicated in figure 1. The left hand curve (A) represents the pH range within which hemolysis of normal corpuscles by Miss B's serum could be demonstrated; the range was approximately pH 6 to pH 8 with an optimum about pH 7. Curve B represents the effect of pH on the absorption of the hemolysin, i.e., the amount of hemolysis observed when corpuscles sensitized in inactivated serum at a range of pH between 6 and 9 were subsequently resuspended and incubated in fresh normal serum at pH 7. The absorption of antibody diminished with increasing alkalinity, and

* Washed normal erythrocytes were suspended at a final concentration of 5 per cent in volumes of patient's inactivated serum whose pH had been adjusted from 9 to 6 by the addition of 10 per cent by volume of HC1 ranging in strength from N/20 to N/7.5 and NaOH ranging in strength from N/20 to N/5. The corpuscle-serum suspensions were centrifuged after thirty minutes at 37 C. and the deposited corpuscles washed once in saline warmed to 37 C. Finally, volumes of fresh normal serum at pH 7.0 were added to the deposited corpuscles and the tubes incubated at 37 C. for 30 minutes. The amount of hemolysis in each tube was dependent upon the amount of hemolysin absorbed in the first stage of the experiment.

![Figure 1](https://example.com/figure1.png)
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...it is this fact that probably reduced to a mere trace the amount of hemolysis caused by unacidified serum. In the right hand diagram in figure 1, curve B is reproduced again. Curve C represents the effect of pH on the absorption of the "cold hemolysin" and the line D shows that pH has no effect on the absorption of anti-A (or B) isohemolysin. In figure 2 are shown as a contrast to curve A of figure 1, pH-hemolysis curves (the summation of effects of pH on the absorption of the antibody and upon the action of human serum complement) for the hemolysis of normal corpuscles by anti-A and anti-B isohemolysins (curves A and B) and by the cold hemolysin (C). The black arrows indicate the amount of hemolysis produced by unacidified serum at approximately pH 8, and show that this is almost maximal. In the case of curves A and B (fig. 2), the relatively wide range of pH within which hemolysis will take place is due to pH affecting the activity of serum complement alone and not the absorption of the antibody. The range for the cold hemolysin (curve C) is slightly more restricted on the alkaline side; in this case, there is some impairment of absorption of antibody between pH 8 and 9. It is noteworthy that the pH range for the action of Miss B's hemolysin quite closely corresponds to the pH range within which the erythrocytes from patients with nocturnal hemoglobinuria will undergo hemolysis in normal serum (Dacie and Richardson).

It is remarkable that the effect of pH on the activity of the three different types of hemolysins described in this paper was different in each case. Such differences no doubt reflect subtle differences in the composition of the protein complexes concerned. From the practical point of demonstrating the hemolytic nature of these antibodies in vitro, the effect of pH cannot altogether be disregarded.

SUMMARY

The presence is recorded of an abnormal hemolysin in the serum of a patient with severe acquired hemolytic anemia. Its activity in vitro was determined by the pH of the corpuscle-serum suspension; the optimum pH was about 6.8 to 7.0 and there was inhibition above pH 8 and below pH 6. This pH range is contrasted with that of other human serum hemolytic systems; it is similar to that found in nocturnal hemoglobinuria.

REFERENCES


HEMOLYSINS IN ACQUIRED HEMOLYTIC ANEMIA: EFFECT OF PH ON THE ACTIVITY IN VITRO OF A SERUM HEMOLYSIN

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