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Received March 31, 1954. P.S.E.B.M., 1954, v85.

Role of Magnesium Ions in Donath-Landsteiner Hemolytic Reaction of Paroxysmal Cold Hemoglobinuria.* (20981)

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The effect of certain metallic ions on complement activity has attracted considerable attention in recent years(1-5). The activity of guinea pig complement against sensitized sheep erythrocytes has been shown to depend on the concentration of Mg^{++} used in the reagents(1,6,7), and it has been stated(5) that calcium as well as magnesium is essential for this immune hemolysis. Other investigators, however, have found that these ions enhance but are not essential for the activity of rabbit(8) or human complements(9). While studying the mechanism of hemolysis in paroxysmal nocturnal hemoglobinuria, Harris, Jordan, Pillemer and Desforges(10) found that Mg^{++} is essential for the activity of the serum factor, or factors, which hemolyse the abnormal erythrocytes. No immune mechanism, however, has been demonstrated in this unusual human hemolytic system(11,12). Accordingly, the role of magnesium was studied in another human hemolytic system, that of paroxysmal cold hemoglobinuria (PCH), in which hemolysin and complement are involved.

The PCH system is unique among the hemolytic systems in that it requires the presence of complement both for antibody fixation in the cold phase of the Donath-Landsteiner reaction as well as for hemolysis in the warm phase(13). The experiments described in this paper examine the part that Mg^{++} plays in these two phases. The sheep cell hemolytic system was used to demonstrate any change which might have occurred in complement activity when Mg^{++} was removed from guinea pig serum by various methods.

Materials and methods. PCH sera. Sera from three patients with paroxysmal cold hemoglobinuria were used. Case No. 1 and case No. 2 have been described previously (13). Case No. 3 was seen in February, 1952, at Crile Veterans Administration Hospital through the courtesy of Dr. Norman Shumway. This 30-year-old colored male had had episodes of cold hemoglobinuria since 1945. Despite a number of courses of penicillin, he was still having frequent episodes of hemoglobinuria. Kahn and Kolmer tests were positive. The Donath-Landsteiner hemolysin titer was 1:4; the PCH antibody titer measured by the indirect Coombs test was 1:16. *Complement titration.* Lyophilized guinea pig serum was used, and was titered with a modification of the method described by Kabat and Mayer(14), using the Coleman Jr. spectrophotometer. The 50% unit of complement was determined by plotting the hemolysis curve on logarithmic paper. All titrations were made in duplicate, and repeated titration showed that the results were reproducible within 5%. *Buffer.* Barbital buffer(1) con-

* This investigation was supported in part by grants from the Brush Foundation, and Mr. Philip R. Mather.

† Study done during tenure of Crile Fellowship.

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taining 1.5 x 10^{-3} M $CaCl_2$, but no Mg^{++}, was used in those experiments designed to test the effect of Mg^{++} depletion. To restore Mg^{++}, similar buffer containing 5 x 10^{-4} M $Mg\ Cl_2$ was used. *Removal of Mg^{++} from reagents.* Mg^{++} was removed from the serum specimens by dialysis, by resin treatment, or by a combination of both procedures. *I. Dialysis.* The serum was placed in cellulose tubing and dialysed against phosphate-buffered saline (pH 7.2) for 24 hours at 4°C, 100 ml of buffer being used for each 1 ml of serum dialysed.

II. Ion-exchange treatment. The sodium cycle of the cation exchange resin, Amberlite IRC-50, was used in all the ion-exchange experiments. Two methods of treatment were used. *A. Ion-exchange column.* A resin column was formed by drawing a narrow neck in a piece of 15 mm glass tubing. A small piece of glass wool was then placed in this neck and a suspension of the resin was poured on the wool. The tube was then placed in an upright position, so that the resin would form an even bed over the glass wool. One half ml of 50% resin in distilled water was used for each 1 ml of serum to be treated, and the column was washed three times with buffered saline before use. The exchange process was carried out at 4°C without suction. *B. Dialysis in presence of ion-exchange resin.* The serum was prepared as previously described for dialysis, but 0.5 ml of the 50% resin suspension was added to each 100 ml of buffered saline and dialysis carried out in the presence of resin for 24 hours at 4°C. The resin was kept in suspension by using a magnetic stirrer throughout the treatment procedure. *Quantitative magnesium determination.* Mg^{++} was determined by a modification of the titan yellow photocolorimetric method described by Orange and Rhein(15). By this method concentrations of between 0.065 μM and 1.0 μM per ml can be determined. *The Donath-Landsteiner test.* A 2.5% suspension of human "O" erythrocytes was standardized to give an optical density of 0.54 when 0.5 ml of the cell suspension was lysed with 2.5 ml of distilled water. This suspension was prepared daily from "O" cells stored in Alsever's solution for not longer than 3 days. For the test the following reagents were added to a tube in an ice bath: 1) 0.25 ml PCH serum; 2) 0.25 ml guinea pig serum (diluted 1:2); 3) 0.50 ml 2.5% "O" cell suspension. Following the addition of the reagents the tubes were shaken, chilled in an ice bath for 30 minutes, and then immediately placed in a 37°C water bath for 30 minutes. Following this incubation, 2.0 ml of barbital buffer was added to each tube, and after centrifugation, the supernates were read for hemolysis in 10 x 75 mm cuvettes at 550 mμ in the Coleman, Jr. spectrophotometer. *Indirect Coombs test.* This test was used in an attempt to quantitate the amount of antibody absorbed by the red cells. The method used was a slight modification of the method previously described [indirect (a)](13).

Results. Effect of Mg^{++} removal on C' titer. Guinea pig serum was treated in 3 ways to determine the method which removed the most Mg^{++} with the least destruction of complement lytic activity. Table I shows the results of such a series of treatments using three different complement pools. The barbital buffer with Mg^{++} was found to contain 0.65 μM of Mg^{++} per ml, and that buffer without Mg^{++}, no measurable amount of Mg^{++}. Thus it is apparent that with the complement diluted 1:100 or 1:50, the buffer and not the complement would become the principal factor in controlling the amount of Mg^{++} present in the titration. For this reason it was felt safe to assume that in the titrations in the presence of Mg^{++} the concentration was about 0.65 μM per ml, and in the titration in the absence of Mg^{++} there was none or at least less than 0.065 μM per ml. Therefore, there was at least 10 times as much Mg^{++} present in the titrations with Mg^{++} as there was in those lacking this ion. An increase in the Mg^{++} concentration above 0.65 μM per ml failed to enhance the complement titer.

Of the various methods used to remove Mg^{++} from serum, passage through a resin column was found to be the most destructive to complement activity even after Ca^{++} and Mg^{++} had been restored to optimum levels. Dialysis in the presence of a resin was found to be less destructive of complement activity,

TABLE I. Effect of Mg^{++} Removal on Complement Titer of Guinea Pig Serum.

Serum pool	Serum treatment	μM of Mg^{++} /ml undiluted serum	Mg^{++} in reagents .65 μM/ml*	<0.065 μM/ml $C'H_{50}$ units/ml	% reduction due to Mg^{++} removal
1	Untreated	1.01	455	327	28
	Resin exchanged	.13	323	323	0
	Dialysed	.47	394	289	27
	Dialysed with resin	.14	361	314	13
2	Untreated	1.06	565	413	27
	Resin exchanged	.12	378	269	29
	Dialysed	.55	459	335	27
	Dialysed with resin	.12	429	300	30
3	Untreated	.61	526	436	17
	Dialysed with resin	.14	343	258	25

* Mg^{++} in the reagents restored Mg^{++} concentration in all specimens to essentially same level. Thus differences in titer between treated and untreated specimens reflect effect of treatment procedure.

but as efficient as the resin column in removing Mg^{++}. Dialysis against buffered isotonic saline was the least destructive of complement activity, but also removed less Mg^{++} than the other methods used. In subsequent experiments, therefore, Mg^{++} was removed from serum by dialysis in the presence of resin.

Effect of Mg^{++} on PCH hemolysis. When at least 90% of the Mg^{++} was removed from the reagents used in the Donath-Landsteiner test, the amount of hemolysis was reduced by amounts varying between 45 to 86% (Table II). When de-ionized human serum was used as a complement source for the serum from case 2 there again was but a slight decrease in the amount of hemolysis. In the PCH hemolytic system, this decrease could have been caused by a reduction in the activity of the lytic components of complement or by a failure of the antibody to unite properly with the red cells.

The indirect Coombs test was used in order to measure the quantity of antibody fixed to the erythrocytes. When reagents lacking Mg^{++} were used, the indirect Coombs titer decreased 4-fold in 2 cases and 2-fold in one case, indicating a reduction in the amount of PCH antibody fixed to the red cells. Since this reduction might have been caused by damage to the antibody during treatment, the Donath-Landsteiner test was set up using PCH serum which had been dialysed with resin, all other reagents containing their original amounts of Mg^{++}. When this was done, it was found that the amount of hemolysis returned to within 10% of the value found prior to treatment of the serum. Thus, the reduction in the amount of PCH antibody fixed as well the decrease in the degree of hemolysis may be attributed to the effect of lack of Mg^{++} on complement activity.

It has been demonstrated that PCH hemolysis depends upon the presence of complement in both the cold and warm phases of the Donath-Landsteiner reaction(13). Accordingly, the need for Mg^{++} during the two phases was investigated, by washing erythrocytes once with a large volume of cold buffer after chilling in complement and sera from case No. 2 or case No. 3 and then adding new lots of complement prior to incubation at 37°C. When Mg^{++} was present in both phases, 93 to 97% hemolysis was obtained. The amount of hemolysis was reduced slightly to 84 to 87% when Mg^{++} was lacking in either the cold phase or the warm phase. As noted previously, hemolysis occurred even though Mg^{++} was absent in both phases.

Discussion. In the experiments reported above the amount of Mg^{++} in the reagents used for complement titration was reduced to very low levels while the amount of calcium was kept constant. Reduction in the amount of Mg^{++} did not prevent hemolysis from occurring in the sheep cell system. Although Mg^{++} was removed to such an extent that it could no longer be detected, the complement titer of guinea pig serum was reduced at most by about 30%. In a hemolytic sys-

TABLE II. Effect of Mg^{++} on PCH Hemolysis.

Case No.	Mg^{++} added		Mg^{++} removed		% reduction due to Mg^{++} removal
	μM Mg^{++}/ml*	% hemolysis	μM Mg^{++}/ml*	% hemolysis	
1 (Serum 1:2)	1.01	86	.052	12	86
2	.84	70	.044	35	50
3	.77	88	.075	48	45

* These values were calculated from quantitative magnesium determinations made on the reagents and thus represent the actual amount of magnesium in the test.

tem free of both Ca^{++} and Mg^{++}, Pillemer, Blum, Pensky and Lepow(9) found that the complement titer of human serum was decreased about 50%. The present study is in agreement with these results in indicating that while Mg^{++} enhances complement activity, it is not an absolute requirement for immune hemolysis.

In the PCH system Mg^{++} removal was not as complete, for in this system both human serum (PCH antibody) and guinea pig serum (complement) had to be used in low dilutions (13). The amount of hemolysis, however, was reduced to a greater extent than in the sheep cell system. This is not a surprising result as it is known that a large amount of complement is required for the lysis of human erythrocytes sensitized with PCH antibody (13). Further, it was shown that suboptimal amounts of Mg^{++} decreased slightly the amount of PCH antibody fixed on the red cells in the cold phase of the Donath-Landsteiner reaction. The addition of reagents containing Mg^{++} after the cold phase reduced the effect of a lack of Mg^{++} in the cold phase, but did not eliminate it. When Mg^{++} was present in the cold phase and reagents lacking Mg^{++} were used in the warm phase, the amount of hemolysis was less than obtained when Mg^{++} was present throughout. Thus Mg^{++} does not appear to be essential for hemolysis in the PCH hemolytic system, although reduction in the concentration of Mg^{++} decreases the activity of complement in both the cold and warm phases of the Donath-Landsteiner reaction.

Summary. 1. Mg^{++} was removed from guinea pig and human sera by dialysing the serum in the presence of an ion-exchange resin. 2. Complete or almost complete removal of Mg^{++} from reagents used in the sheep cell hemolytic system, with the concentration of Ca^{++} being maintained, diminished the complement titer of guinea pig serum by about one-fourth. 3. Antibody fixation was only slightly reduced when Mg^{++} was lacking in the cold phase of the Donath-Landsteiner reaction. Maximum hemolysis was obtained when Mg^{++} was present in both the cold and warm phases. 4. Mg^{++} does not appear to be essential for hemolysis in the Donath-Landsteiner reaction of paroxysmal cold hemoglobinuria.

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Received March 12, 1954. P.S.E.B.M., 1954, v85.