THE ACTIVATING EFFECT OF MAGNESIUM AND OTHER CATIONS ON THE HEMOLYTIC FUNCTION OF COMPLEMENT*

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Efforts to arrive at an understanding of the mechanism of immune hemolysis, whether or not based on the assumption of an underlying enzymic process, have included studies on the influence of numerous salts on complementary activity. The result has been disagreement as to the importance and function of even so ubiquitous an ion as calcium (1–3), an essential inorganic constituent of serum. Complicating all such studies is the enhancing action of some metallic ions, particularly magnesium, in low concentrations, on the lysis of sensitized red cells by complement, and the inhibiting effect of the same ions at higher concentrations (4–6).

The development of a spectrophotometric method for precise measurement of the hemolytic activity of complement (7) provided the opportunity for a reappraisal of the effects of various metallic ions on the lytic process. As a result, important effects of a number of ions on immune lysis have been clearly demonstrated, some of the confusion surrounding the subject has been dispelled, and simple conditions have been found for a considerable enhancement of the lytic potency of complement.

EXPERIMENTAL

Materials and Measurements.—Sheep blood was preserved aseptically in Alsever's solution (8),[1] for it could be shown in agreement with Bukantz and his associates (8) that after 4 days and up to at least 10 weeks sheep cells stored in this medium remain constant in their fragility to lysis by complement (C′) and antibody. A portion of the mixture was removed aseptically and was washed at least 5 times, standardized, and treated with hemolysin as described in a previous paper (7).

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[1] 2.05 gm. glucose, 0.8 gm. sodium citrate, 0.42 gm. sodium chloride per 100 ml. Adjust to pH 6.1 with citric acid. Use equal volumes of this solution and of blood.

Complement, usually pooled from about 8 guinea pigs, was stored in 1 ml. portions in sealed Pyrex glass ampoules and frozen in solid CO_2. Under these conditions the hemolytic activity remained constant for at least several weeks and experiments carried out on different days and with portions of the same lot of C′ from different ampoules were comparable. Dilutions were made with calibrated pipettes and flasks.

The diluent for all reagents was a buffered sodium chloride solution[2] prepared with analytical precision. Veronal buffer was preferred to phosphate, and was used throughout unless otherwise stated, since studies were to be made of the effect of Ca^{++} and Mg^{++}.

In most instances molar stock solutions of the salts to be tested were stored in the cold to avoid contamination with microorganisms. For each experiment a solution of approximately the same ionic concentration as isotonic sodium chloride solution was freshly prepared by dilution with water. Further dilutions were made with veronal-saline so that both salt concentration and pH might remain constant. Solutions of sodium pyrophosphate were neutralized with HCl before dilution with buffer, but this was not necessary with sodium citrate, $CaCl_2$, and $MgCl_2$ in the amounts employed.

Measurements of C′ activity were carried out in duplicate as described previously (7) with 1.0 ml. portions of sensitized cell suspension standardized by colorimetric analysis to contain 500 million cells per ml. (7), 2.5 or 3.0 ml. of buffered saline containing the salt to be tested, and 1.5 ml. or 1.0 ml., respectively, of a dilution of C′ calculated to give partial lysis. A blank containing only cells and veronal-saline, and a tube for complete hemolysis, containing cells and 4.0 ml. of C′ dilution, were included in each set of analyses. After incubation for 45 minutes at exactly 37°C., with occasional mixing, 2.5 ml. of buffered saline were added to make a total volume of 7.5 ml. The tubes were centrifuged, and the optical density of each supernatant was read at a wave length of 550 mμ (7), against the blank supernatant as reference standard.[3, 4]

Calculations.—The degree of hemolysis obtained in each analysis is calculated by dividing the optical density of the supernatant hemoglobin solution by that of completely lysed cells. Two methods are available for obtaining the activity of C′ in terms of 50 per cent units from one or more measurements in the range of partial lysis. Both are based on the characteristic S shape of the curve of lysis obtained with different amounts of C′ and expressed by the equation of von Krogh (9):

$$x = K\left(\frac{y}{1-y}\right)^{1/n} \tag{1}$$

This was derived as an adsorption formula, but owes its characteristics largely to the well known inhomogeneity of red cells (10, 11). In this formula x represents the amount of C′ used and y denotes the degree of lysis expressed as a fraction of 1. For example, $y = 0.80$ indicates 80 per cent lysis. The constant K is the 50 per cent unit of C′ since at this point $y = 0.5$ and the term $\frac{y}{1-y}$ equals unity and therefore $x = K$. The constant $1/n$ which

[2] 85.0 gm. NaCl, 5.75 gm. 5,5-diethyl barbituric acid, 3.75 gm. sodium 5,5-diethyl barbiturate. Dissolve the acid in 500 ml. hot water, add to the solution of the other components, cool, and make up to 2000 ml. with water. Each day dilute accurately 1 part up to 5 with water; pH 7.3 to 7.4. In most instances phosphate buffers were avoided owing to the insolubility of calcium phosphate.

[3] This practice serves to apply a correction for the slight degree of spontaneous lysis which occurs in the absence of C′.

[4] If the C′ dilution contributes any appreciable color, its optical density should be measured and subtracted from the readings. Unless the guinea pig serum is strongly hemolyzed, its color contribution at the dilution generally employed is negligible.

determines the shape of the S curve, has been found in many independent experiments to vary about the value 0.2, in fair agreement with other estimations, widely divergent in time and details of execution, reported by others (12, 13).

If the amount of C′ required for 50 per cent lysis is assigned the value 1, and if $1/n = 0.2$ is substituted in equation (1) the following expression is obtained:

$$x = \left(\frac{y}{1 - y}\right)^{0.2} \tag{2}$$

which relates x, the amount of C′ giving any degree of lysis (y) to 1, the quantity of C′ required for 50 per cent lysis. Table I gives the values of x for the range $y = 0.10$ to $y = 0.90$, calculated from equation (2). With its aid, or more conveniently with a graph constructed from

TABLE I

Conversion Factors Calculated from the von Krogh Equation for $1/n = 0.2$

Degree of lysis	Factor	Degree of lysis	Factor
0.10	0.644	0.55	1.041
0.12	0.671	0.60	1.084
0.14	0.696	0.65	1.132
0.16	0.718	0.70	1.185
0.18	0.738	0.75	1.246
0.20	0.758	0.80	1.320
0.25	0.803	0.82	1.354
0.30	0.844	0.84	1.393
0.35	0.884	0.86	1.438
0.40	0.922	0.88	1.490
0.45	0.961	0.90	1.552
0.50	1.000		

Table I, it is possible to calculate the activity of C′ in terms of 50 per cent units from a single analysis in the range of partial lysis (*cf.* also reference 13). For example, if 1.0 ml. of a 1:100 dilution of C′ produces 30 per cent lysis (*i.e.*, $y = 0.30$), the activity equals $100 \times 0.844 = 84.4$ units.

A more reliable and also more laborious method of application of equation (1) involves experimental determination of two or more points in the range of partial lysis, preferably at least one point below and another above 50 per cent lysis. The logarithmic form of equation (1):

$$\log x = \log K + \frac{1}{n} \log \left(\frac{y}{1 - y}\right) \tag{3}$$

describes a line of intercept log K and slope $1/n$. Since K is the 50 per cent unit of C′ the intercept of the line obtained by plotting log x against $\log\left(\frac{y}{1 - y}\right)$ gives the 50 per cent unit. In applying this method it is convenient to employ logarithmic graph paper (7, 12) so that the only calculation necessary is that of the term $\left(\frac{y}{1 - y}\right)$. This method has been described previously (7). Its main advantage is that it requires no knowledge of the value of $1/n$.

A straight line is also obtained when log x is plotted against the probit of y (14), and this relationship can also be utilized to determine the 50 per cent unit from two or more analyses.

The data presented were calculated from duplicate determinations by the graphical method according to equation (3) when two or more analyses were available. When only a single point could be determined the conversion factors given in Table I were used.

Since calculation of the 50 per cent titer of complement by the methods described above is possible only if the lytic curve follows the von Krogh equation in the presence of the agents added, experiments were carried out with native and dialyzed complement, with and without added optimal Ca^{++} and Mg^{++}. These confirmed the validity of equation (1) and gave essentially uniform values for the constant $1/n$ averaging about 0.2. The conversion factors in Table I are based on this value. Such variations as were encountered were usually within ± 10 per cent and could not be correlated with the treatment of C′ or with the addition of metals, and are therefore ascribed to as yet unknown factors or unavoidable errors. An

TABLE II

Variation of Hemolytic Activity of Guinea Pig C′ with Changes in Temperature, Total Volume of Lytic System, pH, and Salt Concentration

Temperature of incubation, °C.			39.0	38.0	37.0	35.3	32.4
Titer after 45 min.			133	139	144	147	147
Total volume of lytic system, *ml.*			2.0	3.0	5.0	7.5	
Titer, 37.0 °C., 45 min.			272	211	148	108	
pH*			6.9	7.1	7.4	7.6	
Titer			158	155	162	162	
Molarity of NaCl + buffer	0.145	0.151	0.155	0.162	0.168	0.173	0.179
Titer	143	126	118	96	83	75	64

Titers expressed in 50 per cent units per ml. undiluted guinea pig serum.

* Veronal-NaCl buffers, 0.151 M, used.

uncertainty of ± 10 per cent in $1/n$, however, represents not more than a ± 3 per cent error in the titer if analyses are carried out between 20 per cent and 80 per cent lysis.

When deviations markedly greater than 10 per cent from $1/n = 0.2$ were observed, these could be traced to inhibitory or enhancing factors which exerted differential effects on the varying amounts of C′ used in the determination of the lytic curve. This occurs, for example, when the different amounts of C′ distributed in tubes for titration are subjected to incubation before addition of sensitized cells, or when a constant amount of antibody and antigen is added to different amounts of C′ measured out for titration. Serious deviations of $1/n$ are therefore believed to indicate that the titration is improper in that conditions exist which invalidate proportionality between the different quantities of C′ measured out.

Details of Experiments and Data Reported in the Tables

The conversion factors in Table I represent the values of x for the range $y = 0.10$ to $y = 0.90$, calculated from equation (2). The constant $1/n = 0.2$ is the average slope of the lines obtained by plotting experimentally determined values of y against x according to the logarithmic equation (3), page 537. For this purpose analyses were carried out with 1.0 ml. portions of C′ diluted accurately 1:110, 1:120, 1:130, 1:150, 1:180, and 1:220. In the presence of Mg^{++} the C′ dilutions used were 1:180, 1:200, 1:240, 1:270, 1:300, 1:350, 1:400, and 1:440.

Little is known of the effect of variables such as temperature, total volume of the system, pH, and salt concentration on accurate measurements of hemolytic activity. Table II summarizes studies of these effects.

Experiments on the effect of temperature were carried out with duplicate 1.0 ml. portions of C′ diluted 1:130 and 1:175 to yield two points in the range of partial lysis. Until used, the sensitized cells and C′ dilutions were kept in ice water during the entire series; that is, about 5 hours. The determinations were made in the sequence given in Table II, that is, starting at 39.0°C. and finishing at 32.4°C. The reagents for each set of analyses were mixed immediately before incubation. During the 5 hour period the activity of the C′ dilutions held at 0°C. decreased about 3 per cent. It is therefore evident that the increase of C′ activity observed in going from 39°C. to 32.4°C. incubation temperature would probably be somewhat larger if all experiments were performed simultaneously. The values for $1/n$ found at 39.0, 38.0, 37.0, 35.3, and 32.4°C. were, respectively, 0.19, 0.21, 0.20, 0.19, and 0.21, within the normal range.

Determinations at different volumes were set up in duplicate with 1.0 ml. portions each of cells and C′ dilution and with veronal buffer up to the final volume. After incubation all analyses were brought to 7.5 ml. by addition of veronal-saline. The C′ dilutions for the 2 ml. volume were: 1:230, 1:260, and 1:300; for 3 ml.: 1:180, 1:200, and 1:230; for 5 ml.: 1:120, 1:150, 1:180; for 7.5 ml.: 1:80, 1:100, 1:120. The 7.5 ml. set was run for 60 minutes, as well as for 45 minutes, the usual period of incubation, but the difference in activity was only 2 per cent. The usual practice of occasional mixing was compared in this experiment with continuous mixing during incubation. The difference in activity was less than 1 per cent. Values for $1/n$ at 2, 3, 5, and 7.5 ml. volume were, respectively, 0.21, 0.20, 0.21, and 0.18.

In the determination of the effect of varying pH the cells and C′ dilutions were made in the usual veronal-saline of pH 7.3 to 7.4, molarity 0.151. 1.0 ml. portions of sensitized cells were pipetted into the tubes, as well as 2.5 ml. of specially prepared veronal buffers of molarity 0.151 but of different pH, and 1.5 ml. of C′ dilution. The resulting final pH values are recorded in Table II. Three points were set up in duplicate at each pH, with C′ dilutions 1:170, 1:200, and 1:250.

To determine the effect of varying the concentration of salt in the lytic system, dilutions of cells and C′ were made in the 0.151 M veronal buffer. 1.0 ml. portions of cells were pipetted into the tubes. To these were added 3.0 ml. veronal buffers differing in concentration so as to bring the final salt concentration after addition of the C′ dilution to the values given in Table II.

Experiments were then designed for the study of varying additions of Mg^{++} or Ca^{++}, on individual guinea pig and human sera and on pools of sera. The results are summarized in Tables III and IV. The effects of Sr^{++} and Ba^{++} are compared with those of Ca^{++} in Table V.

Table III.—All the complements were diluted 1:160 and 1.0 ml. portions were used. 3.0 ml. of $MgCl_2$ diluted in saline buffered with phosphate were added to each analysis. In similar experiments $MgSO_4$ could be substituted for $MgCl_2$.

Table IV.—1.5 ml. of veronal-saline containing 50 μg. of Ca^{++} or 100 μg. of Mg^{++}, as $CaCl_2$ or $MgCl_2$, respectively, were added to 1.0 ml. of cells, followed by the C′ dilution, which was 1:140 for determinations without added Mg^{++}, 1:250 with Mg^{++}. All analyses were brought to 5.0 ml. with veronal-saline. Co^{++} was added as $CoCl_2$. 100 per cent titrations were made with 0.2 ml. cells in a total volume of 1.0 ml., but the titers were calculated to 1.0 ml. cells as in the 50 per cent titration. Equivalent amounts of Ca^{++} and Mg^{++} were used in both methods of titration.

Table V.—3.0 ml. of a dilution of $CaCl_2$, $SrCl_2$, or $BaCl_2$ in veronal-saline were added to each analysis, followed by 1.0 ml. of C′ dilution 1:121.

Samples of C′ were dialyzed against the veronal-buffered saline in order to remove Mg^{++} and Ca^{++} and thus provide material more sensitive to the addition of minute quantities of these ions. Mg^{++} and Ca^{++} were added separately as well as in combination, and the effects were studied of dialysates of serum and allantoic fluid which had been analyzed for Mg^{++} and Ca^{++}.

TABLE III

*Hemolytic Activity in 50 Per Cent Units per Ml. of Guinea Pig Complement with Varying Amounts of Magnesium Ion**

Magnesium ion added	Guinea pig complements					
	1	2	3	4	5	6 (pool‡)
μg.						
0	93	84	100	114	98	114
0.09	96	86	109	128	107	114
0.46	110	94	118	138	117	123
2.3	133	131	148	179	134	154
12	173	149	206		168	190
58	203	197			206	240
290					208	231

* The diluent was 0.85 per cent saline buffered at pH 7.3 with 0.005 M phosphate.

‡ Sera used did not include 1 to 5.

TABLE IV

Effect of Optimal Amounts of Magnesium and Calcium Ions on the Hemolytic Titers of Guinea Pig and Human Complements

Complement	50 per cent titer			Activation by Mg^{++}	100 per cent titer		Activation by Mg^{++}
	Without additions	With 50 μg. Ca^{++}	With 100 μg. Mg^{++}		Without additions	With Mg^{++}	
				per cent			*per cent*
Guinea pig 7	107	106	194	81	52	80	58
" " 8	163	152	299	83	80	105	31
" " 9	136	135	256	88	80	105	31
" " 10*	143	136	254	78	80	105	31
" " pool	129	127	234	81	80	105	31
Human	36	27	46	28	16	20	25

Units based on 1.0 ml. sensitized cell suspension containing 500×10^6 erythrocytes.

* 50 per cent titer with 50 μg. Co^{++}: 174. 50 per cent titer with 50 μg. Co^{++} and 100 μg. Mg^{++} : 236.

TABLE V

Effect of Alkaline Earth Cations on the Hemolytic Activity of Guinea Pig C'. (Activity without Additions: 127 Units)

Cation	Ca^{++}					Sr^{++}					Ba^{++}				
Amount added, μg	0.77	3.8	19	96	480	1.7	8.4	42	210	1050	2.6	13.2	66	330	1650
Titer	134	137	132	107	75	125	125	120	104	78	127	128	121	109	70

Titers in 50 per cent units per ml.

Table VI.—1.5 ml. portions of $CaCl_2$ and $MgCl_2$, or of the dialysates, diluted in veronal-saline, were used. The dialysates of serum and allantoic fluid were the outside portions from dialysis in cellophane against equal volumes of veronal-saline (pH 7.3 to 7.4) for 3 days in the

TABLE VI

Enhancement of Hemolytic Activity of Dialyzed Guinea Pig Complement, Lot 1, by Ca^{++}, Mg^{++}, Serum Dialysates, and Dialysate of Chick Embryo Allantoic Fluid*

(Values given are 50 per cent units per ml.)

	Mg^{++} added, μg.								Dialysate of		
									Human serum	Sheep serum	Guinea pig serum
Ca^{++} added, μg.	0	0.2	1.0	5.0	25	50	100	200	0.15 ml.	0.15 ml.	0.15 ml.
0	14	19	24	29	35	38	39	41	37	39	42
0.2	18	22	26	35		42	44				
1.0	22	26	32	43	54	49	51				
5.0	25	30	37	49	59	56	58	62			
25	26					60	62	63			
50						60	62	64			
100	25					58	61	65	38	38	40
200						55	57	59			
100 μg.Mg^{++} added									53	56	56
100 μg.Mg^{++} + 100 μg. Ca^{++} added	60								58	60	60

Dialyzed complement, lot 2
Additions of cations

Guinea pig dialysate‡ added	None	100 μg. Ca^{++}	100 μg. Mg^{++}	100 μg. Ca^{++} + 100 μg. Mg^{++}
None	48	80	111	142
0.15 ml.	95	92	143	148

Dialyzed complement, lot 3
Additions of cations

Allantoic fluid dialysate added	None	50 μg. Ca^{++}	100 μg. Mg^{++}	50 μg. Ca^{++} + 100 μg. Mg^{++}
None	18	28	48	70
0.15 ml.	41	46	64	72

Preliminary experiments, also with dialyzed C′, indicated that Co^{++} and Ni^{++} are potent activators in amounts of 1 to 50 μg., while Mn^{++} and Ba^{++} activated less well, best between 0.1 and 0.5 μg., and slight activations resulted from similarly small amounts of Sr^{++}, Cd^{++}, and Fe^{++} (*cf.* also (5)). Al^{+++} did not activate, while as little as 0.4 μg. La^{+++} inhibited strongly.

* Chemical analyses of dialysates:

	Human	Sheep	Guinea pig
Ca, μg. per 0.15 ml.	5.2	5.8	6.2
Mg, μg. per 0.15 ml.	0.6	1.5	2.0

‡ Same dialysate as above.

cold, with frequent mixing. The dialyzed C′ was obtained by dialysis of fresh guinea pig serum in cellophane against 30 to 50 volumes of veronal-saline for 1 week in the cold with 2 changes of dialysate daily. The dialyzed C′ in the cellophane tube contained less than 6 μg. Ca and about 6.5 μg. Mg in 5 ml. analyzed according to the method described in reference 16.[5] The low titer of this dialyzed C′ was largely due to deterioration as a result of exposure to ice box temperature for an entire week (*cf.* Table VII).

The experiments in Table VII were carried out with rapidly dialyzed C′ in order to separate, if possible, the effect due to the loss of Mg^{++} from the decrease in activity caused by deterioration of C′ during the longer period of dialysis (Table VI).

Guinea pig C′ was dialyzed in cellophane for 1 day in the ice box against a slow stream of about 700 volumes of veronal-saline with gentle mechanical agitation. Ca^{++} was less than 6 μg. and Mg^{++} was about 3 μg., respectively, in 5 ml. The volume increased 12 per cent during dialysis and the titers reported are corrected accordingly. The control C′ was kept in cellophane in a stoppered glass tube and was agitated with the dialyzing C′. There was no change in volume.

TABLE VII

Effect of Dialysis on Complement Activity of Guinea Pig Serum with and without Added Ca^{++} and Mg^{++}

	Titer in 50 per cent units per ml.	
	Dialyzed complement	Control complement
Without addition	88	110
With 50 μg. Ca^{++}	102	110
With 100 μg.Mg^{++}	211	213
With 50 μg.Ca^{++} and 100 μg.Mg^{++}	251	233

Since it is difficult to remove all traces of bivalent cations from C′ by dialysis, recourse was had to addition of anions with which Mg^{++} and Ca^{++} are known to form complex ions with low dissociation constants.

Table VIII.—Approximately isotonic and neutral solutions of sodium citrate and freshly prepared sodium pyrophosphate were diluted in veronal-saline. Ca^{++} and Mg^{++} were supplied as $CaCl_2$ and $MgCl_2$. It should be noted that the values given are degrees of lysis expressed in per cent.

Allantoic fluid and tissue extracts are frequently used in complement fixation tests and are known to enhance C′ activity (19). Since this effect, due to Mg^{++} and Ca^{++}, is undesirable if uncontrolled, the experiment recorded in Table IX is of value in showing that there is no supplementary enhancing action when the system contains optimal Mg^{++} and Ca^{++}.

These experiments were carried out with whole guinea pig C′ while those in Table VI were performed with dialyzed C′. The allantoic fluid was collected from three 13 day old chick embryos. Serum dialysates were made as for Table VI. 1.5 ml. of a dilution of $MgCl_2$ or $CaCl_2$ in veronal-saline containing 100 or 50 μg. of Mg^{++} or Ca^{++}, respectively, and 1.5 ml.

[5] For the determination of total Ca in serum, the proteins were removed with trichloroacetic acid, and calcium oxalate was precipitated and washed once by decantation, with thorough draining. The precipitate was dissolved in N H_2SO_4 and the oxalic acid titrated with 0.01 N $KMnO_4$ (16*a*).

Mg was precipitated from the calcium-free filtrate as in the method of Briggs (16*b*) and the washed precipitate was analyzed for P as in the method described by Bodansky (16*c*).

of serum dialysate or allantoic fluid, also diluted in veronal-saline, were added to 1.0 ml. of sensitized cells, followed by 1.0 ml. of C′ dilution. Different lots of C′ were used in the three experiments, so that the values reported are not comparable.

TABLE VIII

Inhibition of Lysis by Citrate or Pyrophosphate and Its Reversal by Mg^{++} and Ca^{++}

Concentration of anion, molar × 10^3	1.0 ml. C′ dilution used	No cation added	Final concentration of added cation,* molar × 10^3							
			Mg^{++}				Ca^{++}			
			0.17	0.83	4.2	21	0.19	0.83	4.2	21
Citrate		Per cent lysis								
None	1:150	43								
0.9	1:100	34	97				65			
0.9	1:150	7	86	92	92	7	20	16	4	1
9.0	1:100	0	1				2			
9.0	1:150	0	0	1	4	83	1	1	2	1
			Final concentrations							
Pyrophosphate			0.17	0.83	4.2	21	0.10	0.50	2.5	12.5
		Per cent lysis								
None	1:151	41								
0.2	1:151	10	96	92	93	6	19	15	6	0
2.0	1:151	0	0	44	92	11	1	2	1	7

* Concentrations of Mg^{++} and Ca^{++} normally present were estimated at approximately 0.005×10^{-3} molar each.

TABLE IX

Enhancement of C′ Activity by Guinea Pig Serum Dialysate or by Chick Embryo Allantoic Fluid with and without Addition of Optimal Quantities of Ca^{++} and Mg^{++}

Cation added	Serum dialysate 1			Serum dialysate 2			Allantoic fluid		
	None	1:10	1:35	None	1:10	1:25	None	1:10	1:25
None	125	181	128	104	148	122	117	203	174
50 μg. Ca^{++}							116	194	168
100 μg. Mg^{++}	245	255	254	190		230	226	237	232
50 μg. Ca^{++} + 100 μg. Mg^{++}							238	236	243

Values given are 50 per cent units per ml.

RESULTS AND DISCUSSION

Measurements of the activity of C′ by the spectrophotometric method appear to have a precision of about ± 5 per cent if analyses are performed within the range of 20 to 80 per cent lysis and provided the factors considered

in Table II are controlled. The most important of these is the total salt concentration since a deviation of 1 per cent in the latter causes an error of over 2 per cent in hemolytic activity. Careful control of temperature is also desirable since the change in activity is about 3 per cent per degree Centigrade. Considerable latitude is permissible with respect to pH, no significant difference being noted between pH 6.9 and 7.6, the limits of the range investigated. If activity measurements in different experiments are to be comparable the total volume of the lytic system should be the same. The volume of 5 ml. used throughout this work was selected arbitrarily.

A striking feature of the present experiments is the marked enhancing effect of Mg^{++} on the hemolytic activity of C′. Data on the effect of Mg^{++} on five different individual guinea pig sera and one pool are assembled in Table III. An increase of activity generally becomes noticeable with as little as 0.1 to 0.5 μg. of Mg^{++} and reaches an optimum of 100 per cent or more with about 50 μg. of Mg^{++} per test. Additional data on individual sera and a pool (Table IV) indicate enhancement of somewhat less than 100 per cent, the lower effect possibly being due to the use of veronal-buffer in this instance instead of phosphate as in Table III. While measurements in terms of complete lysis are much less precise than those at 50 per cent lysis, they are included for comparison. Enhancement of the 100 per cent titer was less pronounced than at the 50 per cent level. The reason for this was not readily ascertained.

Any possible change in the character of the lytic curve on addition of Mg^{++} was investigated by determination of $1/n$ in the presence of optimal Mg^{++} but the values found were similar to those obtained without added Mg^{++}.

The enhancing effect of Mg^{++} on the lytic function of complement has long been known (4, 6), but its utility and significance appear largely to have been overlooked. Much complement could undoubtedly be conserved by the use of Mg^{++} in optimal amounts (*cf.* also references 6 and 15) but its effects on the sensitivity and reliability of any given diagnostic test would need to be explored. The activation of the lytic power of complement by Mg^{++} at once suggests the parallel activation of numerous enzyme systems by this ion, and, indeed, it was a search for possible clues concerning the enzymatic nature of complement which led to the current experiments.

When about 1 to 20 μg. of Ca^{++} are added to guinea pig C′, no enhancement or only about 5 to 10 per cent increase of activity results (Table V). Larger amounts are inhibitory as are also quantities of Mg^{++} as large as 500 μg. (*cf.* also reference 6). However, with C′ which has been depleted of its natural content of Ca^{++} and Mg^{++} by dialysis against saline-buffer at pH 7.3 the enhancing action of Ca^{++} is more marked and the optimal level appears to be between 5 and 100 μg. (Table VI). The sample of $CaCl_2$ used contained not more than 0.5 per cent Mg (Merck's reagent grade), but the increase in activity

was not due to this since data in Table VI show that Ca^{++} activates dialyzed complement even in the presence of optimal Mg^{++}.

Analyses of guinea pig C′ for calcium and magnesium[5] yielded values of 103 and 33 μg. per ml., respectively. Since a lytic determination, as carried out in these studies, usually contains about 1 ml. of 1:150 guinea pig C′ the amounts of Ca and Mg contributed by C′ are about 0.7 and 0.2 μg., respectively. The contribution by the hemolysin is very much smaller because only 0.5 ml. of a 1:500 dilution is employed. Washed red blood cells contribute about 0.3 μg. Mg and not more than 0.1 μg. Ca. The total amounts of Ca and Mg normally present in the hemolytic system would then be approximately 1 and 0.5 μg., respectively. It is obvious then that the usual hemolytic system contains not much less than the optimal level of Ca (*ca.* 5 μg.) while there is a 100-fold deficit between the amount of Mg naturally present (0.5 μg.) and that required for optimal activity (*ca.* 50 or 100 μg.). In order to demonstrate activation with Ca^{++} it is therefore necessary to work with a lytic system made deficient with respect to Ca^{++}.

The decrease of C′ activity which occurs on dialysis (4, 17) was ascribed to loss of a dialyzable component of C′. Cernovodeanu and Henri (4) found that the activity of dialyzed C′ could be increased by Mg^{++}, but Jones and Ecker (18) believed the decrease during dialysis to be due to deterioration of C′. If Ca^{++} and Mg^{++} are to be considered additional components of C′ or essential adjuvants, their removal should lead to a decrease in C′ activity or even complete loss of function. Experiments were carried out in order to evaluate the relative importance of C′ deterioration during dialysis. In each test, two portions of C′ were treated similarly in all respects except that one sample was dialyzed, the other not. The hemolysin and red cells used were also dialyzed in order to remove as much Ca^{++} and Mg^{++} as possible. The averages of the results of two such experiments (Table VII) indicate that the dialyzed C′ lost somewhat more activity than could be accounted for by deterioration alone. Addition of Ca^{++} and Mg^{++} resulted in a slightly higher titer in the dialyzed C′ than in the control, suggesting that deterioration was slightly less in the dialyzed portion. While the decrease in activity due to dialysis is so small that the function of Ca^{++} and Mg^{++} as essential adjuvants or coenzymes does not emerge clearly, all reagents used in the test, including the water and salt, contain traces of these metals which might suffice for the levels of activity observed.

Because of this it is much simpler to bind Ca^{++} and Mg^{++} by addition of an excess of citrate or pyrophosphate (19). As shown in Table VIII, both of these anions inhibit C′ activity, pyrophosphate being effective in lower concentrations than citrate. Since this inhibition is readily overcome by addition of Mg^{++} and since the data in Table VIII indicate a relation between the amount

of anion necessary for inhibition and the quantity of Mg^{++} which restores lytic activity, the essential rôle of Mg^{++} is evident. While Ca^{++} also activates the system, it is inferior to Mg^{++} and its rôle might merely be that of a substitute for Mg^{++}. Ni^{++} and Co^{++} also activate the system but are less effective than Mg^{++}.

In C′ fixation tests involving the use of reagents which bind Ca^{++} and Mg^{++} and therefore depress the activity of C′, the addition of optimal amounts of Ca^{++} and Mg^{++} should be advantageous. For example, in an experiment similar to those in Table VIII addition of 1.5 ml. of 0.003 M sodium citrate solution to the lytic system depressed the activity of guinea pig C′ from 129 to 84 units. However, in the presence of 50 μg. of Ca^{++} and 100 μg. of Mg^{++}, the activity of the C′ was 266 units without added citrate and 260 units with addition of citrate.

The high degree of enhancement with Mg^{++} suggests this metal as the real cofactor which may, however, be replaced more or less effectively by other divalent metals. It is also possible that the Ca^{++} acts by displacement of the equilibrium of the complex Mg-citrate ion with release of Mg^{++}. The data in Table VI which show enhancement by Ca^{++} and Mg^{++} beyond the level reached by optimal Mg^{++} alone might, however, be taken to indicate a direct rôle of Ca^{++} as well as of Mg^{++}. On the basis of an enzymatic hypothesis the existence of two cofactors might be an indication that more than a single enzyme system is involved in the lytic process, and this would not be in disagreement with the complex nature of C′ in terms of its four components.

The dialyzable and heat-stable component of C′ observed by Friedewald (20) in serum and in allantoic fluid can now be identified as consisting of Ca^{++} and Mg^{++}. As shown in Tables VI and IX the effects noted by Friedewald (20) are confirmed, and it is also evident that whole allantoic fluid, or dialysates of serum or allantoic fluid fail to exert any supplementary enhancing action when the lytic system contains optimal quantities of Ca^{++} and Mg^{++}. When whole C′ is used, as in Table IX, the lytic system contains almost enough Ca^{++} for optimal activity and the enhancing effect of serum dialysate or allantoic fluid is largely due to the Mg^{++}. In C′ fixation with antigens derived from allantoic fluid the use of optimal levels of Ca^{++} and Mg^{++} would be advantageous, since the C′ titer would then be unaffected by the antigen.

To determine whether or not the enhancing action of Mg^{++} could be due to increased fragility of the red cells other modes of lysis were tested. While Mg^{++} failed to affect lysis by sodium taurocholate, enhancement of lytic activity was observed with saponin. On the other hand, lysis by Duponol was diminished by Mg^{++}. This variable behavior of Mg^{++} with respect to different kinds of hemolysis permits no conclusion as to any relation between the effect and the fragility of red cells. It appears significant, however, that neither citrate nor pyrophosphate caused inhibition of lysis by Duponol, saponin, or

sodium taurocholate, indicating that Mg^{++} or Ca^{++} play an essential rôle only in lysis by C′ and antibody.

Since the Ca and Mg ions in serum are in equilibrium with Ca and Mg bound to protein the present experiments indicate that changes in the distribution between ionized and bound Ca and Mg must be considered in measurements of C′ activity When a reagent like pyrophosphate, which binds these ions (19), is added the equilibrium is shifted and the result is not only a lowering of the level of free metal ions but also release from the proteins of bound cations and combination of these with pyrophosphate. The effects noted should therefore be considered not in terms of free and bound cations as static entities (1) but from the point of view of chemical equilibria. In this dynamic sense the rôle of metals in C′ lysis can be expressed by stating that the process requires the presence of free bivalent cations. This was stressed by Wadsworth, Maltaner, and Maltaner (2) for Ca^{++} in its relation to cephalin, but the experiments recorded in the present paper would tend to indicate the more decisive importance of Mg^{++} in C′ activity.

SUMMARY

1. The evidence presented indicates that Mg^{++}, or other cation such as Ca^{++}, Ni^{++}, or Co^{++}, is essential for the hemolytic action of C′. Ca^{++}, Ni^{++}, and Co^{++} are less effective than Mg^{++}. The hemolytic system usually does not contain sufficient Mg^{++} for optimal hemolytic activity so that a marked enhancement can be obtained by addition of extra Mg^{++}.

2. The enhancing action of tissue fluids can be ascribed to their contribution of Mg^{++}.

3. Substances which bind Mg^{++} and Ca^{++} are anticomplementary when added to the usual hemolytic system which contains only a small quantity of Mg^{++}. This type of anticomplementary effect can be overcome by addition of extra Mg^{++}.

4. Ca^{++} may also be essential to the lytic process but its action is much less pronounced than that of Mg^{++}.

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