and ocular defects. Multiple malformations were rare. The teratogenic dose of Congo red did not produce any alteration in maternal serum protein components during the expected period of teratogenic activity. This is contrasted to the situation found following treatment with trypan blue.

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Role of Calcium in Complement Dependent Hemolysis.* (29529)

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Mayer and his co-workers(1,2) have defined the role played by divalent cations in complement (C′) dependent immune red cell (EA) lysis. Their investigations clearly show that the need for Ca^{++} occurs earlier in immune hemolysis than the need for Mg^{++}. This observation enabled the same investigators to establish techniques for preparation and study of sensitized red cell complement component intermediates (EAC′....)(3,4). As a result it is now known that Ca^{++} participates in the attachment of the first component of C′ (C′1) to EA, while Mg^{++} is required for the attachment of C′2. C′4 and C′3 can function in the absence of both Mg^{++} and Ca^{++}. The various salts of EDTA have been of great practical importance in studies on EAC′.... lysis. Addition of Na_3HEDTA to a source of C′ binds both Mg^{++} and Ca^{++}; such a reagent, containing fully active C'_3, will support the lysis of $EAC'_{1,4,2}$. Addition of Na_2Mg EDTA to a C′ source binds Ca^{++}, but the Mg^{++} present preserves the ability of C′4, C′2, and C′3 to function; such a reagent will support the hemolysis of EAC'_1, $EAC'_{1,4}$, and $EAC'_{1,4,2}$, but will not support lysis of EA or EAC'_4 when C′ is employed in the usual concentrations(5,6).

The *in vitro* hemolysis of red cells (E) from patients afflicted with paroxysmal nocturnal hemoglobinuria (PNH) occurs best in undiluted human serum which has been slightly acidified (pH 6.5), but requires the presence of all 4 components of C′(7). In addition it can be shown that following exposure to acid serum, those cells which have resisted hemolysis bear on their surface components of C′(8). Nevertheless, PNH-E do not require Ca^{++} for lysis in serum depleted of Ca^{++} and Mg^{++} by IRC-50 resin treatment, since the readdition of Mg^{++} only will reconstitute the PNH hemolytic system(7). The appropriate use of EDTA salts establishes the same principle (Table I) since serum chelated with Na_2Mg EDTA is fully capable of supporting PNH-E lysis, while Na_3HEDTA is almost totally inhibitory.

If PNH-E lysis is truly C′ dependent, these results might suggest that PNH-E are in the functional state $PNH\text{-}EC'_1$, or $PNH\text{-}EC'_{1,4}$. If this were so, pre-exposure of PNH-E to

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TABLE I. Hemolysis of PNH Erythrocytes in Whole Serum with and without Addition of Various Salts of EDTA.

	PNH cells 20% suspension	Serum pH 6.5	Na_3HEDTA (.15 M pH 6.5)	Na_2Mg EDTA (.15 M pH 6.5)	Saline	O.D. 540 mμ	% Lysis
Blank	—	1.0	—	—	.2	—	—
Control lysis	.05	1.0	—	—	.15	.564	46
Na_3HEDTA lysis	.05	1.0	.15	—	—	.066	5
Na_2Mg EDTA lysis	.05	1.0	—	.15	—	.562	46
Complete lysis (freeze and thaw)	.05	1.0	—	—	.15	1.197	—

The final concentration of EDTA was 1.9×10^{-2} M. Hemolysis performed at 37° for 30 min under oil. Serum pH adjusted to 6.5 with .3 N HCl.

Na_3HEDTA(9) or polyinosinic acid(10), followed by washing, should decrease their susceptibility to hemolysis on subsequent exposure to acid serum containing Na_2Mg EDTA. An experiment testing this possibility showed no loss of hemolytic susceptibility (Table II).

PNH hemolysis must be carried out in a relatively high C′ concentration;—serum diluted 1:4 will no longer sustain PNH-E lysis effectively (Fig. 1). Since most studies on the role of Ca^{++} in EA lysis have been done at much higher C′ dilutions, the ability of EDTA salts to inhibit EA lysis over a wide range of C′ concentrations starting with undiluted human serum was tested (Fig. 2). The EA used in this experiment were sensitized in the presence of Na_3HEDTA, and subsequently washed once in the same buffer and twice in normal saline, in order to prevent attachment of the C′1 sometimes found in commercially obtained glycerinated ambo-

TABLE II. Effect of Preexposure of PNH Cells to Na_3HEDTA or Polyinosinic Acid upon Their Subsequent Lysis in Acid Serum Containing 1.9×10^{-2} M Na_2Mg EDTA.

Red cell preexposure	Hemolysis in Na_2Mg EDTA human serum pH 6.5 (O.D. 540 mμ)
Saline	1.08
Na_3HEDTA	1.03
Polyinosinic acid	1.048

One-tenth ml aliquots of a 20% suspension of PNH cells were exposed to (a) saline (30 min, 37°); (b) poly I (.5 ml 4 μmoles P/ml in saline—37°), and (c) 5 ml .0075 M Na_3HEDTA in saline (2 15-min incubation). They were then washed 3 times and 1.5 ml Na_2Mg EDTA serum added to each cell button. Supernatant O.D. read after 30 min incubation at 37°, under oil.

ceptor(6). While Na_3HEDTA completely inhibited lysis of EA even in the undiluted serum, EA were capable of 100% hemolysis in both the undiluted and 1:2 diluted serum containing Na_2Mg EDTA. Further dilution (1:4) resulted in abrupt decline of hemolytic C′ activity. Human serum diluted 1:128 in buffered saline containing optimal Ca^{++} and Mg^{++} gave approximately 50% hemolysis.

These results indicate that the capacity of PNH-E to undergo lysis in undiluted human serum despite the absence of Ca^{++} in no way distinguishes these cells from the more classical complement dependent hemolytic system involving EA, and suggests that the requirement for Ca^{++} in complement dependent hemolytic systems is not absolute, while the requirement for Mg^{++} is. The role of Ca^{++} in relationship to C′1 function has recently been clarified; Ca^{++} acts as a ligand which serves to stabilize the 3 subcomponents (C′1q, C′1r, C′1s) of the C′1 macromolecule (6). It would appear from these results that despite the inability to detect the C′1 macromolecule by density gradient centrifugation or ultracentrifugation of human serum in the presence of EDTA(11), the ability of a very small fraction of the 3 subcomponents to associate and produce a hemolytically functional C′1 molecule persists in a 1:2 dilution of serum. This hypothesis is made more tenable by the fact that very small amounts of C′1 are needed to initiate the sequence of events leading to complement dependent lysis; Borsos *et al* have shown that a 1:80,000 dilution of guinea pig complement suffices to convert EAC'_4 to $EAC'_{1a,4}$(12). Since most investigations of the mechanism

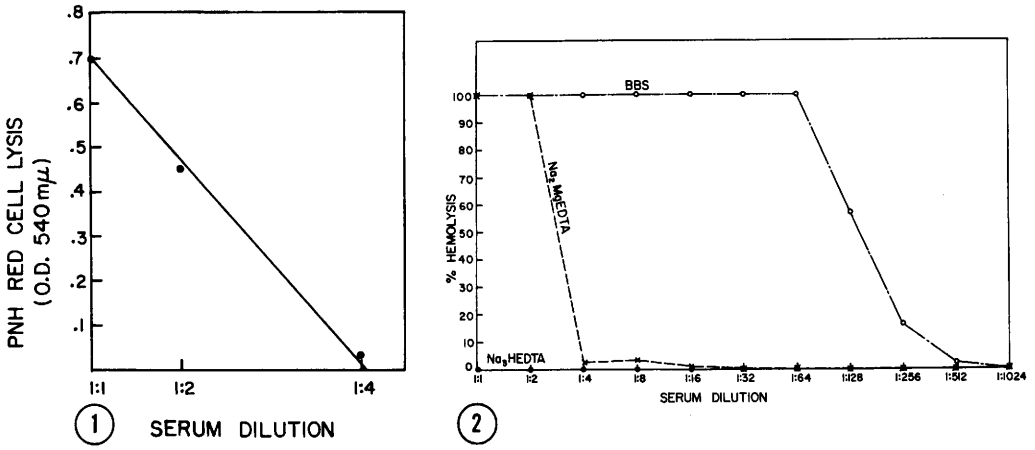

FIG. 1. Effect of serum dilution upon PNH red cell lysis. Human serum adjusted to pH 6.5 with 0.3 N HCl was diluted with .15 M NaCl. Following dilution, 0.05 ml of a 20% suspension of washed PNH cells was added to 1 ml of each serum dilution and incubated under oil at 37° for 30 min.

FIG. 2. Effect of various EDTA salts upon ability of human serum to support hemolysis of sensitized sheep red cells (EA). Sufficient 0.15 M EDTA solution, pH 7.4, was added to human serum to give a final EDTA concentration of 2×10^{-2} M. Serial 2-fold dilutions were then made in barbital buffered saline (BBS), pH 7.4, containing the appropriate EDTA salt (also at 2×10^{-2} M). Control serum was equally diluted with BBS containing 5×10^{-4} Mg^{++} and 1.5×10^{-4} Ca^{++}. To 1 ml of each serum dilution was added 0.05 ml of a 20% suspension of EA. Degree of hemolysis was determined after 60 min incubation at 37°C.

of C′ action involve the use of EA and much more dilute C′ reagents than those discussed in this paper, the results reported here concerning the role of Ca^{++} in immune lysis are not meant to detract from the practical or theoretical value of Ca^{++} depletion by means of Na_2Mg EDTA in the study of EAC'_1 and $EAC'_{1,4}$ lysis.

Summary. The cation requirements of two complement dependent hemolytic systems, one involving red cells from patients with paroxysmal nocturnal hemoglobinuria, and the other involving antibody coated sheep red cells, are similar when studied in undiluted human serum as a source of complement. Both are inhibited by the presence of Na_3HEDTA, but not by Na_2Mg EDTA. These results suggest that the need for Ca^{++} in complement dependent hemolysis is not absolute, while the need for Mg^{++} is.

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