

The Action of Two Sulfhydryl Compounds on Normal Human Red Cells

Relationship to Red Cells of Paroxysmal Nocturnal Hemoglobinuria

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PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) is an uncommon hemolytic disorder caused by an intrinsic defect of the erythrocytes about which very little is known. This may, at least in part, depend on the relative rarity of the disease and on the impossibility of reproducing the characteristic lesion of the erythrocytes in the laboratory.

Attempts along this line were made by Rodbard¹ who, assuming that the erythrocytic defect was localized in the lipid fraction of the stroma, succeeded on a few occasions in obtaining lysis of normal red cells in acidified serum—which occurs also under the same conditions to PNH red cells²—by means of repeated washing with dilute alcoholic solutions. However, the results were not constant and reproducible.

By contrast, the results obtained by Metz et al.,³ using an acetylcholinesterase inhibitor (OMPA), and by Ghiotto et al. (quoted by De Sandre et al.⁴) using another acetylcholinesterase inhibitor (physostigmine), were complete failures.

So far, the best results have been obtained by Yachnin et al.,⁵ who have reported that, when normal red cells are altered by means of various proteolytic enzymes (trypsin, ficin, bromelain and papain), cholera vibrio filtrate, influenza virus or sodium periodate, it is possible to obtain erythrocytes whose in vitro behaviour is similar to that of PNH red cells, although differing from the latter in some respects.

The purpose of this report is to describe the results obtained by treating normal human red cells with AET (2-amino-ethylisothiuronium bromide) and with cysteine; both substances contain SH groups, belong to the group of sulfhydryl compounds, but are not enzymes in nature.

MATERIALS AND METHODS

(1) Treatment of Red Cells with AET

We used AET of the Nutritional Biochemicals Co. for our experiments. An 8 per cent solution of this substance in distilled water was prepared immediately before use and its pH adjusted to 8.0 with 5 N NaOH. Four volumes of this solution were then added to 1 volume of normal packed erythrocytes, collected into acid-citrate-dextrose anticoagulant (ACD) and washed twice in saline. The mixture, prepared in a flat-bottomed container in order to form a thin layer, was gently mixed, with the container stoppered and im-

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mediately incubated in a water bath at 37 C. for exactly 19 minutes. At the end of this period, the red cells were immediately washed with large volumes of saline. After the first centrifuging, carried out at low speed for a short time, the red cells formed an agglomerate at the bottom of the tubes, and therefore these had to be shaken to obtain resuspension of the erythrocytes. Washing was repeated until the supernatant was completely free from hemoglobin. At this stage care was taken to remove as much supernatant as possible, and the red cells were ready for use.

(2) Treatment of Red Cells with Cysteine

In this case we used L-cysteine hydrochloride of the British Drug House, and the same method adopted with AET, except for the fact that a 10 per cent cysteine solution was used and that the incubation period at 37 C. was prolonged to exactly 15 minutes.

For both altering substances, the aforesaid concentrations of the solutions, their pH, the solution-volume/packed-red-cell-volume ratio, the incubation times and temperature were used because they had proved to be optimal on a series of preliminary tests. In fact, by keeping constant the incubation temperature (37 C.), the pH of the solutions (pH 8.0) and the pH of the serum used for the *in vitro* hemolysis tests (pH 6.1), the intensity of the hemolysis increased proportionately to the concentration of the substances and to the incubation period. The best results were obtained when the red cells were incubated with the altering substances at 37 C., although similar results could be obtained on incubating at room temperature for longer periods.

We were unable to obtain satisfactory results incubating at 4 C. Moreover, the susceptibility of the treated red cells to hemolysis depended on the pH of the altering solutions: it was nil if the pH was kept below 7.5, whereas spontaneous lysis of the erythrocytes occurred when the pH exceeded 8.5.

Observed under the microscope, the red cells treated with both substances appeared nonagglutinated. They were used within 1 hour from preparation.

The *in vitro* hemolysis experiments were in all cases performed as follows: the packed red cells and the compatible normal human serum (fresh or kept at -25 C. and thawed but once) were preincubated at 37 C. for 15 minutes; 1 volume (0.1 ml.) of red cells was then added to 10 volumes (1 ml.) of serum and the mixtures were thoroughly mixed and incubated at 37 C. for 45 minutes. The percentage of hemolysis was calculated after photometric determination (Beckman C, filter 413 m μ) on the supernatant of the hemoglobin as cyanmethemoglobin.

Because of the results of the pH-hemolysis curves (see below), except where otherwise stated, the pH of serum was adjusted to 6.1 in all experiments with altered red cells, whereas in tests with PNH red cells the pH of serum was adjusted to 6.5.

Our experiments were carried out according to the Authors hereinafter cited: the pH-hemolysis curves according to Dacie et al.,⁶ the thrombin tests (Thrombin Hoffmann-La Roche & Co., final concentration 100 N.I.H. units/ml.), the tests with inactivated serum and with heparin and the dextran tests (Macrodex Don Baxter Inc.) according to Crosby et al.^{7,8,9,10}

The role of Ca⁺⁺ and Mg⁺⁺ in the hemolytic system was studied on citrated serum and on oxalated serum according to Clapp et al.¹¹ The tests of sensitivity to iso-antibodies¹² were performed with selected anti-A and anti-B sera. The Hegglin-Maier tests¹³ with altered red cells were carried out as follows: 1 ml. of packed red cells were placed in a test tube together with 3 ml. of compatible whole blood from a healthy donor withdrawn at the moment and without adding of anticoagulants. The mixture was thoroughly mixed and immediately incubated at 37 C. for 60 minutes. At the end of this period the blood had coagulated and the hemolysis was evaluated, comparing the serum with the supernatant of the control which consisted of 1 ml. altered red cells incubated at 37 C. for 60 minutes in 3 ml. of unacidified serum (from the same donor).

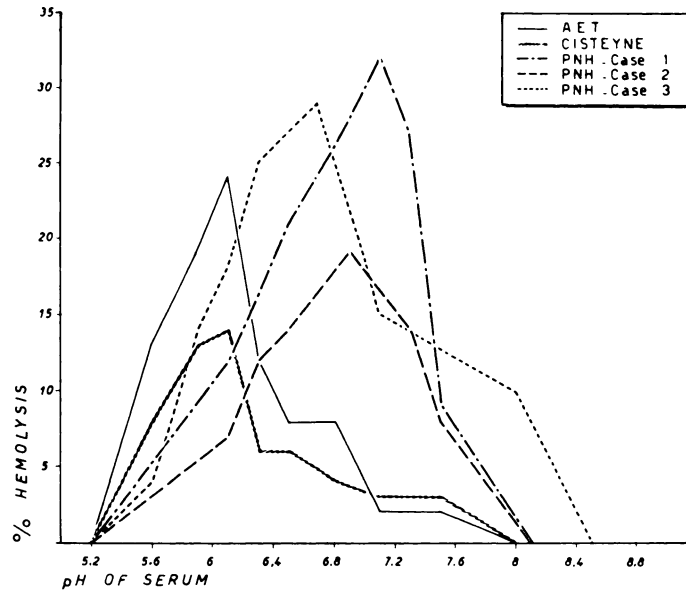


Fig. 1.—pH-hemolysis curves of PNH and altered red cells.

RESULTS

In order to better illustrate our results, we report in parallel the *in vitro* behavior of normal red cells treated with AET, of normal red cells treated with cysteine and of red cells from three cases of PNH that we had the opportunity to study. The best known methods of investigation performed in PNH are taken into account.

(1) *pH-hemolysis curve.* Figure 1 is a graphic representation of the percentage hemolysis of red cells incubated with compatible normal serum at different pH values. The optimal pH for hemolysis of altered red cells was in the region of pH 6.0, whereas hemolysis did not occur if the medium was too acid. Figure 2 shows the pH-hemolysis curve with addition of thrombin. Comparing figures 1 and 2, it can be seen that the course of the curves is very similar, and that the addition of thrombin intensifies hemolysis.

(2) *Hegglin-Maier test.* As in the case of PNH, the test was always positive with altered red cells, although the intensity of the hemolysis varied according to the various red cells used.

(3) *Test of sensitivity to iso-antibodies.* Unlike what occurs with PNH red cells, the results with altered red cells appeared not constant, in spite of the fact that altered red cells showed a certain aptitude to be hemolyzed by anti-A and anti-B agglutinins. Moreover, in those cases where hemolysis occurred, this was limited to the first dilutions of the antisera, whereas agglutination continued up to much higher dilutions.

(4) *The role of complement in the hemolytic system.* Altered red cells do not undergo hemolysis in acidified compatible normal serum heated to 56 C. for 30 minutes, similarly to what occurs for PNH red cells.¹⁴ Figure 3 illus-

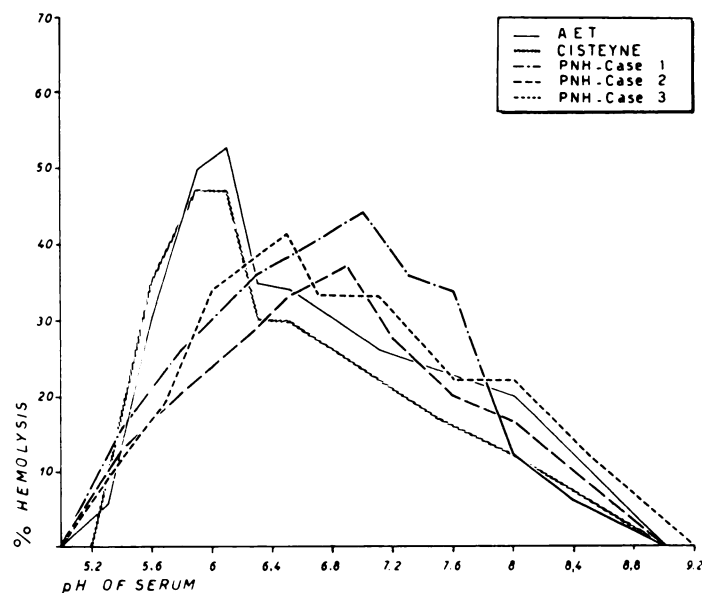


Fig. 2.—pH-hemolysis curves of PNH and altered red cells when thrombin is added.

trates the behavior of red cells incubated with varying proportions of heated and fresh serum. Similar curves were obtained by performing the same experiment with the addition of thrombin; only the hemolysis percentages were higher.

Most probably on account of its anticomplementary effect, we observed that sodium thiosulphate inhibits hemolysis of PNH red cells; the addition of decreasing quantities of 0.6 M sodium thiosulphate to the serum (final concentrations of 60, 30, 15, 7.5 and 3.75 mM/L) inhibits also hemolysis of altered red cells, in a similar way. Similar curves were also obtained by performing the same experiment with the addition of thrombin; in this case, the hemolysis percentages were higher.

It was observed that also heparin exerts a similar action using PNH and altered red cells.

(5) *The role of Ca^{++} and Mg^{++} in the hemolytic system.* The addition to the serum of sodium citrate or sodium oxalate inhibits hemolysis of both PNH¹⁵ and altered red cells. For both cells, the addition to citrated serum of progressive quantities of Ca^{++} (final concentrations of 0.625, 1.25, 2.5, 5, 10 and 20 mM/L) does not restore the hemolytic activity of serum. On the contrary, the addition of progressive amounts of Mg^{++} (final concentrations of 0.625, 1.25, 2.5, 5, 10 and 20 mM/L) does restore the hemolytic activity of serum: optimal hemolysis occurs at a final concentration of 5 to 10 mM/L, beyond which any further increase of Mg^{++} causes inhibition (fig. 4).

For both cells, addition of small amounts of Ca^{++} (up to a final concentration of 3 mM/L) to the oxalated serum causes the appearance of slight hemolysis.

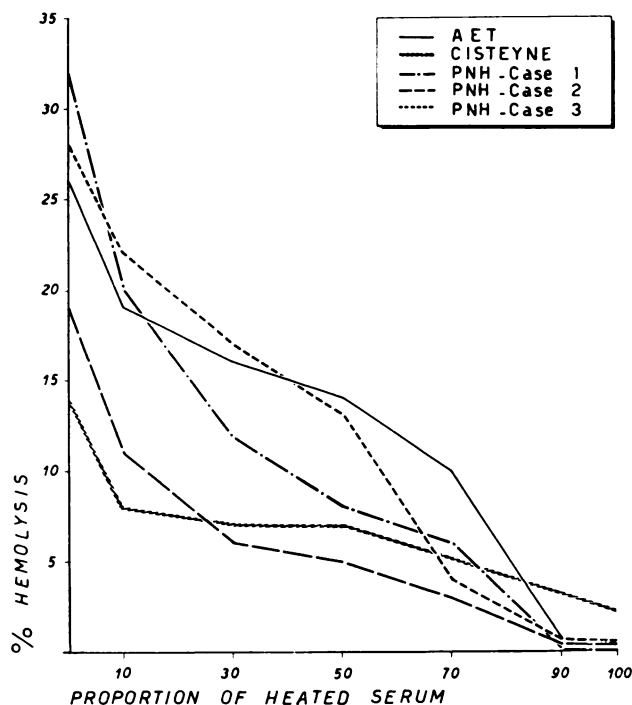


Fig. 3.—The effect of heat on the activity of serum against PNH and altered red cells. Where 90 per cent heated serum is indicated, 10 per cent fresh serum is present.

ysis; by contrast, higher concentrations (exceeding a final concentration of 3 mM/L) inhibit the hemolysis. Addition to the oxalated serum of progressive amounts of Mg^{++} restores the hemolytic activity of serum: optimal hemolysis occurs at a final concentration of 5 to 10 mM/L, beyond which any further increase of Mg^{++} causes inhibition (fig. 5).

(6) *Influence of dextran on the hemolytic activity of serum.* The addition to serum of increasing concentrations of clinical dextran has led to progressive inhibition of the hemolysis of PNH and, although to a lesser extent, of altered red cells.

Depending upon the different sera used, a varying susceptibility to hemolysis of the same altered red cells was observed, just as in the case of PNH red cells.¹⁶ Likewise, altered red cells from different healthy donors have shown a different susceptibility to hemolysis caused by the same serum, as can be observed when incubating the same serum with the red cells of different patients suffering from PNH.¹⁷

DISCUSSION

From the above referred data, it is possible to observe that treatment of normal human red cells with AET or cysteine changes them in such a way that their behavior in in vitro hemolysis tests is similar to that of PNH red cells. In fact, in both cases:

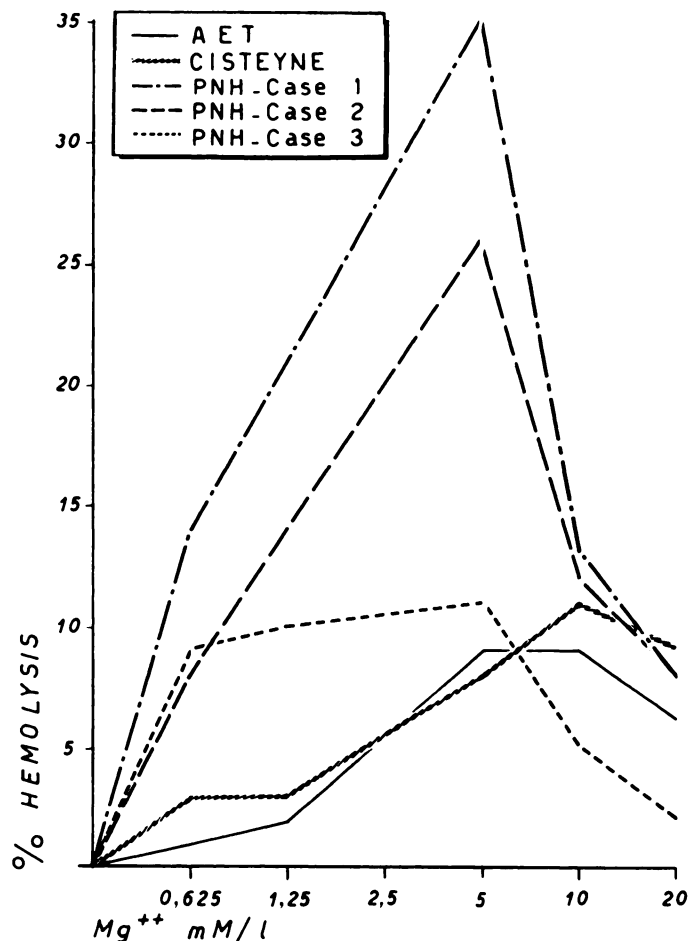


Fig. 4.—The effect of increasing concentrations of Mg^{++} on PNH and altered red cells lysis in the Ham test performed with citrated serum.

- (a) Red cells hemolyze in acid compatible normal sera.
- (b) Hemolysis is enhanced by thrombin.
- (c) Hemolysis does not occur in heat-inactivated acid serum (or in serum inactivated by addition of heparin or sodium thiosulphate) even if thrombin is added.
- (d) Mg^{++} is indispensable for hemolysis, whereas Ca^{++} does not seem so strictly necessary. Excess of Mg^{++} and/or Ca^{++} inhibits hemolysis.
- (e) Clinical dextran inhibits hemolysis and the degree of inhibition is proportional to dextran concentration.
- (f) All the compatible normal sera used caused hemolysis and the intensity of the latter differed from serum to serum. Red cells from different persons have shown different susceptibility to hemolysis caused by the same serum.
- (g) The Hegglin-Maier test is positive.
- (h) Red cells do not agglutinate in compatible normal sera.

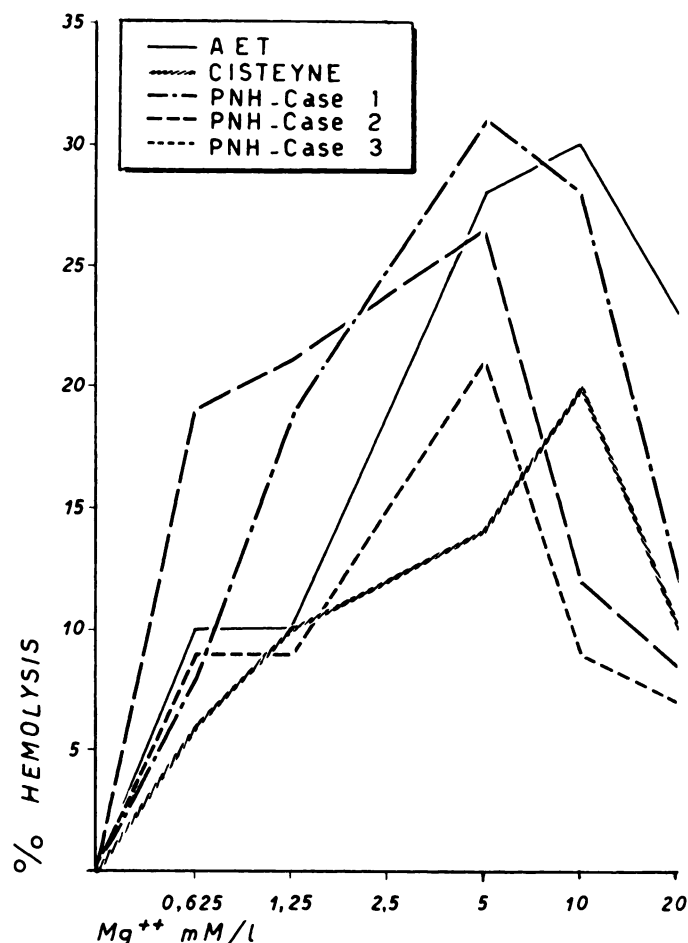


Fig. 5.—The effect of increasing concentrations of Mg^{++} on PNH and altered red cell lysis in the Ham test performed with oxalated serum.

The behavior of altered red cells in the *in vitro* hemolysis tests differs from that of PNH red cells because of the following aspects:

- pH-hemolysis curve: PNH red cells hemolyze in compatible normal sera with an optimum around pH 7.0. Altered red cells hemolyze in compatible normal sera with an optimum around pH 6.0.
- Inhibition of hemolysis with clinical dextran: this is more marked in the case of PNH than of altered red cells.
- Test of sensitivity to iso-antibodies: the results of this test appear inconstant and unsatisfactory with altered red cells, whereas it is always positive with PNH red cells.

In regard to the mechanisms involved in the modification of the erythrocytes by means of AET and cysteine, we feel that this change is due to the -SH groups of the two substances. More specifically, if oxidation of their -SH groups with formation of S-S bonds occurs as a result of the adjustment

to pH 8.0 of the solutions,¹⁸ the resulting compounds might alter the erythrocytes by oxidizing the -SH groups of the stroma. With regard to this, it is interesting to note that Vaccari and Baldini¹⁹ and Resegotti and Givone²⁰ have reported that PNH red cells show a diminution of stromal -SH groups.

SUMMARY

Treatment of normal human red cells with AET and cysteine, under suitable experimental conditions, modifies them in such a way that their behavior in *in vitro* hemolysis tests becomes similar to that of the erythrocytes of paroxysmal nocturnal hemoglobinuria.

It is felt that alteration of the red cells is due to the -SH groups possessed by both substances. A possible mechanism of action is hypothesized.

SUMMARIO IN INTERLINGUA

Le tractamento de normal erythrocytos human con bromuro de 2-aminoethylisothiuronium e cysteina—sub appropriate conditiones experimental—modifica los in un tal maniera que lor comportamento in tests *in vitro* de hemolyse deveni simile a illo de erythrocytos ab subjectos con paroxysmic hemoglobinuria nocturne.

Es postulate que le alteration del erythrocytos es possiblementemente le effecto del gruppos SH que es presente in ambe le mentionate substantias. Un possibile mecanismo del action es presentate.

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REFERENCES

1. Rodbard, J. A.: Chronische haemolytische anaemie met nachtljke haemoglobinurie (ziekte van Marchiafava-Micheli). Thesis, University of Amsterdam, 1950. *In*: The Haemolytic Anaemias, Congenital and Acquired, J. V. Dacie, ed. London, J. & A. Churchill, 1954.
2. Ham, T. H.: Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria. Study of the mechanism of hemolysis in relation to acid-base equilibrium. *New England J. Med.* 217:915, 1937.
3. Metz, J., Stevens, R., Rensburg, N. J., and Hart, D.: Failure of *in-vivo* inhibition of acetylcholinesterase to affect erythrocyte life-span: the significance of enzyme defect in paroxysmal nocturnal haemoglobinuria. *Brit. J. Haemat.* 7:458, 1961.
4. De Sandre, G., Ghiotto, G., and Perone, G.: The acetylcholinesterase activity of paroxysmal nocturnal haemoglobinuria erythrocytes (abstract). Communication to the 9th Cong. European Soc. Haemat., Lisbon, 1963.
5. Yachnin, S., Laforet, M. T., and Gardner, F. H.: pH dependent hemolytic system. I. Their relationship to paroxysmal nocturnal hemoglobinuria. *Blood* 17:83, 1961.
6. Dacie, J. V., and Richardson, N.: The influence of pH on *in vitro* haemolysis in nocturnal haemoglobinuria. *J. Path. Bact.* 55:375, 1943.
7. Crosby, W. H.: Paroxysmal nocturnal hemoglobinuria. A specific test for the disease based on the ability of thrombin to activate the hemolytic factor. *Blood* 5:843, 1950.
8. —, and Dameshek, W.: Paroxysmal nocturnal hemoglobinuria. The mechanism of hemolysis and its relation to the coagulation mechanism. *Blood* 5:822, 1950.

9. —: Paroxysmal nocturnal hemoglobinuria. Plasma factors of the hemolytic system. *Blood* 8:444, 1953.
10. —, and Benjamin, N. R.: Paroxysmal nocturnal hemoglobinuria. The effect of dextran on hemolysis in vitro. *Acta haemat.* 19:193, 1958.
11. Clapp, M. P., Williams, M. J., and Mendel, J. L.: Observations on the hemolytic mechanism of paroxysmal nocturnal hemoglobinuria. Demonstration of the activity of at least two serum factors in PNH hemolysis. *Blood* 7:1117, 1952.
12. Dacie, J. V.: Diagnosis and mechanism of hemolysis in chronic hemolytic anemia with nocturnal hemoglobinuria. *Blood* 4:1183, 1949.
13. Hegglin, R., and Maier, C.: The 'heat resistance' of erythrocytes. A specific test for the recognition of Marchiafava's anemia. *Am. J. Med. Sc.* 207: 624, 1944.
14. Hijmans, van den Berg, A. A.: Ictère hémolytique avec crises hémoglobini-riques. *Fragilité globulaire. Rév. Méd.* 31:63, 1911.
15. Jordan, F. L. J.: Études sur l'hémoglobinurie. *Acta Med. Scandinav.* 95: 319, 1938.
16. Dacie, J. V., and Mollison, P. L.: Survival of transfused erythrocytes from a donor with nocturnal haemoglobinuria. *Lancet* 1:390, 1949.
17. Ham, T. H.: Studies on destruction of red blood cells. I. Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria: an investigation of the mechanism of hemolysis, with observations on five cases. *Arch. Int. Med.* 64:1271, 1939.
18. Clarke, H. T.: Natural amino acids. *In Organic Chemistry. An advanced treatise*, 2nd ed., H. Gilman, ed. New York, J. Wiley & Sons Inc., 1947, p. 1132.
19. Vaccari, F., and Baldini, E.: Ricerche sulle natura della lesione intrinseca dei globuli rossi nella emoglobinuria parossistica notturna. *Atti 7° Cong. Int. della Int. Soc. Haemat., Rome, 1958.* Rome, Il Pensiero Scientifico 1960, p. 445.
20. Resegotti, L., and Givone, S.: Paroxysmal nocturnal haemoglobinuria. Report of a case in a two-year-old boy. *Acta haemat.* 27:120, 1962.

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