# IMMUNE LYSIS OF NORMAL HUMAN AND PAROXYSMAL NOCTURNAL HEMOGLOBINURIA (PNH) RED BLOOD CELLS

## III. The Membrane Defects Caused by Complement Lysis*

By WENDELL F. ROSSE,‡ M.D., ROBERT DOURMASHKIN, M.D., and JOHN H. HUMPHREY, M.D.

*(From the Medicine Branch, National Cancer Institute, Bethesda, Maryland, the Department of Haematology, Postgraduate Medical School, London, the Division of Experimental Biology and Virology, Imperial Cancer Research Fund, and the Immunology Division, National Institute for Medical Research, Mill Hill, London, England)*

Plates 86 to 90

(Received for publication 7 January 1966)

The action of antibody and complement (C′) upon red blood cells results in the formation of discrete holes in the cell membrane. The existence of such holes was first inferred from experiments in which cell lysis was found to be prevented by a sufficiently high concentration of osmotically active macromolecules (1, 2). These experiments indicated that with most hemolytic systems tested, the holes must be smaller than the diffusion radius of hemoglobin and albumin.

The holes were first demonstrated directly on electron photomicrographs by Dourmashkin and Humphrey (3). When membranes of sheep red cells lysed by rabbit antibody and guinea pig complement were negatively stained, the holes were seen as distinctive defects filled with negative stain and scattered randomly over the red cell surface. In collaboration with Borsos, these workers were able to show that with the antibody, cell, and C′ used, one such hole was necessary and sufficient for lysis of the cell (4). Each defect was about 88 A in mean diameter, which corresponds well with estimates made using macromolecules.

The purpose of the present paper is to present data comparing quantitatively and qualitatively the formation of membrane defects on human red cells both from normal donors and from patients with paroxysmal nocturnal hemoglobinuria using a variety of antibodies and complements. These experiments show that the size of the hole appears to depend upon the source of the complement used and not upon the source of antibody or the type of cell. They also show that a great many holes are formed under some circumstances in

* This work was presented in part to the annual meeting of the American Federation for Clinical Research at Atlantic City, New Jersey on 2 May 1965.

‡ Present address: Department of Medicine, Duke University Medical Center, Durham, North Carolina.

which human complement is used for lysis. We suggest that this phenomenon is responsible for certain apparent differences in hole size noted by others using osmotically active macromolecules.

We have also found that under the same conditions of complement and antibody concentration, the same number of holes per membrane are found on PNH or normal cells lysed by complement when only those membranes containing holes are considered. When the amount of complement is limited more PNH cells are lysed, due to their peculiar sensitivity to the action of C′ (5). Hence a larger proportion of such cells show these holes.

## *Materials and Methods*

*Red Blood Cells.*—Human and sheep blood cells, human and rabbit antibodies, and human and guinea pig complement were obtained, stored, and prepared for use in the same manner as outlined in reference 6.

*Antibodies Used.*—(*a*) Human anti-I, an autoantibody from a patient with "idiopathic" cold agglutinin disease. (*b*) Donath-Landsteiner antibody from a patient with paroxysmal cold hemoglobinuria associated with congenital syphilis. (*c*) Rabbit antibody against purified somatic O antigen from *Shigella* which could be adsorbed to red cells for passive lysis experiments. The 19S antibody component was isolated by density gradient centrifugation (6). (*d*) Rabbit antibody to washed whole human red cells which were injected intravenously 4 times over a 2 wk period. (*e*) Purified macroglobulin anti-A antibody (kindly supplied by Dr. T. Borsos). (*f*) Rabbit antibody to sheep red cells.[1]

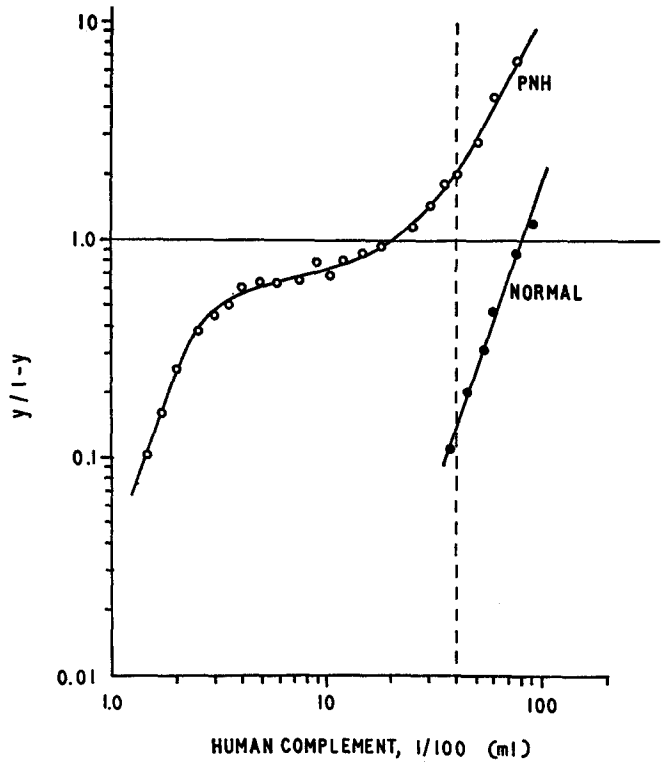
Isotonic veronal-buffered saline (VBS) with 0.00015 M $Ca^{++}$ and 0.0005 M $Mg^{++}$ was used throughout unless otherwise stated.

Red cells were lysed by antibody and complement in the same buffers, relative cell concentration, reaction volumes, and incubation times and temperatures as used in the complement lysis sensitivity test (6). ½ ml of a standard suspension of cells (containing about $2 \times 10^8$ cells/ml) was mixed (at 0°C for cold reacting antibodies) with 0.5 ml of antibody in the optimal dilution for the sensitization of normal cells (see reference 6). Complement was added and the total volume of the reaction mixture was brought up to 7.5 ml with veronal-saline buffer (VBS) (at 0°C for cold reacting antibodies). After incubation at 0°C for 12 min the tubes were placed at 37°C for 1 hr, at which time lysis was maximal.

Complement lysis sensitivity tests to determine the amount of C′ required for lysis were performed on normal and PNH cells as previously outlined (6). Equal volumes of cell suspensions containing $2 \times 10^8$ cells and antibody diluted to the "optimal" degree were mixed (at 0°C for cold reacting antibodies) and 1 ml was pipetted into each of six tubes containing 4.0, 3.5, 3.0, 2.5, 1.5, and 0.5 ml of VBS. Fresh human serum diluted appropriately was added to each tube in sufficient quantity to bring the total volume of 7.5 ml (a diltuion of 1/3 to 1/6 was used for testing normal cells; because of the wide range of sensitivity of PNH cells dilutions of 1/100, 1/33.3, 1/10, and 1/3.33 were used in four groups of 6 tubes each when these cells were tested). The mixtures were incubated at 0°C for 12 min (for cold reacting antibodies) and then at 37°C for 60 min. They were centrifuged and the optical density of the supernatant fluid at 541 mμ was determined. Corrections for complement color and spontaneous lysis were applied and the fraction lysed, $y$, determined. The logarithm of $[y/(1 - y)]$ was plotted against the logarithm of C′ concentration. The curves of normal and PNH cells used in these tests are shown in Text-fig. 1.

[1] Obtained from Staynes Laboratories, Inc., High Wycombe (Bucks), England.

Membranes of cells treated with antibody and complement were prepared for electron microscopy as follows (7, 8). For experiments using 2% potassium phosphotungstate at pH 6.4, the membranes were first sedimented in an isosmotic buffer, then washed in 0.01 M phosphate buffer at pH 6.4 and resedimented. Sedimentation in each case was carried out in a

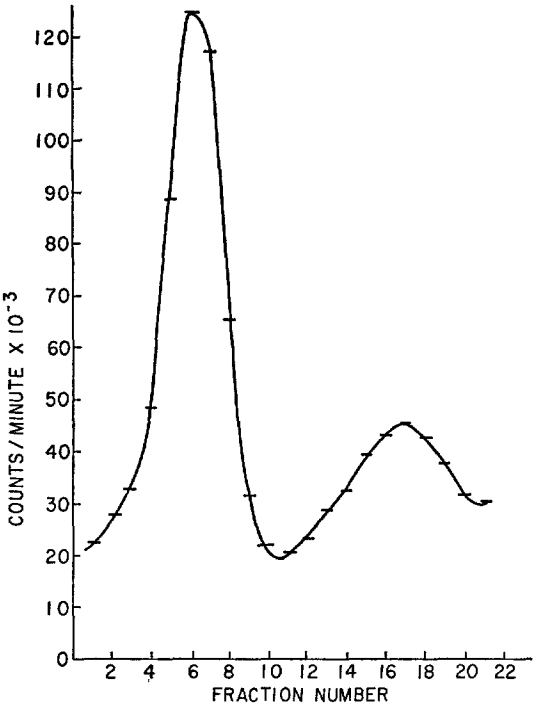



TEXT-FIG. 1. The lysis of normal and paroxysmal nocturnal hemoglobinuria (PNH) cells sensitized with anti-I antibody and lysed with human complement. The logarithm of the complement concentration is plotted against the logarithm of the lysed fraction, $y$, divided by the unlysed fraction, $(1 - y)$. The straight line seen when normal cells are used indicates that these cells consist of a single population which is relatively insensitive to complement lysis. The complex curve seen when PNH cells are used indicates that these cells consist of two populations, one of which is sensitive, the other less sensitive to C′ lysis. The dashed line indicates the level of C′ used in the experiments illustrated in the lower part of Table II and in Text-fig. 3.

Spinco untracentrifuge at 100,000 $g$. In experiments using 4% sodium silicotungstate, the membranes were washed the second time with distilled water. In either case, the sedimented membranes were resuspended in varying amounts of the negative stain, depending on the amount of material present, and sprayed with a fine nebulizer on carbon-coated copper grids. (The carbon film was prepared by evaporation on freshly cleaved mica *in vacuo* and stripped on a water

bath.) Spraying tended to separate the membranes into small fragments permitting better visualization of the membrane defects. No fixatives were employed in these experiments.

For counting holes, membrane fragments were selected at random for photography at a magnification of 20,000. The total number of holes in each piece of membrane photographed was counted using a low power microscope and a ruled grid. The total area of the fragments examined was determined by planimetry. The mean number of holes per square micron of red cell surface was determined from the ratio of total holes counted to the total area measured.




TEXT-FIG. 2. The distribution of $I^{131}$-labeled protein following sucrose gradient ultracentrifugation of partially purified anti-I antibody. The macroglobulin peak (fractions 2 to 9) contained protein 78% of which had antibody activity and was used in the experiments estimating antigen sites number.

Alternatively, where large numbers of holes were present, the holes in a 0.5 cm square (an area equivalent to $\frac{1}{16}$ square micron of cell surface) was counted. Each fragment of cell membrane seen on the electron micrographs was tallied according to the concentration of holes on the cell membrane surface. Histograms were drawn representing the number of fragments of membrane showing similar concentrations of holes.

For each different combination of red cell, antibody, complement tested, the greatest and least diameter of 20 holes on different fragments of red cells were determined by measurement on carefully calibrated photographs with a fine rule and magnifying glass. The mean diameter was calculated by averaging these measurements. The total area of the red cells surface as seen in the electron microscope was calculated from low power (5000 ×) electron micrographs of cell membranes lysed by gradual osmotic lysis. Normal or PNH cells were suspended in

0.1 M NaCl and placed in dialysis bags. The suspension was dialyzed against 0.025 M NaCl for 1 hr. The heaviest (i.e. most complete) membranes were removed by slow centrifugation, washed in 0.045 M NaCl, suspended in neutral potassium phosphotungstate, and applied to carbon-coated grids with a fine pipet. The areas were measured by planimetry and the result doubled to include the membrane of both sides of the cell. The area of normal and PNH cell membranes obtained in this way was 117 $\mu^2$ and 122 $\mu^2$ respectively.

*Determination of Anti-I Sites.—*

The number of anti-I antigen sites per red cell was estimated by the technique of Hughes-Jones, Gardner, and Telford (9). Purified anti-I antibody was prepared by reacting 2 ml of high titer antiserum at 0°C for 1 hr with 2 ml of red cell stromata. The stromata were prepared by gradual osmotic lysis of normal human cells and were washed 4 times with water and once with 0.14 M NaCl. After the completion of the incubation, the stromata and antibody were washed with ice cold saline 3 times, and the antibody was then eluted by incubating at 37°C for 30 min and centrifuging at 37°C to remove the stromata. The proteins of the supernatant were labeled with $I^{131}$ by a modification of the iodine monochloride method of McFarlane (10). Approximately 1 g atom iodine$^{131}$ was added per 100,000 g protein. The proteins were then centrifuged twice in a sucrose gradient 30 to 10% w/v in 0.1 M phosphate buffer at pH 7.0 at an average of 41,000 *g* for 15 hr at 13°C. The radioactivity due to $I^{131}$ in fractions of 40 drops each was determined in a well type scintillation counter. The fraction with a sedimentation coefficient of about 19S was collected, concentrated by pressure dialysis, and centrifuged again under the same conditions. The distribution of radioactivity after the second centrifugation is shown in Text-fig. 2. Samples containing the macroglobulin peak (fractions 2 to 9) were pooled and concentrated by pressure dialysis against veronal-saline buffer. The optical density of the solution was read at 280 m$\mu$ on a Unicam spectrophotometer. The protein concentration was estimated assuming that each unit of optical density corresponds to 0.7 mg of protein. The number of protein molecules was estimated assuming that each mg of macroglobulin contains $6.7 \times 10^{14}$ molecules and that the molecular weight of the macroglobulins is 900,000.

Dilutions of antibody were mixed with $2.2 \times 10^7$ red cells (normal) or $1.7 \times 10^7$ (PNH) at 0°C for 2 hr in a total volume of 1.0 ml. After centrifugation, the supernatant fluid was removed carefully and the cells were lysed with 1.0 ml of 0.01% $Na_2CO_3$. The radioactivity of the superantant and the lysed cells was determined as above.

The method of analysis of the number of antibody molecules able to be accommodated on antigen sites was that used by Hughes-Jones et al (9).

If $N$ = total number of cells in the system
$M$ = maximal fraction of labeled immunoglobulin which could be attached to red cells in great excess

then, assuming that all the immunoglobulin (Ig) molecules are an average equally labeled,

$$T = \text{molecules of antibody/ml} = \text{mg protein/ml} \times M \times 6.7 \times 10^{14}.$$

At each dilution of antibody the following parameters were measured where CPM = counts of $^{131}$I/min above background.

$$r = \text{number of attached antibody molecules/cell.}$$
$$= \text{CPM cells} \times \text{number of antibody molecules per count} \times 1/N$$
$$= \frac{\text{CPM cells}}{N} \times \frac{\text{total number of Ig molecules} \times M}{\text{total number of counts} \times M}$$
$$= \frac{\text{CPM cells}}{N} \times \frac{T \times \text{volume of Ig}}{\text{CPM total} \times M}$$

$A$ = number of free antibody molecules in the system.

= total antibody molecules − antibody molecules on cells

$$= T \times \text{volume of Ig} - \frac{\text{CPM cells} \times T \times \text{volume of Ig}}{\text{CPM total} \times M}$$

$$= T \times \text{volume of Ig}\left(1 - \frac{\text{CPM cells}}{\text{CPM total} \times M}\right)$$

$$r/A = \frac{\text{CPM cells}}{N(\text{CPM total} \times M - \text{CPM cells})}$$

## RESULTS

*Electron Microscopy of Defects Produced by Complement on Human Red Cells.* —The defects produced by human and guinea pig complement in the membrane of human red cells, both from normal donors or from patients with paroxysmal nocturnal hemoglobinuria are illustrated in Figs. 1 to 8. Fragments of human cells lysed with human complement showed defects ("holes") filled with negative stain, which were round to ovoid in outline and scattered without order over the surface of the fragment. These varied in size from 93 to 112 A in diameter. Human erythrocytes lysed with guinea pig complement showed defects identical to those previously described from sheep cells lysed with guinea pig complement. They were surrounded by a "ring" of about 20 A thickness; occasionally, the ring had a double contour and small projections

TABLE I

*The Size of the Membrane Defects Produced by the Action of the Complement on Sensitized Red Cells*

| Cell | Antibody | Complement source | Mean diameter of membrane defects | SD |
|---|---|---|---|---|
| | | | *A* | |
| Normal human | Anti-I | Human | 99.7 | 5.6 |
| PNH | Anti-I | Human | 100.2 | 4.2 |
| PNH | Normal serum* | Human | 101.3 | 6.0 |
| PNH | Donath-Landsteiner | Human | 97.7 | 5.6 |
| PNH | *Shigella*-anti-*Shigella* | Human | 103.8 | 5.6 |
| Normal human | Anti-A | Human | 104.0‡ | — |
| Normal human | Rabbit-anti-human red cell | Human | 102.8‡ | — |
| PNH | Anti-I | Guinea pig | 88.0 | 5.1 |
| Sheep | Rabbit(anti-Forssman) | Guinea pig | 88.2 | 5.0 |
| Sheep | Rabbit(anti-Forssman) | Rabbit | 88.6 | — |

* Cells lysed by acidified normal human serum.

‡ Electron microscopy kindly performed by Dr. Brian Bull, Clinical Center, National Institutes of Health.

surrounded the ring in an asymmetric fashion. On the edge of the membrane fragments, the defects could be seen as indentations and parts of the rings could be seen as strands extending from the rims of the defects. These defects were 80 to 100 A in diameter, which was significantly different from those produced by human complement; but were qualitatively similar to the defects produced by human C′ in all other respects. Such defects were not seen in cells treated with antibody and C′ in the presence of 0.009 M EDTA. When partial lysis occurred, these defects were not seen in the unlysed cells.

As shown on Table I, the mean diameter of the membrane defects produced by human complement was the same regardless of whether normal or PNH cells were used and regardless of whether the antibody used for sensitization

TABLE II

*The Number of Membrane Defects Produced in Normal Human and PNH Cells Sensitized with Anti-I Antibody and Lysed with Human Complement (C′)*

| Cell | C′ | Cells lysed | Fragments* with membrane defects | Mean No. of defects per cell* |
|---|---|---|---|---|
| | *ml* | % | % | |
| Normal | 3.5 | 96 | 89 | 92,000 |
| PNH | 3.5 | 100 | 97 | 85,000 |
| Normal | 0.4 | 11 | 12.5 | 9,800 |
| PNH | 0.4 | 67 | 70 | 52,000 |

* Calculated from analysis of *all* membrane fragments whether or not they contained holes.

was autologous (anti-I, Donath-Landsteiner) or heterologous (*Shigella*- anti-*Shigella* system); 7S IgG (Donath-Landsteiner) or 19S IgM (anti-I, anti-*Shigella*). Further, PNH cells lysed by acidified normal serum showed the same defects as those lysed by specific antibody and human complement.

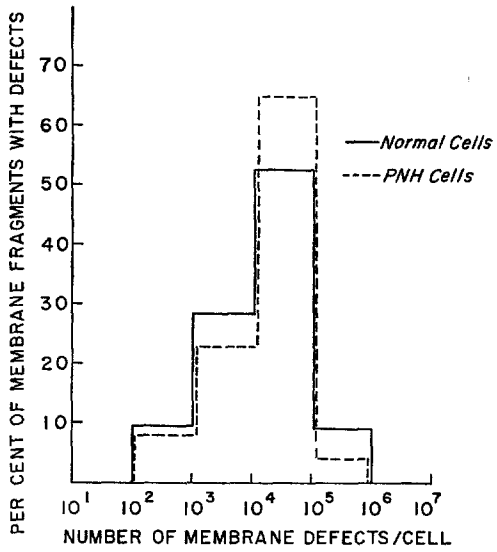
However, when guinea pig serum was used to lyse the red cells sensitized with anti-I antibody, the resultant defects were significantly smaller ($0.05 < P < 0.1$) and were the same size as those produced in sheep cells sensitized with rabbit antibody and lysed with guinea pig serum, or with rabbit serum. It appears, therefore, that minor but significant differences in the size of the defects produced in sensitized cells is more related to the type of complement used rather than the cell or antibody used.

When PNH cells sensitized with the same amount of anti-I antibody were lysed by the same amount of human and of guinea pig serum, there appeared to be many more defects formed in those cells lysed by the human serum.

*Enumeration of the Membrane Defects Produced by Complement.*—Red cell membranes from normal and PNH cells sensitized with the same amount of

anti-I antibody and lysed with different amounts of human complement were examined by electron microscopy and the number of defects produced per cell membrane was enumerated and calculated. The results are tabulated on Table II and Text-fig. 3.

The proportion of fragments showing holes depended on the percentage of cells lysed; since more PNH cells were lysed because of their sensitivity to C′ lysis, a larger percentage of fragments showed holes. However, the numerical



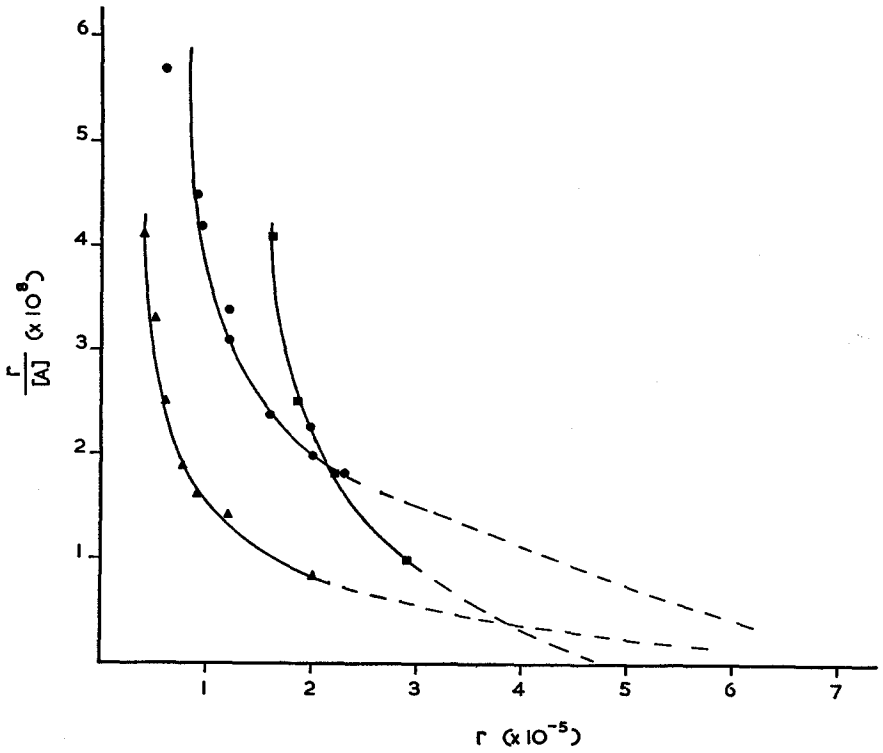

TEXT-FIG. 3. The numerical distribution of holes on membranes of normal and PNH cells lysed by anti-I antibody and limiting amounts of complement. The number of holes per membrane is plotted on a logarithmic scale against the per cent of those membranes having holes which have that number of holes.

distribution of holes on the membranes of the lysed cells was the same for both types of cells.

These findings suggest that when a human cell is lysed by human C′, a "surplus" of holes is formed and that the magnitude of this "surplus" is the same for normal and PNH cells.

*Estimation of the Number of I-antigen Sites.*—In order to relate the number of holes noted on lysed normal and PNH cells to the number of antibody molecules which could be attached to antigen sites on the cell surface, an estimation of the number of available antigen sites for the anti-I antibody on the red cell surface was made, using the method of Hughes-Jones, Gardner, and Telford (9). This method depends upon estimations of the proportion of antibody attached and free at different concentrations of antibody. If the number

of molecules of antibody attached to each red cell is plotted against this number divided by the number of free antibody molecules at several different initial concentrations of antibody, a line results which is theoretically straight but is, in all systems so far tested, curved (9, 11). The curvature is thought to be due to heterogeneity of the antibody molecules with respect to the equilib-

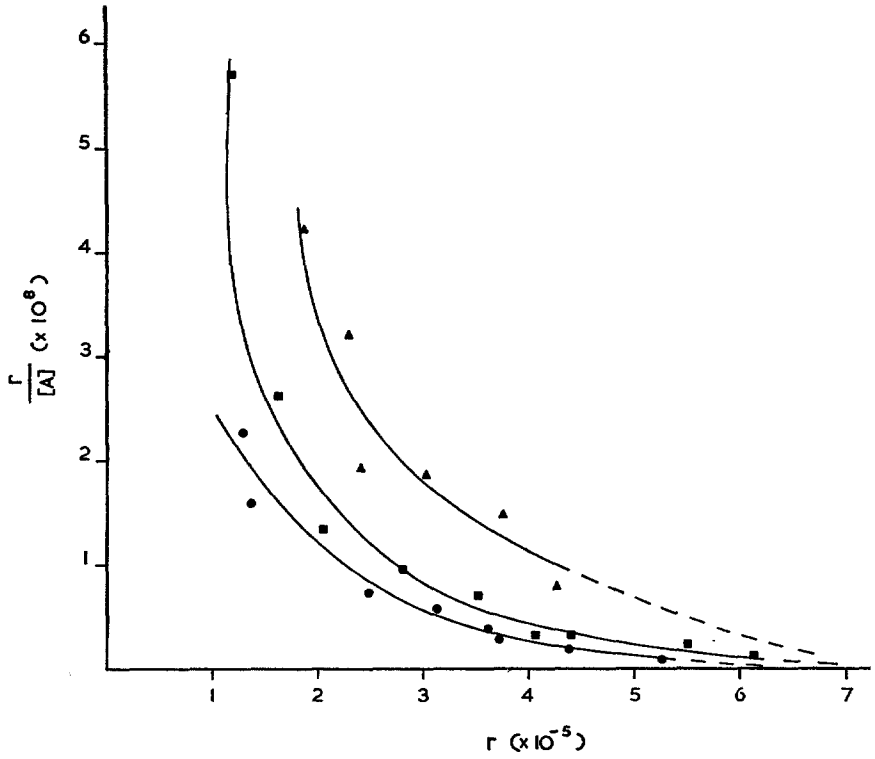



TEXT-FIG. 4. The estimation of the number of anti-I antibody molecules which may theoretically combine with normal red cells from three donors. $r$ = the number of molecules of antibody attached per cell, and $A$ = the number of antibody molecules free at equilibrium. The analysis of the data is by the method of Hughes-Jones, Telford, and Gardner (9).

rium coefficient of attachment to antigen. If the curve is extrapolated to the point where all the antibody is theoretically attached ($r/A = 0$), the estimate of the total number of potential available sites of antibody attachment are given.

The results of such experiments using anti-I antibody and both normal and PNH cells is shown in Text-figs. 4 and 5. Although it is somewhat difficult to make precise extrapolations from such curves, it is evident that the number

of anti-I antibody molecules which can be attached to either normal and PNH cells is about the same and is probably in excess of 500,000 per cell. The maximum number of holes produced by C′ is apparently not limited by the number of possible sensitized sites.




TEXT-FIG. 5. Same as Text-fig. 4 except that the red cells of three patients with paroxysmal nocturnal hemoglobinuria were used for analysis.

## DISCUSSION

The production of discrete membrane defects by the action of complement upon sensitized cells appears to be a cytolytic effect of complement. Such holes have been demonstrated in red cell membranes, Krebs ascites tumor cells, mast cells, and bacterial cell walls (3, 12, and unpublished data). The size of the defect made in these cases by guinea pig complement is relatively constant.

In human red cells, no significant difference can be seen in the size of the holes produced in the membranes of sensitized normal and PNH red cells. This would suggest that the mechanism producing the holes in these two

types of cells was probably similar and that differences in sensitivity to complement of these two types of cells are more likely due to differences in the efficiency of complement in producing these defects, as suggested in a previous paper (5).

Lysis of PNH cells in acidified serum produced holes in the membrane which are entirely similar to those produced by specific antibody and C′. Ham and Dingle (13) as well as later workers (14) have shown that procedures which destroy components of complement destroy the ability of serum, when acidified, to lyse PNH cells. These findings suggest that lysis in acidified serum in vitro and by extension hemolysis in vivo of PNH cells is due to the action of complement.

The defects produced in human red cell membranes by human complement appear to be the same regardless of the antigen or antibody involved. In the present experiments, a heterologous antigen and antibody produced the holes of the same size as both a 19S macroglobulin antibody (anti-I) and a 7S autoantibody (Donath Landsteiner antibody) as well as an unknown "antibody" or sensitizer present in acidified normal serum. It is unlikely that the cold reacting antibodies (anti-I, Donath Landsteiner) could influence the formation of the hole since under the conditions of the test, the antibody is probably dissociated from the cell at the time the hole is made. In both cases, cells, complement, and antibody are incubated at 0°C for a period during which time no lysis takes place; they are then incubated at 37°C, during which time the antibody rapidly dissociates from the cell (15) and complement complex (16) and the cells are lysed. It seems, therefore, unlikely that the nature of the antibody could have any influence on the nature of the defect produced by complement.

The only variation in size of the membrane defect produced by complement was noted when guinea pig and rabbit complement were used to lyse red cells. In this instance, the hole is significantly smaller than that produced by human complement under the same conditions. Membranes lysed by guinea pig C′ were also different from those lysed by human C′ in that for the same amount of antibody and of fresh serum, the number of holes produced by guinea pig C′ was much less than that produced by human serum. In a previous paper, it was shown that normal cells were relatively more sensitive to guinea pig serum compared to human complement than PNH cells (6). These differences suggest that the mechanism of defect production by guinea pig complement may be different in detail from that of human complement. In any event, the size of the hole produced by immune lysis in human red cell membranes is most probably related to the complement used rather than to the antibody, the antigen or the cells.

By the ingenious use of albumin, dextran, or hemoglobin in the external medium, Sears, Weed, and Swisher predicted that the effective diameter of

the holes produced by human complement on cell sensitized with antibody to whole human cells produced in the rabbit was about 65 A (2). The present finding that the holes as visualized on electron microscopy are about 100 A in diameter are not in exceptional disagreement with these findings if the effect of the absorbed ionic cloud in diminishing the effective diameter of a small aperture for penetration by albumin and dextran on the one hand and possible slight alterations of the size of the hole during the process of preparation for electron microscopy on the other are considered.

However, these authors have shown that using certain antibodies and human red cells, holes larger than the diffusion radius of albumin are formed. The argument used in drawing these conclusions is as follows. If the defect is smaller than the diffusion radius of hemoglobin, lysis is thought to be due to osmotic disequilibrium following the free passage of ions through the defects. The hemoglobin within the cell exerts osmotic pressure, resulting in a net influx of water which caused the cell to swell and burst. The influx of water may be counteracted if an osmotically active macromolecule which is unable to pass the hole is present in the external medium in sufficient concentration to balance the osmotic effect of the hemoglobin.

When Sears et al. lysed human cells with a rabbit antiwhole cell antibody and human C′, albumin and hemoglobin prevented the lysis of the cells. When anti-A antibody was used, these substances did not prevent the lysis of the cells, suggesting that holes large enough to permit the inward diffusion of albumin and/or the direct leak of hemoglobin had been formed. They suggested that the differences in size might be due to difference in the quality of the antibody molecule or antigen site. The findings here presented (Table I) indicate that the size of the primary defect is the same when either of these two antibodies are used for lysis. Therefore, it appears likely that the difference noted is a secondary effect and is probably related to the number of holes generated rather than to the size of the primary defect. If more holes were made by the anti-A antibody, then holes large enough to permit the passage of albumin and hemoglobin could result from tearing of the membrane between holes or the near superposition of primary holes.

The experiments of Frank, Rapp, and Borsos (17) indicate that the lysis of sheep cells sensitized with rabbit antibody to the Forssmann antigen and treated with guinea pig complement can be prevented by albumin, suggesting that the number of holes so produced is relatively small. This is in accord with the present finding that guinea pig complement does not produce the great excess of holes that human complement does under the same conditions. The reasons for variations in the number of holes in different lytic systems remains to be investigated.

The enumeration of holes caused by the action of complement shows a marked difference between the sheep cell lysed with guinea pig C′ and the human cell

lysed with human C′. In the former case, Borsos, Dourmashkin, and Humphrey have shown that each completed C′ sequence results in the formation of a single hole and that hole is sufficient and necessary for lysis (4). When this is the case, if 50% of the cells are lysed, then a mean of 0.7 holes per cell are present. However, when only 12% of a standard number of normal human cells are lysed by human complement, a mean of over 10,000 holes were formed per cell.

This does not mean, however, that more than one hole is necessary for lysis of the human red cell. There is no reason to believe that the "one-hit theory" of immune hemolysis (18) is not valid for human cells and complement. In all likelihood, one hole is sufficient and necessary for lysis of the cell and the other holes formed upon the completion of that C′ activation sequence are superfluous.

It is possible, despite the evidence quoted above (4), that the defects seen on electron microscopy are not true holes but are defects which only partly penetrate the membrane. Alternatively, the human red cell may be able to "repair" the hole and the repaired hole might have the same electron microscope appearance as the "unrepaired" hole. This is not likely to be the case since the repaired holes ought to be evenly distributed on all cells but the proportion of cell membranes prepared from the unlysed cells have no such membrane defects. Hence, the most likely alternative is that the completion of a single complement sequence is responsible for the formation of more than one hole although one hole would be sufficient for lysis. This effect could be due to the production of a locally transferable product responsible for hole-formation or to alterations in the cellular membrane so that subsequent holes are formed more efficiently by neighboring complement complexes or to some locally self-propagating micellar formation in the bimolecular lipid layer of the membrane. The final explanation of the excessive number of defects in human cells lysed with human complement as well as the explanation of the differences between human and guinea pig complement will depend upon a better understanding of the last stages of complement lysis.

The wide variability in the number of holes is difficult to account for. If the holes are related to the antigen sites on the cell, the number of these sites is probably not limiting in either the normal or PNH cell since there appear to be no less than 500,000 such sites in each cell. Few membranes have nearly so many holes.

It is unlikely that even under theoretically optimum conditions (that is, when each available antigen site is occupied by an antibody molecule which is able to fix all components of complement) that the number of membrane defects could conform to the number of antibody-binding sites. Assuming the area of the membrane defect (including the hole and surrounding the ring) to be $1.5 \times 10^{-4}$ sq $\mu$ (as measured in the present studies) and the number of

antigen sites per cell to be 500,000, then the total area occupied by the membrane defects if each antigen site were to produce one would come near to the total area of the red cell. Under the conditions of the present experiments where "maximal" lysis took place, about one hole for every six antibody-binding sites was produced in both normal and PNH cells.

The action of complement on the PNH cell appears to produce the same number of holes on the lysed cell as on the normal human cell. Thus, when lysis is complete (that is, when C′ is present in excess) the mean number of holes is the same for normal and PNH cells. When the amount of C′ is limited, more PNH cells are lysed due to their remarkable sensitivity to C′ and hence a larger proportion of cell membranes have holes. But the number of holes per lysed membrane is the same. It therefore appears that complement is able to produce the first hole (i.e. bring about lysis) of the PNH cell more efficiently than the normal cell but the subsequent proliferation of holes occurs similarly under the same conditions in both types of cells.

## SUMMARY

1. The defects produced on the membrane of the human red blood cell by the action of complement and antibody have been studied by the use of the electron microscope. These are round to slightly ovoid holes and are surrounded by an irregular ring, about 20 A thick. The mean diameter of the holes is about 103 A if human complement is used (regardless of the antibody used for sensitization) and about 88 A if guinea pig complement is used.

2. The holes in normal and PNH red cells appear to be identical, under the same conditions. The membrane defects produced by lysis of PNH cells with acidified normal serum (the Ham's test) are identical to those produced by complement lysis with specific antibody, indicating that complement is undoubtedly the cause of such lysis.

3. Evidence is presented that when human complement acts on human red cells sensitized with anti-I antibody, each complete activation of complement leads to the production of a cluster of holes. This contrasts to the action of guinea pig complement, on sheep cells, each activation of which leads to a single hole.

4. The maximum number of anti-I antibody molecules which can attach to a human red cell (i.e. the minimum number of antigen sites) is about 500,000 for both normal and PNH cells.

5. The number of holes produced during lysis of the PNH cell is the same as that of the normal cell. When all cells are lysed by an excess of C′, a mean of about 90,000 holes are present on each membrane. When complement is limited, a larger proportion of PNH cells are lysed due to their peculiar sensitivity to C′ but the number of holes on each lysed cell is the same as for normal cells lysed by the same concentration of C′.

The authors wish to thank Professor J. V. Dacie, Postgraduate Medical School, London, for his support and aid during the time spent by one of us (W. F. R.) in his department as Visiting Fellow. They also wish to thank Dr. Brian Bull of the Clinical Pathology Section, National Institutes of Health for his help in taking the electron photomicrographs of human cells lysed by anti-A antibody and rabbit anti-human red cell antibody which were used for estimation of hole size in these systems.

## BIBLIOGRAPHY

1. Green, H., Barrow, P., and Goldberg, B., Effect of antibody and complement on permeability control in ascites tumor cells and erythrocytes, *J. Exp. Med.*, 1959, **110,** 699.
2. Sears, D. A., Weed, R. I., and Swisher, S. N., Differences in the mechanism of *in vitro* immune hemolysis related to antibody specificity, *J. Clin. Inv.*, 1964, **43,** 975.
3. Dourmashkin, R. R., and Humphrey, J. H., in preparation.
4. Borsos, T., Dourmashkin, R. R., and Humphrey, J. H., Lesions in erythrocyte membranes caused by immune haemolysis, *Nature*, 1964, **202,** 251.
5. Rosse, W. F., and Dacie, J. V., Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. II. The role of complement components in the increased sensitivity of PNH red cells to immune lysis, *J. Clin. Inv.*, in press.
6. Rosse, W. F., and Dacie, J. V., Immune lysis of normal human and paroxysmal nocturnal hemoglobinuria (PNH) red blood cells. I. The sensitivity of PNH red cells to lysis by complement and specific antibody, *J. Clin. Inv.*, in press.
7. Brenner, S., and Horne, R. W., A negative staining method for high resolution electron microscopy of viruses, *Biochim. et Biophysica Acta.*, 1959, **34,** 103.
8. Hall, C. E., and Litt, M., Morphological features of DNA macromolecules as seen with the electron microscope, *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 1.
9. Hughes-Jones, N. C., and Gardner, B., and Telford, R., Studies on the reaction between the blood-group antibody anti-D and erythrocytes, *Biochem. J.*, 1963, **88,** 435.
10. McFarlane, A. S., Efficient trace-labelling of proteins with iodine, *Nature*, 1958, **182,** 53.
11. Hughes-Jones, N. C., Gardner, B., and Telford, R., The kinetics of the reaction between the blood-group antibody anti-c and erythrocytes, *Biochem. J.*, 1962, **85,** 466.
12. Humphrey, J. H., and Dourmashkin, R. R., Electron microscope studies of immune cell lysis, *In* Complement, (G. E. W. Wolstonholme and J. Knight, editors), London. J. & A. Churchill, Ltd., 1965, 175.
13. Ham, T. H., and Dingle, J. H., Studies on destruction of red blood cells. II. Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria: certain immunological aspects of the hemolytic mechanism with special reference to serum complement, *J. Clin. Inv.*, 1939, **18,** 657.
14. Hinz, C. F., Jr., Jordan, W. S., and Pillemer, L., The properdin system and immunity. IV. The hemolysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria, *J. Clin. Inv.*, 1956, **35,** 453.

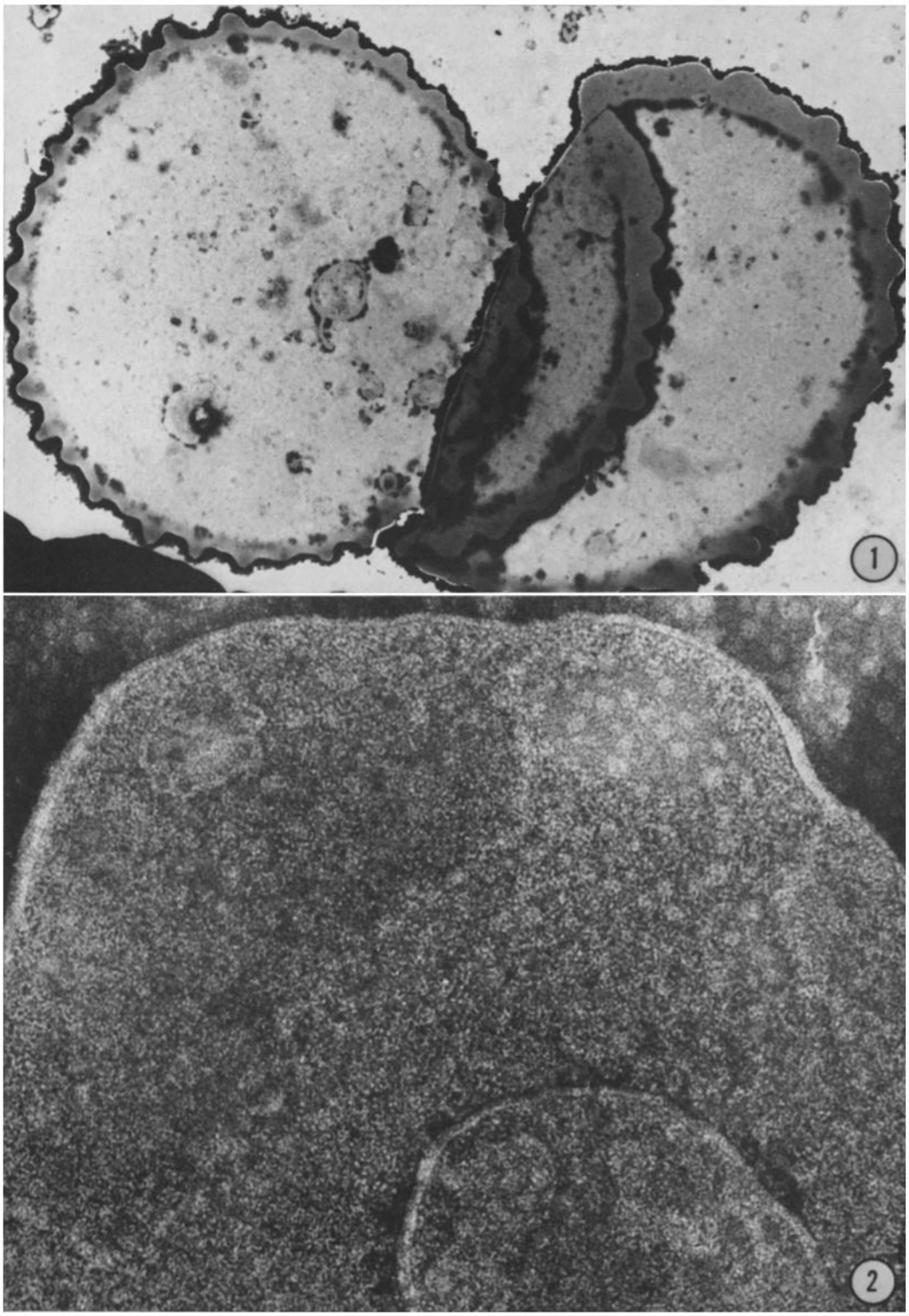
15. Evans, R. S., Turner, E., and Bingham, M., Studies with radioiodinated cold agglutinins of ten patients, *Am. J. Med.*, 1965, **38,** 378.
16. Harboe, M., Interactions between $^{131}I$ trace-labelled cold agglutinin, complement and red cells, *Brit. J. Haematol.*, 1964, **10,** 339.
17. Frank, M. M., Rapp, H. J., and Borsos, T., Studies on the terminal steps of immune hemolysis. II. Resolution of the E* transformation reaction into multiple steps, *J. Immunol.*, 1965, **94,** 295.
18. Rapp, H. J., The nature of complement and the design of complement fixation test, *in* Immunological Methods, (J. F. Ackroyd, editor), Oxford, England Blackwell Scientific Publications, 1964, 1.

## EXPLANATION OF PLATES

### PLATE 86

FIG. 1. Electron micrograph of erythrocytes from a patient with paroxysmal nocturnal hemoglobinuria. The cells were dialyzed against hypotonic saline until hemolyzed and negatively stained using 2% neutral potassium phosphotungstate. × 9400.

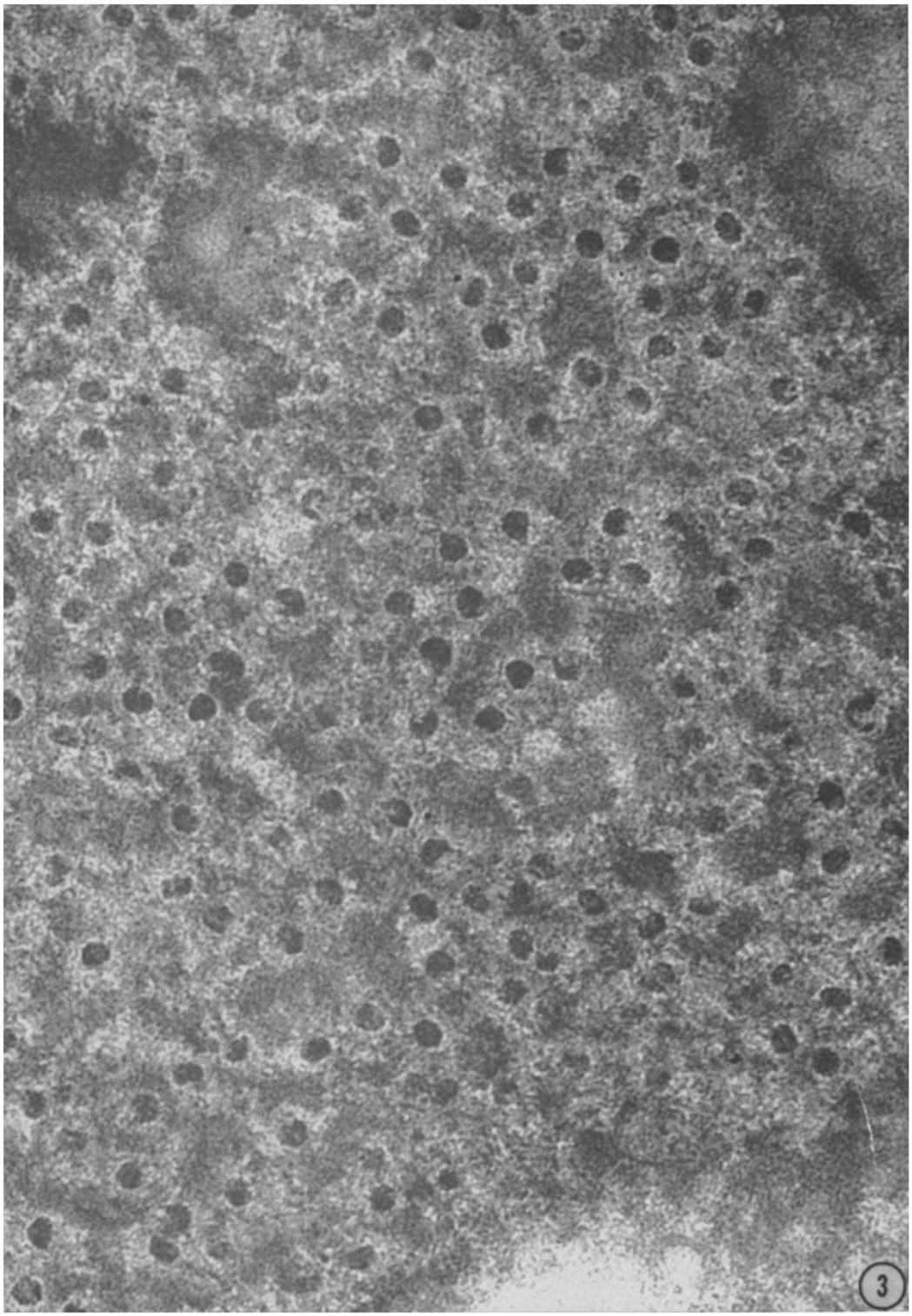
FIG. 2. Negatively stained membrane of erythrocytes from normal person. In this and subsequent photomicrographs 4% sodium silicotungstate was used as the negative stain. × 187,000.




(Rosse et al.: Immune lysis of red blood cells. III)

PLATE 87

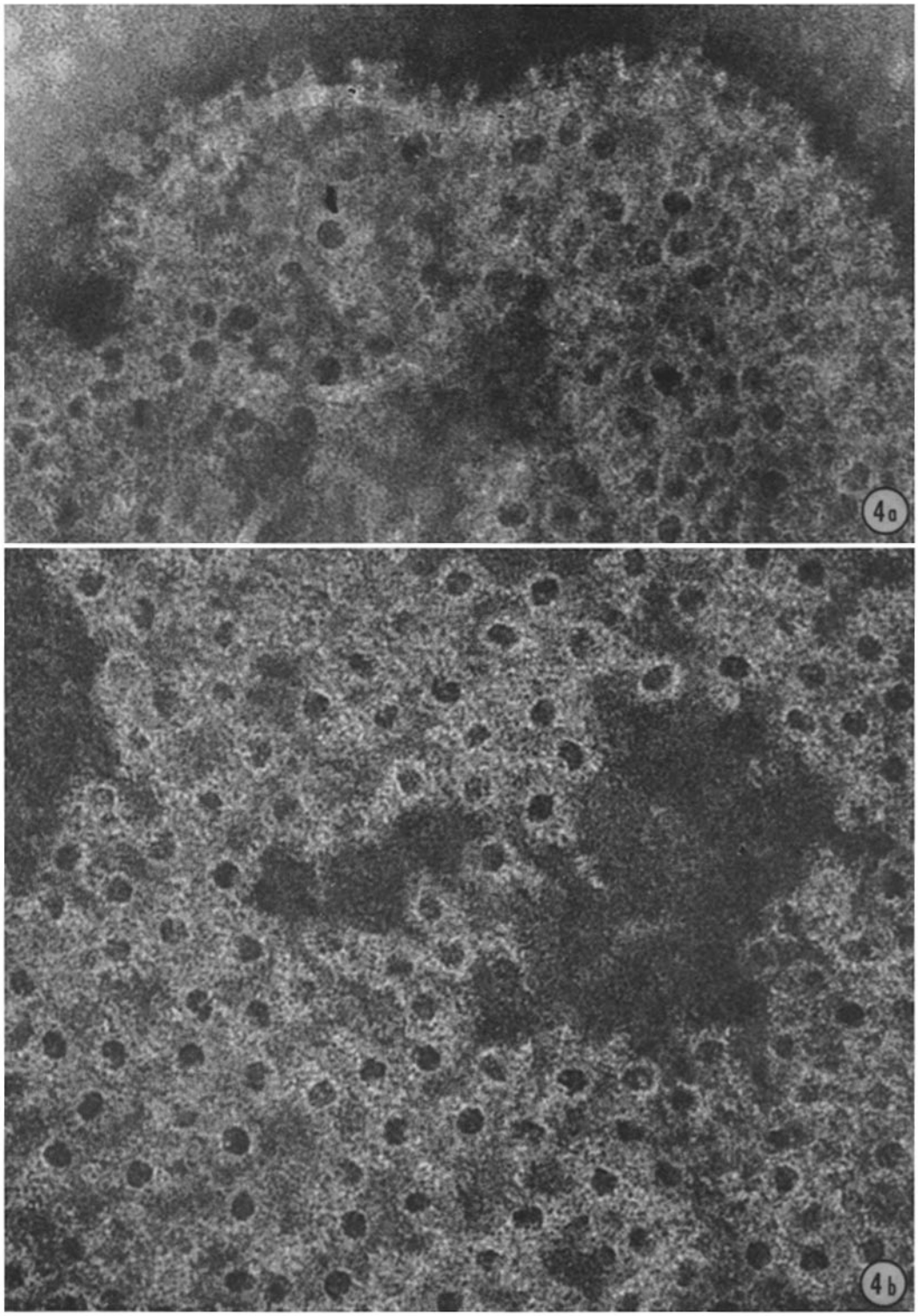
FIG. 3. A fragment of a normal red cell membrane which had been sensitized with anti-I antibody and lysed with human complement present in large amount. Large numbers of holes are seen as nearly circular dark areas filled with negative stain; surrounding each hole is a clear ring. × 374,000.




(Rosse et al.: Immune lysis of red blood cells. III)

PLATE 88

FIGS. 4 *a* and 4 *b*. A fragment of a red cell membrane from a patient with paroxysmal nocturnal hemoglobinuria; the cells had been lysed by the same amount of human C′ and antibody as those illustrated in Fig. 3 and the appearance of the holes is entirely similar. Note the indented appearance of the holes which are viewed on edge at the rolled margin of the membrane fragment. × 374,000.

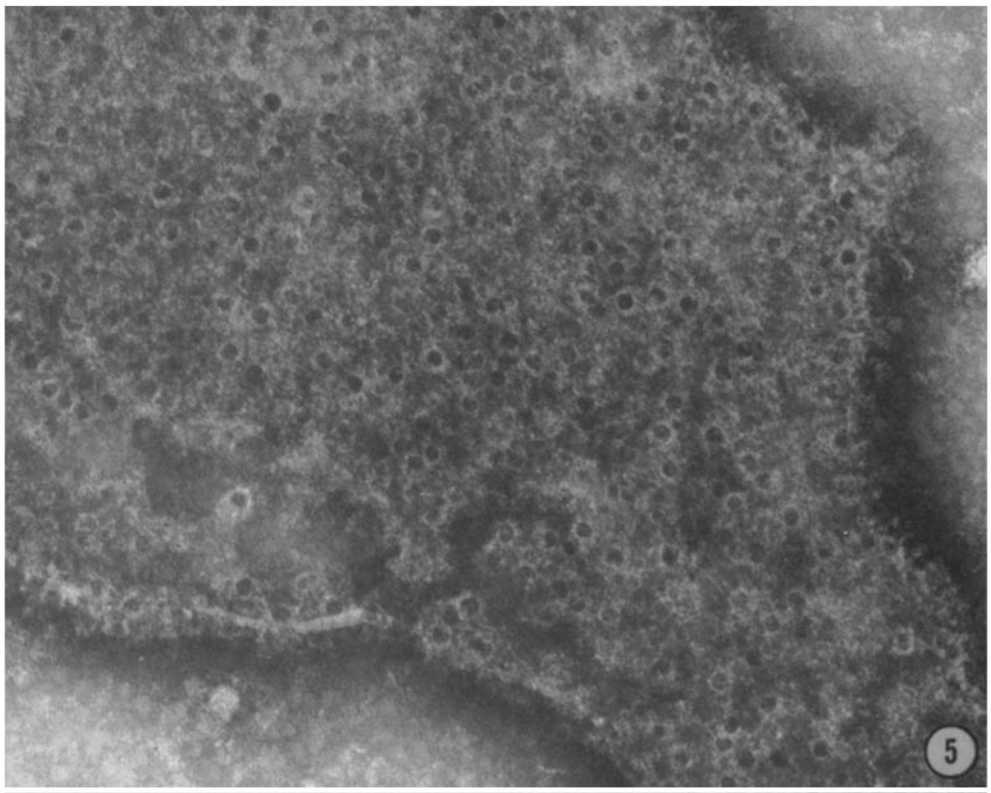


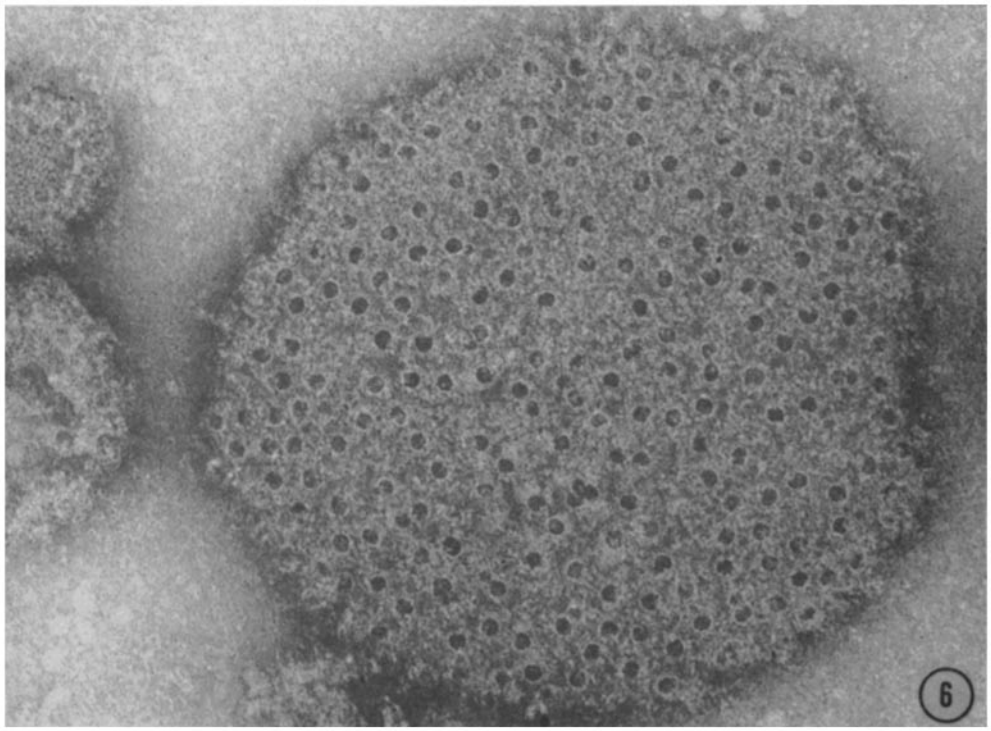

(Rosse et al.: Immune lysis of red blood cells. III)

PLATE 89

FIG. 5. Fragment of a membrane of PNH red cell lysed with human C′ and Donath-Landsteiner antibody from a case of congenital syphilis. × 187,000.

FIG. 6. Fragment of a membrane of PNH cell treated with somatic polysaccharide from *Shigella dysenteriae* and lysed by antibody produced in rabbits to this antigen and human C′. × 187,000.



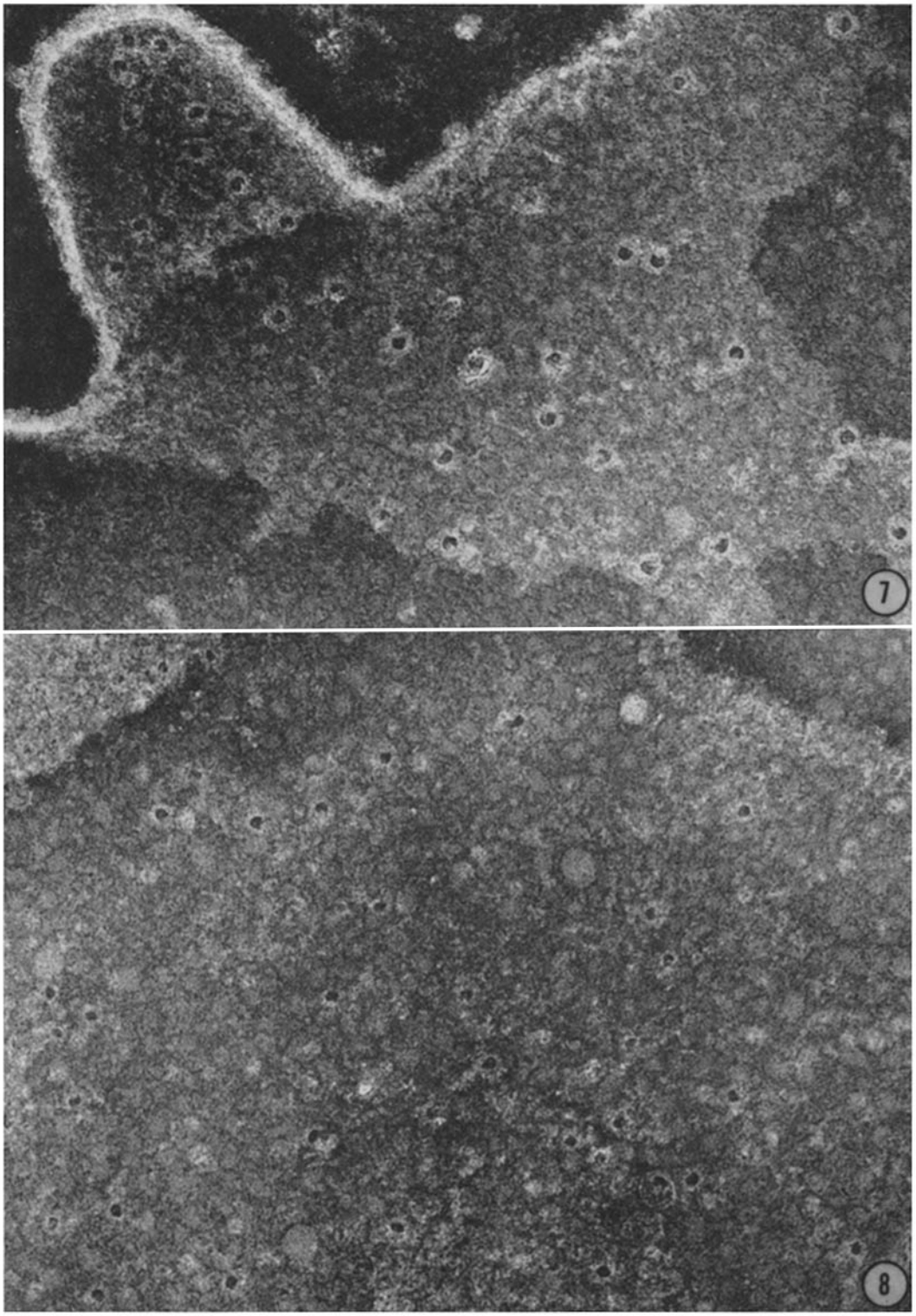




(Rosse et al.: Immune lysis of red blood cells. III)

PLATE 90

FIG. 7. Fragment of a PNH red cell membrane lysed by acidified normal human serum. In cells lysed by acidified serum, the distribution of holes in different membrane fragments varies in a manner similar to that seen when specific antibody and human complement are used for lysis. × 187,000.

FIG. 8. Fragment of a PNH cell lysed with anti-I antibody and guinea pig C′. Note the smaller size of the defects when compared to those made by human C′ (see Figs. 5 and 6). The same amount of antibody and fresh serum was used as in lysing the cells shown but many fewer defects than in Figs. 3 and 4 are seen. × 187,000.




(Rosse et al.: Immune lysis of red blood cells. III)