MEMBRANE LESIONS IN IMMUNE LYSIS

Surface Rings, Globule Aggregates, and Transient Openings

G. H. ILES, P. SEEMAN, D. NAYLOR, and B. CINADER

From the Departments of Pharmacology, Medical Cell Biology and Clinical Biochemistry,
Institute of Biomedical Electronics and Institute of Immunology, University of Toronto,
Toronto 5, Ontario, Canada

ABSTRACT

It is known that there are 100 Å-wide circular structures associated with the erythrocyte membrane in immune lysis. To determine whether these structures were functional holes extending through the membrane, freeze-etch electron microscopy was carried out. Sheep erythrocytes incubated with either rabbit complement or rabbit antibody (anti-sheep erythrocyte antibody) did not hemolyze and did not reveal any abnormalities in freeze-etch or negative-stain electron microscopy. Erythrocytes incubated with both complement and antibody revealed rings on the extracellular surface (etch face) of the cell membrane. Allowing for the 30 Å-thick Pt/C replica, the dimensions of the surface rings were similar to those seen by negative staining. The ring's central depression was level with the plane of the membrane; some rings were closed circles, others were crescent shaped. The cleavage face of the extracellular leaflet revealed globule aggregates, each aggregate appearing to be composed of about four fused globules. The cleavage face of the cytoplasmic leaflet was normal. When immune lysis was carried out in the presence of ferritin, ferritin was subsequently detected in all lysed erythrocytes. If ferritin was added *after* immune lysis was complete, only 15% of the cells were permeated by ferritin, indicating that transient openings exist in the cell membrane during immune lysis. No abnormal structures were detected when C6-deficient rabbit serum was used as a source of complement. It is concluded that antibody and complement produce surface rings, prelytic leakage of K^+, colloid osmotic swelling, membrane disruption, and membrane resealing; the surface rings persist after these events.

INTRODUCTION

In the membranes of erythrocytes, lysed by antibody and complement, circular structures (about 100 Å in diameter) can be observed (2, 33, 17, 34) by electron microscopy of negatively-stained preparations. Larger structures (150 Å) have been observed after reactive lysis, dependent only on late-acting complement components, activated by inulin (31). Liposomes prepared from pure lipid can be damaged by antibody and complement (1, 13–15, 18, 20–22, 25). Circular structures have been found in such liposomes in some circumstances (15) but not in others (23).

The question arises whether such structures represent functional holes which extend through the full thickness of the membrane. The strongest argument against such a functional hole comes from experiments in which complement components up to and including C5 can induce the circular structure, though they do not cause hemolysis (32). This has been demonstrated with

isolated complement components and with C6-deficient rabbit serum as a complement source (32), but the latter part of the experimental evidence has remained controversial (15).

Hitherto, the circular structures have only been observed with negative-staining techniques and not by thin-section electron microscopy. It is known that negative staining can produce structural artifacts, such as mitochondrial membrane ATPase "projecting headpieces" which are found not to project from the membrane when monitored by freeze-etch electron microscopy (29, 45).

The present study, employing freeze-cleavage and thin-section electron microscopy, provides further evidence that the circular structures are not holes through the membrane, but rather are raised rings on the external surface of the membrane, and that the loss of hemoglobin occurs through other large, transient openings in the cell membrane.

MATERIALS AND METHODS

Complement Lysis of the Cells

SHEEP ERYTHROCYTES: Citrated blood from a single sheep (Woodlyn Farms, Guelph, Ontario) was spun at 2000 rpm at 4°C for 10 min to remove as much plasma as possible. The cells were washed five times in Gl·GVB^{++}; a 2.5% solution was then prepared by suspending 1.25 ml of the packed cells in 48.75 ml of Gl·GVB^{++} (packed sheep erythrocytes 24 × 10^9 cells/ml).

GL·GVB^{++}: (See reference 19). Fresh gelatin glucose Veronal buffer was prepared on the day of experiment as follows: stock concentrate of Gl·GVB^{++} was thawed and diluted 1:5 with gdw (glass-distilled water), gelatin was added to give 0.1% gelatin (the solution was warmed to dissolve gelatin), and this solution was then mixed with an equal volume of 5% dextrose. The Gl·GVB^{++} contained 3 × 10^{-4} M Ca^{2+} and 1 × 10^{-3} M Mg^{2+}.

MODIFIED EARLE'S BALANCED SALT SOLUTION (BSS) FOR COMPLEMENT ABSORPTION: A modification of Earle's BSS was used to wash erythrocytes after initial washing of erythrocytes with Gl·GVB^{++}. This procedure was carried out in order to remove Ca^{2+} and Mg^{2+} from the erythrocytes which would cause loss of complement as a result of fixation by immune complexes. Modified Earle's BSS consisted of: NaCl 6800 mg/liter, KCl 400 mg/liter, NaH$_2$PO$_4$·H$_2$O 140 mg/liter, glucose 100 mg/liter, NaHCO$_3$ 2200 mg/liter, Na$_2$HPO$_4$ 283 mg/liter. The pH of this solution was 7.95 at

25°C and it was adjusted to pH 7.35 with isotonic (i.e. 0.147 M) NaH$_2$PO$_4$.

RABBIT ANTIBODY TO SHEEP ERYTHROCYTES (HEMOLYSIN): A group of eight rabbits was used. Three intravenous injections of 6 × 10^9 sheep erythrocytes in a volume of 1.5 ml of phosphate-buffered saline were given on days 0, 2, 4, 28, 30, 32, 56, 58, and 60, using the same number of sheep erythrocytes, but in a volume of 10.0 ml phosphate-buffered saline. Bleedings were taken on days 65 and 71; the sera from the bleedings were pooled. This antiserum was heated at 56°C for 1.5 h to inactivate the rabbit complement before using it as an antibody source.

RABBIT COMPLEMENT: Rabbits (3–4 kg) (Rieman's Farm, Ontario) were bled and the blood was allowed to clot at room temperature for 2 h. Individual sera were used to lyse sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibody. On negative staining a marked heterogeneity in the number of complement-induced lesions (per p) was observed. We selected a rabbit serum producing large numbers of lesions for further work. This rabbit was bled at weekly intervals and the sera were pooled for use as a complement source. Pooled sera were stored at −70°C.

In addition to the selection of a constant source of rabbit serum, it was necessary to absorb rabbit complement with sheep and goat erythrocytes in order to remove nonspecific antibody directed against sheep erythrocyte determinants. The method of absorption was as follows: 0.1 M ethylenediaminetetraacetic acid (EDTA) (di-Na$^+$) was added to the serum to be absorbed in a ratio of one part EDTA: 10 parts serum. 1 ml of packed sheep erythrocytes (washed three times with Gl·GVB^{++}, one time with Earle's modified BSS) was then added to 10 ml of rabbit serum. Absorption was carried out at room temperature with occasional shaking for 15 min. At the end of this time, the cells were centrifuged at 2000 rpm (0°C, 10 min), and the supernatant was retained. This absorption procedure was repeated a further six times with sheep erythrocytes. Even after this extensive absorption, undiluted absorbed rabbit serum lysed more than 50% of sheep erythrocytes in the absence of external antibody. We decided, therefore, to carry out further absorption with packed goat erythrocytes. Seven absorptions were carried out in a similar manner to the absorption with sheep erythrocytes. At the end of this, absorbed undiluted rabbit serum did not lyse (<5%) in the absence of external antibody.

RED CELL STROMA TREATED WITH RABBIT ANTIBODY TO SHEEP ERYTHROCYTES AND RABBIT COMPLEMENT: Conditions of lysis were such that a great excess of complement and antibody were used. 100% lysis occurred within 4 min at 37°C. The reaction mixture consisted of: 3.75 ml of 2.5% sheep erythrocytes, 11.25 ml of rabbit serum

undiluted, 3.00 ml of Gl·GVB^{++}, and 4.00 ml of rabbit antibody to sheep erythrocytes diluted 1:400.

The lysed cells were washed three times in 10 mM sodium phosphate buffer, pH 7.0. The final pellet of packed cells was suspended in the phosphate buffer to a dilution of approximately 1:1 for freeze fracture and 1:100 for negative staining.

For control purposes, some sheep erythrocytes were treated as above except that the rabbit serum (i.e. complement) was omitted and several additional washes in 10 mM sodium phosphate buffer were needed in order to remove most of the hemoglobin.

Addition of Ferritin

Ferritin (previously dialyzed against the medium) was added before lysis had taken place in one sample and after lysis had taken place in another sample. The erythrocytes were then fixed in 2% glutaraldehyde, postfixed in OsO$_4$, stained en bloc in uranyl acetate, dehydrated in ethanol, and embedded in Epon 812 (see 28, 37, and 38 for details).

NEGATIVE STAINING: A 2% solution of sodium silicotungstate was mixed with the suspended cells in a 1:1 ratio, and a drop of the suspension placed on a carbon-coated grid (28).

Freeze-Etch Procedures

The majority of the experiments were done using a Bullivant-Ames-Weinstein Type II freeze-cleave apparatus (6, 7, 42) which was modified to permit the concomitant preparation of replicas of five different samples. The erythrocyte membranes, washed and suspended in 10 mM sodium phosphate buffer (pH 7), were pipetted into the five specimen wells where they were rapidly frozen in Freon 22. Complete further details are described in reference 39.

In order to observe membrane lesions successfully it was essential to produce very thin platinum/carbon replicas. The replica thickness was monitored by using a Hewlett-Packard photodiode (HP 5082-4220) (Hewlett-Packard Co., Palo Alto, Calif.) to measure the amount of light received from a light-emitting diode (HP 5082-4120) after the light had passed through a glass slide. The entire device was independently calibrated by measuring various film thicknesses with a Taylor and Hobson "Talystep". The replica thicknesses ranged from 33–55 Å (see reference 39). Thinner films produced poor contrast, while thicker films obscured surface detail.

Results using a Balzers 360M Freeze-Etch instrument with quartz-crystal thin-film monitor were identical.

The Epon sections were picked up on uncoated 400-mesh grids, while the platinum/carbon replicas were picked up on Formvar-coated 400-mesh grids. Electron micrographs were taken at an original magnification of 39,900 using a Philips EM 300 electron microscope.

RESULTS

The Normal Erythrocyte Membrane of the Sheep

The cell membrane of a normal erythrocyte of the sheep appears indistinguishable from that of the normal human erythrocyte, as examined by freeze-cleavage electron microscopy. The cleavage plane of the membrane, or the central hydrophobic plane of the cell membrane, contains numerous globules each about 85–105 Å in diameter. (See references 3–5, 10, 12, 30, 39, and 41 for the interpretational basis of the cleavage and etch faces).

The Appearance of the Sheep Erythrocyte Membrane in the Presence of Only Antibody or Complement

Fig. 1 shows the appearance (cf. references 30, 39, and 41) of a freeze-etched sheep erythrocyte membrane which had been incubated only with antibody (anti-sheep erythrocyte).

Fig. 2 reveals the cleavage face of the extracellular leaflet of the sheep erythrocyte mem-

---

FIGURE 1   The electron microscope appearance of a platinum/carbon replica of a sheep erythrocyte membrane which had been incubated only with antibody (anti-sheep erythrocyte; no complement added), and then freeze-etched in a modified Bullivant-Ames-Weinstein Type II freeze-cleave device. The extracellular surface ("etch face" at bottom) is smooth; the cleavage plane (top half) reveals 90 Å-wide globules attached to the cytoplasmic leaflet of the split membrane. No abnormalities are seen. × 210,000. The direction of platinum/carbon shadowing for all the micrographs is from the bottom upwards.

FIGURE 2   Showing the cleavage face of the extracellular leaflet of the sheep erythrocyte membrane which had been incubated only with antibody to sheep erythrocytes (no complement). The globules are normal in size and shape, and are normally separated from one another. × 210,000.
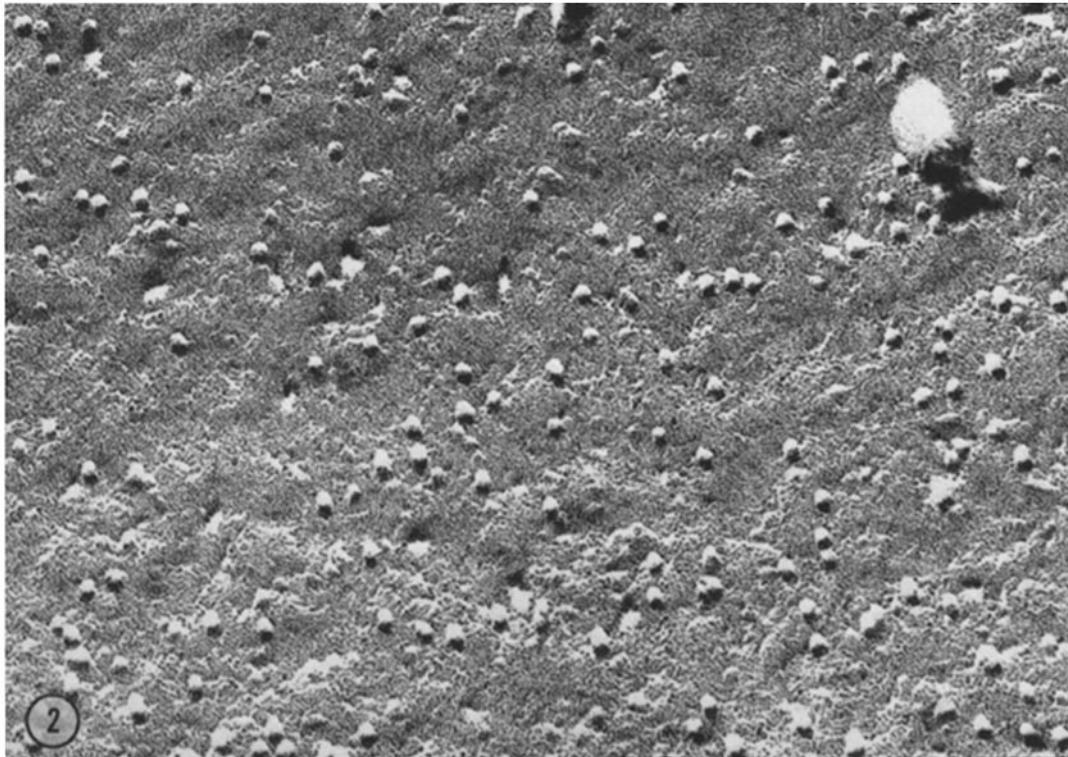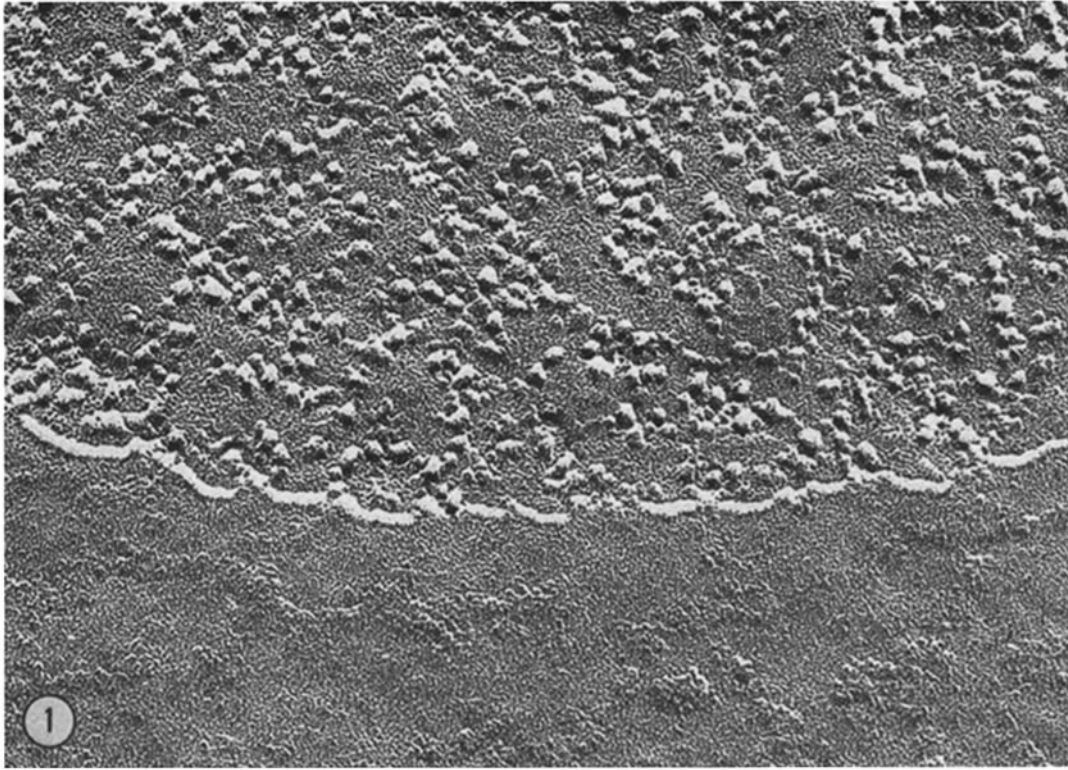
brane after exposure to antibody only. The globules are one fourth to one fifth less numerous than the cytoplasmic leaflet, which is normally the case for the extracellular leaflet (12, 43, 44).

Negative staining and freeze-etching showed that erythrocytes exposed to antibody only (Fig. 3) or complement only (Fig. 4) were indistinguishable from normal.

Structure of Cell Membranes Lysed by the Complete System of Antibody and Complement

Surface rings were observed on the extracellular surface of erythrocytes which had been incubated with both antibody and complement (Figs. 5, 7, and 8). Some of the rings are closed while others are crescent shaped. The surface rings do not have deep depressions in the center. In a few instances the centers appeared to be devoid of platinum, giving the illusion of a hole through the entire thickness of the membrane. In fact the angle of shadowing was extremely shallow in these cases, so that the raised edge or "lip" of the surface ring cast a long shadow preventing deposition of platinum in the center.

Surface rings, as seen in freeze-cleavage microscopy (Fig. 5), were compared with the circular lesions as seen in negatively-stained preparations of erythrocytes from the same experimental incubation mixture (Fig. 6). The surface rings have an outer and inner diameter of about 235 and 90 Å, respectively, observed in freeze cleavage. The circular structures, seen in negatively-stained preparations, had the corresponding diameters of 195 and 105 Å. The dimensions, observed by the two techniques, are compatible since the platinum/carbon film is approximately 30 Å and will tend to increase the outer dimensions of the rings and fill in the center of the surface ring.

Treatment with antibody and complement did not affect the gross structure seen in the cleavage face of the cytoplasmic leaflet which remained unchanged and free of holes (Fig. 9; compare with Fig. 1).

The cleavage face of the extracellular leaflet, however, was abnormal and revealed "globule aggregates" (Fig. 10). Each globule aggregate appeared to be composed of three or four globules tightly fused to one another. The number of globule aggregates were counted on micrographs

printed at a magnification of 119,000. There were 284 globule aggregates per square micron of cell membrane. This compares with 255 surface rings per square micron.

Membrane Structure in the Presence of C6-Deficient Serum

No membrane lesions of any kind were detected in the cell membranes of erythrocytes incubated with antibody and with rabbit complement which was deficient in the sixth component.

Transient Openings in the Cell Membrane during Immune Lysis

It is known that transient holes or defects appear in the erythrocyte membrane during the course of hypotonic hemolysis (see references in 37, 38). These membrane defects are very large (200–500 Å wide, and about 0.1–1 μm long) and can admit colloidal gold, ferritin, and other macromolecules (37, 38).

In order to test whether such membrane lesions occurred in immune lysis, ferritin was added to the incubation medium containing antibody and complement. When ferritin was present from the very beginning of the incubation, ferritin was subsequently detected in all the erythrocyte ghosts (see Fig. 11). When ferritin was added after immune lysis had taken place, however, 85% of the erythrocyte ghosts did not contain any ferritin (Fig. 12).

DISCUSSION

There appear to be three types of membrane changes in antibody-mediated complement lysis: surface rings, globule aggregates within the membrane, attached to the extracellular leaflet, and transient openings which are only patent during the course of immune lysis. The rings seen on the extracellular surface of the complement-lysed membrane are *not* holes which penetrate the cell membrane, since the centers of the rings appear to be at the same level as the plane of the membrane (Figs. 5, 7, and 8). The rings correspond in shape, dimensions, and frequency to the circular structures seen by negative staining (2, 17, 33). It is possible that the surface rings may have been patent during immune lysis, and that these rings represent the residual state of the transient openings. It is also possible, how-
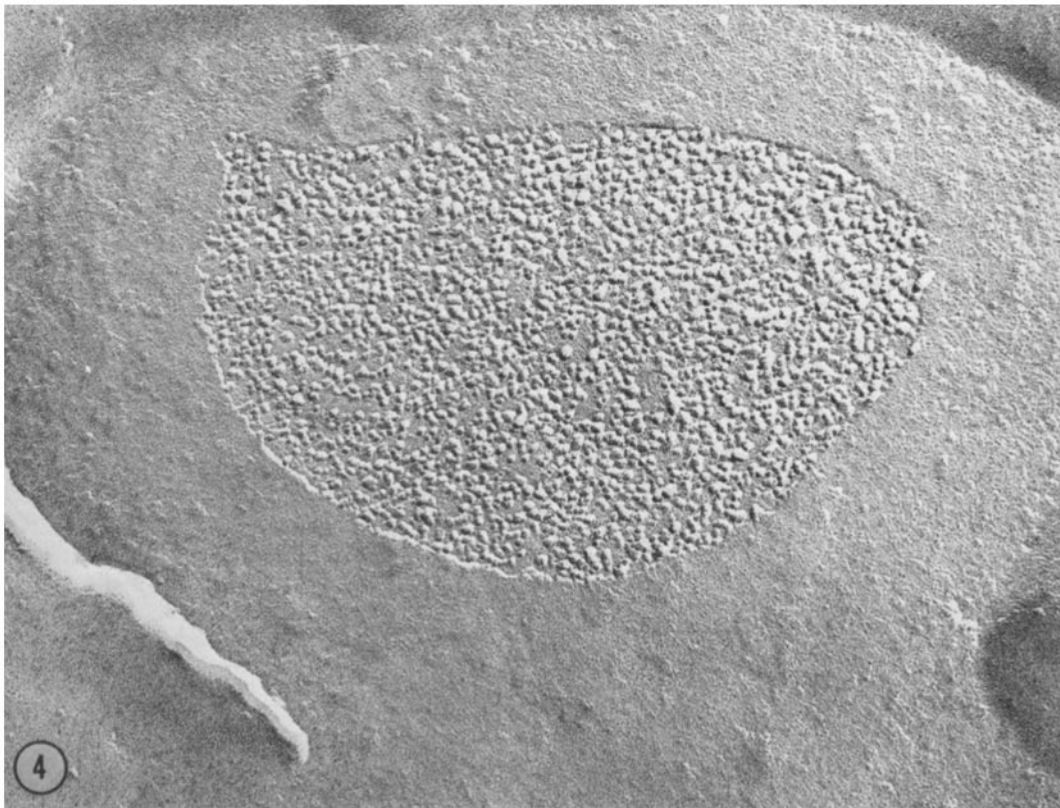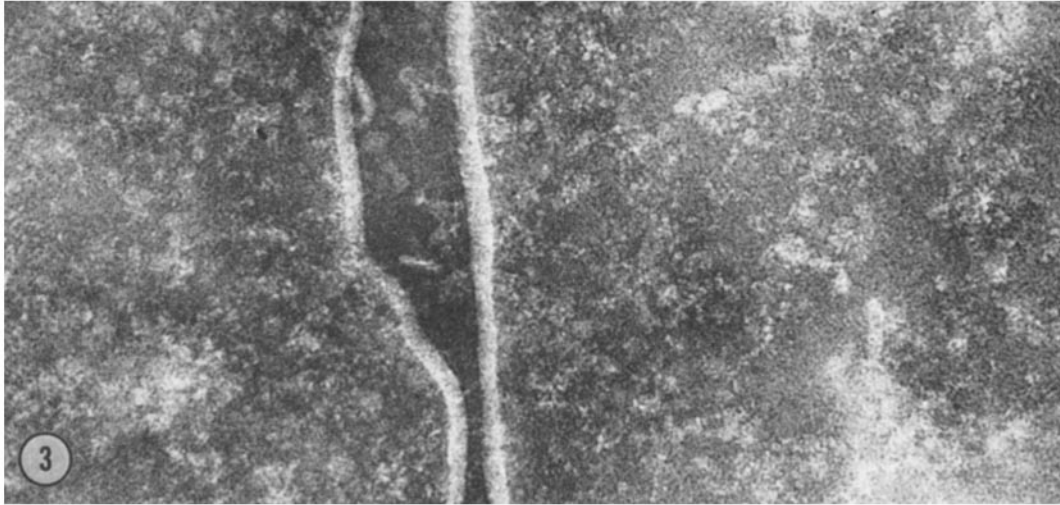
FIGURE 3   Negatively-stained image of sheep erythrocyte membranes which had been incubated with anti-sheep erythrocyte antibody only (no complement). No abnormalities are seen. The edges of the two cell membranes (in the center of the photo) appear thick presumably because of curvature at the edge of the cells. × 210,000.

FIGURE 4   Freeze-etch micrograph of a sheep erythrocyte membrane which had been incubated with rabbit serum complement only (no antibody added). The extra-cellular surface (etch face) is smooth and normal; the cleavage face of the cytoplasmic leaflet appears normal and contains numerous globules. The globules appear to be crowded because the angle of Pt/C shadowing was very low in this case. × 114,000.
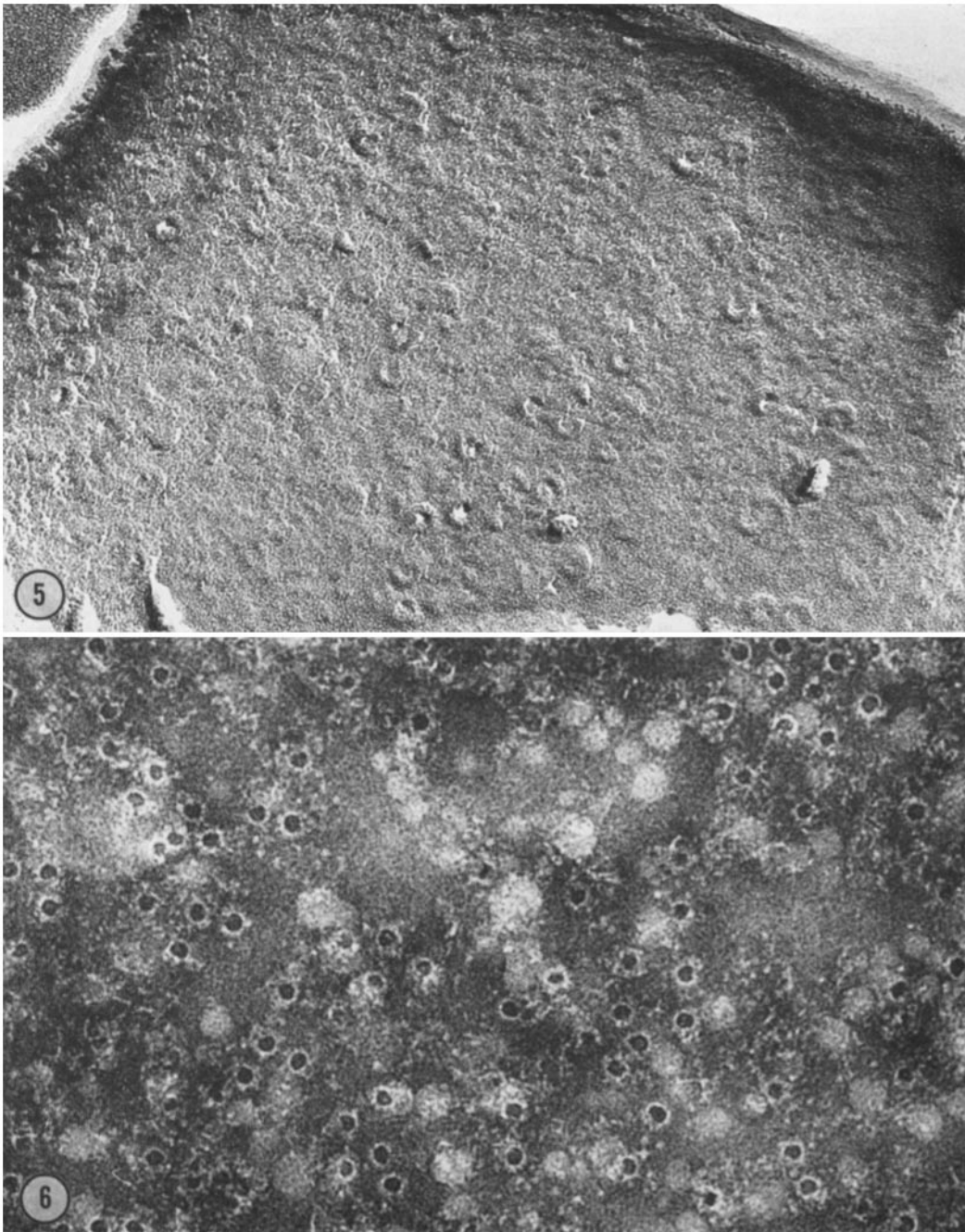
FIGURE 5 Extracellular surface (etched) of a sheep erythrocyte membrane which had been lysed by the complete system of rabbit complement and anti-sheep erythrocyte antibody. Note the surface rings, having outer and inner diameters of about 235 and 90 Å, respectively. Some rings are closed, some are crescent shaped. There are no deep depressions in the centers of the rings. In some cases long, white shadows (simulating "holes") are cast by the edges of the rings. Since the Pt/C film is of the order of 30 Å, thick, the rings' dimensions are similar to those shown in Fig. 6. × 210,000.

FIGURE 6 Negatively-stained membranes of cells from the same incubation mixture as in Fig. 5. The outer and inner diameters of the rings are about 195 and 105 Å, respectively. × 210,000.

534
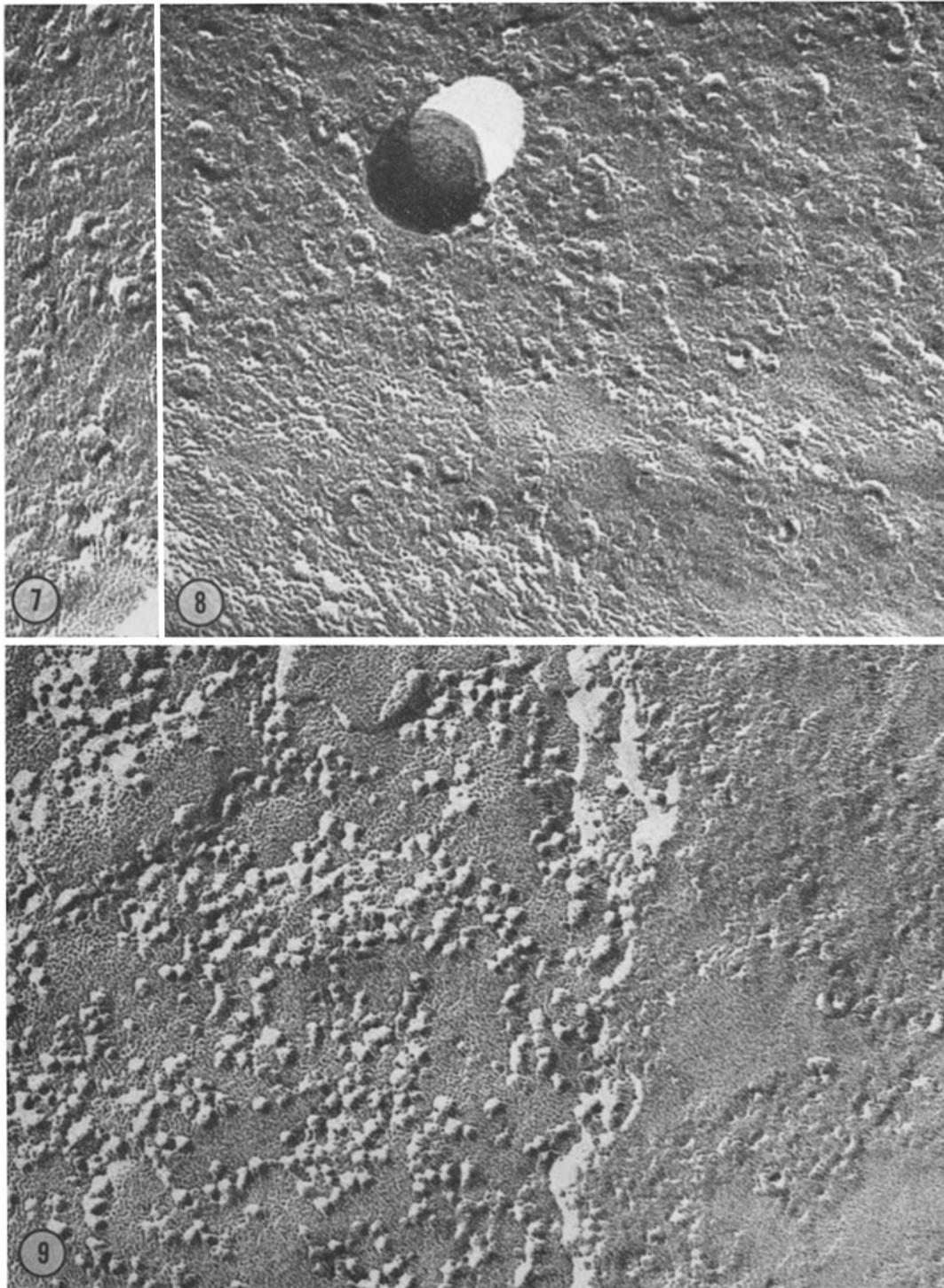
Figures 7–8 Extracellular surface (etch face) of sheep erythrocyte membranes lysed by the complete system of antibody and complement. × 209,000.

Figure 9 Freeze-etch appearance of sheep erythrocyte membranes lysed by complete system of antibody and complement. The extracellular surface on the right contains several surface rings. The cleavage face on the left shows the normal pattern of globules on the cytoplasmic leaflet. × 210,000.
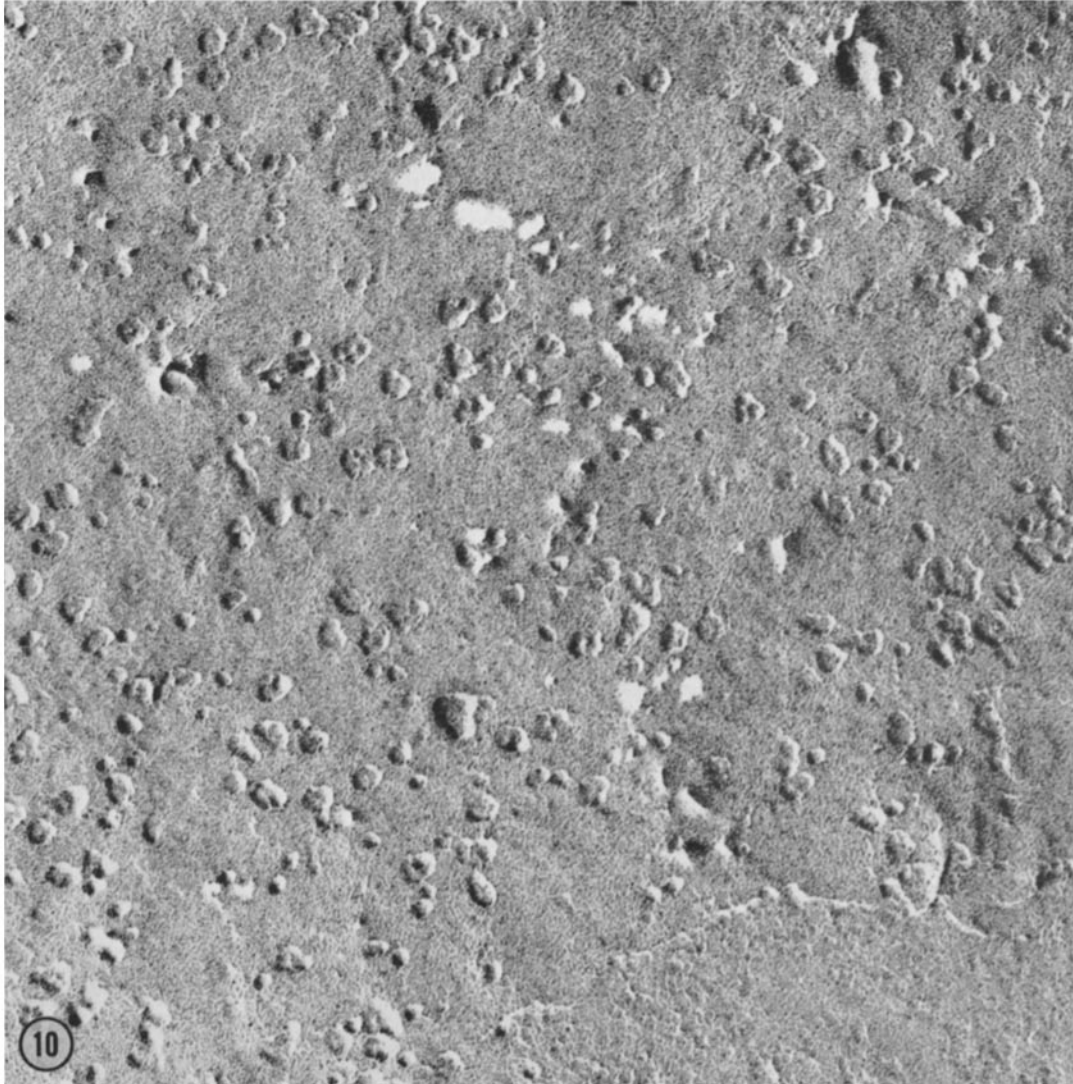
Figure 10 Freeze-etch appearance of sheep erythrocytes which had been lysed by the complete system of antibody and complement. The lower right-hand portion shows the intracellular surface of the cell membrane. The majority of the micrograph reveals the cleavage face of the extracellular leaflet, containing globule aggregates. Each aggregate appears to consist of three or four globules fused together. × 210,000.

ever, that the transient openings may be stretched equivalent pores (8) or may be identical to the openings in osmotic hemolysis (38).[1]

Complement-mediated hemoglobin release is preceded by leakage of K^+ (16, 35–40) and involves the development of surface rings (32),

[1] It has been argued that the membrane openings are less than 64 Å wide since albumin and dextran 40 inhibit the release of hemoglobin (36). However, high concentrations of a macromolecule can reduce the diffusibility of a second macromolecule (9, 11, 26, 27) and thus can prevent escape of hemoglobin from holes which can be much larger than the interfering macromolecules (see reference 9 and also Seeman, P. 1973, Can. J. Physiol. Pharmacol., submitted for publication).
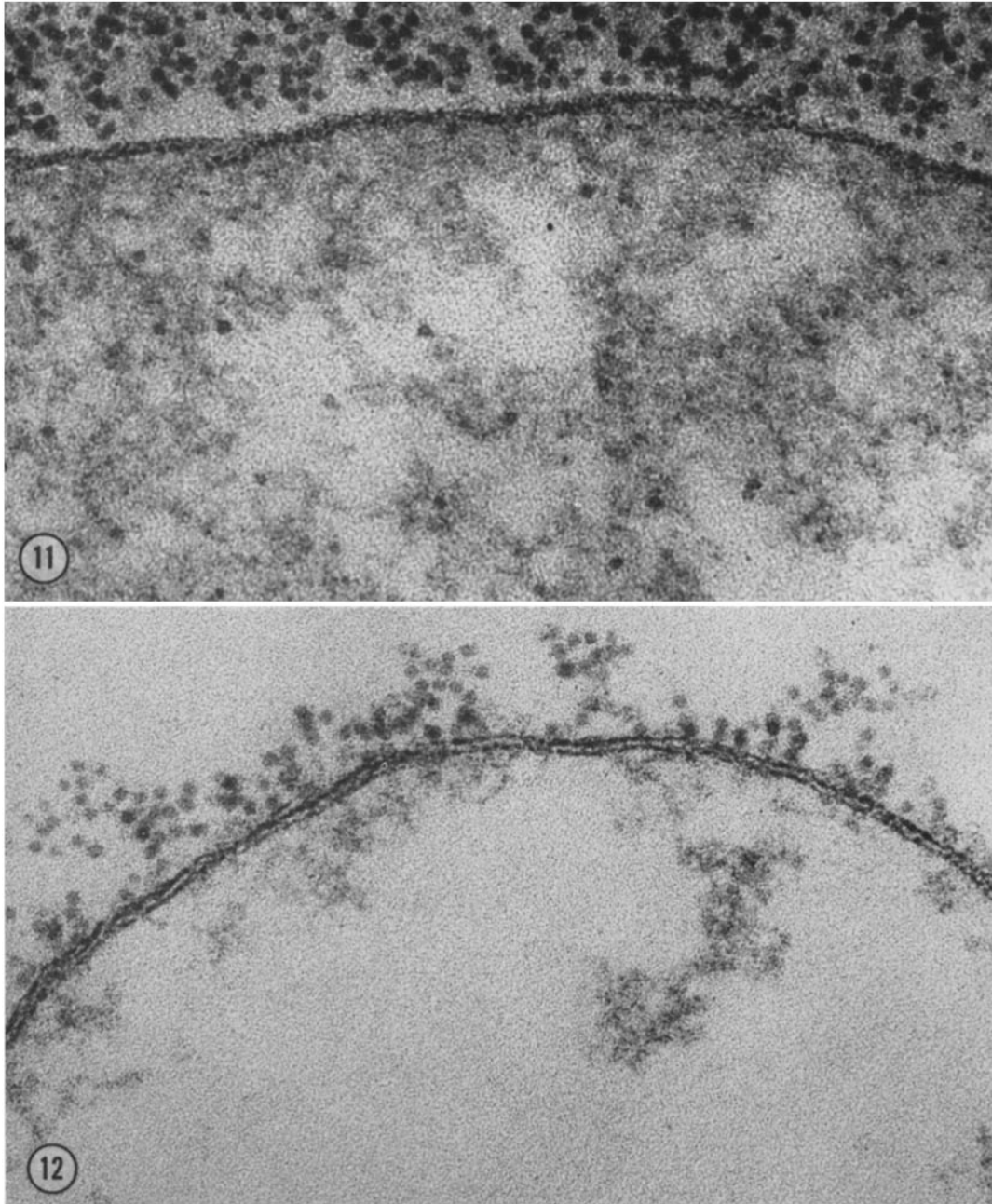
FIGURE 11 Thin-section appearance of a sheep erythrocyte which had been hemolyzed by antibody and complement in the presence of ferritin. The extracellular space (top of photo) contains numerous ferritin molecules. The cytoplasmic space of the erythrocyte ghost contains many ferritin particles as well as residual hemoglobin. $\times$ 204,000.

FIGURE 12 Thin-section appearance of a sheep erythrocyte which had been hemolyzed by antibody and complement *before* being exposed to ferritin. The ferritin did not enter the cytoplasmic space. $\times$ 204,000.
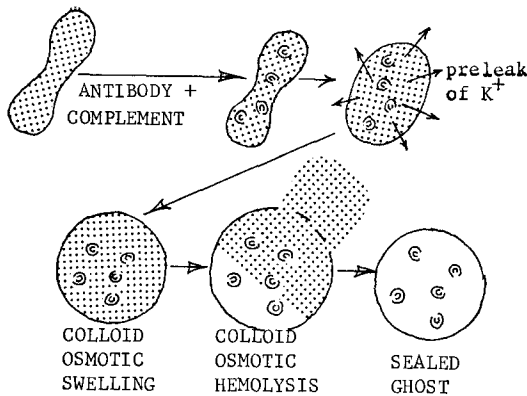
FIGURE 13 A summary and hypothetical chain of the ultrastructural events which may occur in complement-mediated lysis. Surface rings develop upon addition of complement and antibody. The membrane becomes leaky to K^+. The cell then swells by colloid osmotic hemolysis, reaches the critical volume, and bursts. After egress of the hemoglobin, the transient openings in the membrane seal over, still leaving the surface ring lesions.

colloid osmotic swelling, and hemolysis (36) associated with the development of transient openings in the membrane. The precise sequence in which these events occur and the causal relation between them is not known but one hypothetical scheme is suggested in Fig. 13 which also serves as a pictorial summary. It remains for future research to determine (a) the structure of the transient openings, and (b) whether there is a relation between the observed globule aggregates, the observed surface rings, and the hypothetical decamolecular complement complex of Kolb et al. (24).

REFERENCES

1. ALVING, C. R., S. C. KINSKY, J. A. HAXBY, and C. B. KINSKY. 1969. Biochemistry. 8:1582.
2. BORSOS, T., R. R. DOURMASHKIN, and J. H. HUMPHREY. 1964. Nature (Lond.). 202:251.
3. BRANTON, D. 1966. Proc. Natl. Acad. Sci. U. S. A. 55:1048.
4. BRANTON, D. 1969. Annu. Rev. Plant Physiol. 20:209.
5. BRANTON, D. 1971. Philos. Trans. R. Soc. Lond. Ser. B. Biol. Sci. 261:133.
6. BULLIVANT, S., and A. AMES. III. 1966. J. Cell Biol. 29:435.
7. BULLIVANT, S., R. S. WEINSTEIN, and K. SOMEDA. 1968. J. Cell Biol. 39(2, Pt. 2):19 a (Abstr.).
8. BURTON, A. C. 1970. In Permeability and Function of Biological Membranes. L. Bolis, A. Katchalsky, R. D. Keynes, W. R. Loewenstein, and B. A. Pethica, editors. North Holland Publishing Co., Amsterdam. 1.
9. DAVIES, H. G., N. V. B. MARSDEN, S. G. ÖSTLING, and A. M. M. ZADE-OPPEN. 1968. Acta Physiol. Scand. 74:577.
10. DEAMER, D. W., and D. BRANTON. 1967. Science (Wash. D. C.). 158:655.
11. DUDMAN, W. F. 1966. Nature (Lond.). 211:1049.
12. ENGSTROM, L. H. 1970. Structure in the Erythrocyte Membrane. Ph.D. Dissertation. University Microfilms Ltd., Ann Arbor, Mich.
13. HAXBY, J. A., C. B. KINSKY, and S. C. KINSKY. 1968. Proc. Natl. Acad. Sci. U. S. A. 61:300.
14. HAXBY, J. A., O. GÖTZE, H. J., MÜLLER-EBERHARD, and S. C. KINSKY. 1969. Proc. Natl. Acad. Sci. U. S. A. 64:290.
15. HESKETH, T. R., R. R. DOURMASHKIN, S. N. PAYNE, J. H. HUMPHREY, and P. J. LACHMANN. 1971. Nature (Lond.). 233:620.
16. HINGSON, D. J., R. K. MASSENGILL, and M. M. MAYER. 1969. Immunochemistry. 6:295.
17. HUMPHREY, J. H., and R. R. DOURMASHKIN. 1969. Adv. Immunol. 2:75.
18. INOUE, K., T. KATAOKA, and S. C. KINSKY. 1971. Biochemistry. 10:2574.
19. KABAT, E. A., and M. M. MAYER. 1961. Experimental Immunochemistry. 2nd edition. 149.
20. KINSKY, S. C., J. A. HAXBY, D. A. ZOPF, C. R. ALVING, and C. B. KINSKY. 1969. Biochemistry. 8:4149.
21. KINSKY, S. C., P. P. M. BONSEN, C. B. KINSKY,

L. L. M. VAN DEENEN, and A. F. ROSENTHAL. 1971. *Biochim. Biophys. Acta.* **233**:815.

22. KINSKY, S. C. 1972. *Biochim. Biophys. Acta.* **265**:1.

23. KNUDSON, K. C., D. H. BING, and L. KATER. 1971. *J. Immunol.* **106**:258.

24. KOLB, W. P., J. A. HANBY, C. M. ARROYAVE, and H. J. MÜLLER-EBERHARD. 1972. *J. Exp. Med.* **135**:549.

25. LACHMANN, P. J., E. A. MUNN, and G. WEISS-MANN. 1970. *Immunology.* **19**:983.

26. LAURENT, T. C. 1963. *Biochem. J.* **89**:253.

27. LAURENT, T. C. 1963. *Acta Chem. Scand.* **17**:2664.

28. MacLENNAN, D. H., P. SEEMAN, G. H. ILES, and C. C. YIP. 1971. *J. Biol. Chem.* **246**:2702.

29. MUHLETHALER, K. *In* Recent Physical Studies of the Structure of Bio-Membranes. D. Chapman, editor. In press.

30. PINTO DA SILVA, P., and D BRANTON. 1970. *J. Cell Biol.* **45**:598.

31. POLLEY, M. J. 1972. *Fed. Proc.* **31**:788 (Abstr.).

32. POLLEY, M. J., MÜLLER-EBERHARD, and J. D. FELDMAN. 1971. *J. Exp. Med.* **133**:53.

33. ROSSE, W. F., R. DOURMASHKIN, and J. H. HUMPHREY. 1966. *J. Exp. Med.* **123**:969.

34. ROSSE, W. F., and P. K. LAUF. 1970. *Semin. Hematol.* **7**:323.

35. ROTH, S., and P. SEEMAN. 1972. *Biochim. Biophys. Acta.* **255**:190.

36. SEARS, D. A., R. I. WEED, and S. N. SWISHER. 1964. *J. Clin. Invest.* **43**:975.

37. SEEMAN, P. 1967. *J. Cell Biol.* **32**:55.

38. SEEMAN, P., D. CHENG, and G. H. ILES. 1972. *J. Cell Biol.* **56**:519.

39. SEEMAN, P., and G. H. ILES. 1972. *Nouv. Rev. Fr. Hematol.* In press.

40. SEEMAN, P., T. SAUKS, W. ARGENT, and W. O. KWANT. 1969. *Biochim. Biophys. Acta.* **183**:476.

41. TILLACK, T. W., and V. T. MARCHESI. 1970. *J. Cell Biol.* **45**:649.

42. WEINSTEIN, R. S., R. A. WILLIAMS, and K. SOMEDA. 1968. *Lab. Invest.* **18**:21.

43. WEINSTEIN, R. S., and S. BULLIVANT. 1967. *Blood.* **29**:780.

44. WEINSTEIN, R. S. and N. S. McNUTT. 1970. *Semin. Hematol.* **7**:259.

45. WRIGGLESWORTH, J., L. PACKER, and D. BRANTON. 1970. *Biochim. Biophys. Acta.* **205**:125.