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The M-Antigen in HK and LK Sheep Red Cell Membranes*

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Summary. Red cells of all high-potassium-type (HK) sheep and of more than one half of all low-potassium-type (LK) sheep contained the M-antigen and were hemolyzed by iso-immune anti-M antiserum in presence of a guinea pig serum complement. It was characteristic for the hemolysis of HK red cells by the M-antiserum the all HK cells were ultimately hemolyzed at suboptimal antibody concentrations, provided the time of incubation at 37 °C was sufficiently long. Thus, the M-antigen appears to be expressed on all red cells of an individual HK sheep. The M-antibody was absorbed by HK red cells and their membranes with a high affinity, whereas M-negative LK red cells and their membranes did not bind the antibody. The ratio of the number of antibody units absorbed per cell or membrane to the number of antibody units required for lysis approached unity. The amount of antibody absorbed per membrane was unaffected by ouabain in the presence of ATP, Mg^{++}, Na^{+}, and K^{+}. The M-antigen activity depends on the integrity of the red cell membrane and was not detectable after lyophilization of HK membranes or in the membrane protein solubilized by n-butanol. The major M-antibody activity was found among the high molecular weight plasma proteins and may be attributed to the β_2M globulins. Heterogeneity within the antibody fraction cannot be excluded since some hemolytic activity was detected in a chromatographic fraction containing predominantly γ-globulin. The relationship between the M-antigen and the Na^{+}-K^{+} transport system in sheep red cell membranes is discussed.

The observation of Rasmusen and Hall (1966*a*) that all red blood cells of high-potassium-type (HK) sheep but only two-thirds of the red cells of low-potassium-type (LK) sheep contain the M-antigen suggested a close genetic relationship between potassium transport and M-antigen activity. It has been shown by Tosteson and Hoffman (1960) that both the active and passive transport of sodium and potassium are different in HK and LK sheep red cells. In particular, active transport of these ions is about four times greater in HK than in LK cells. HK cells also contain a Na^{+}-plus-K^{+}-stimulated and ouabain-sensitive adenosine triphosphatase (S-ATPase) which is about four times more active than in LK red cells, suggesting a close association between the two processes (Tosteson, 1963).

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Efforts were undertaken, therefore, to characterize the relationship between the M-antigen and the Na^+-plus-K^+ transport system in HK and LK sheep red cells. This paper reports the characteristics of the immune hemolysis of HK red cells by anti-M antiserum, the absorption of the antibody by HK and LK red cells and their membranes, and the effect of ATP and ouabain on the binding of the antibody by HK membranes. The studies indicate that the M-antigen activity depends on the integrity of the red cell membrane, and that the antibody activity is largely present in the macroglobulin fraction of the M-antiserum.

Materials and Methods

For each experiment, blood from HK and LK sheep was freshly drawn and heparinized (10 USP units sodium heparin/ml). The red cells were washed in 150 mM NaCl, and the cells were counted with a model F Coulter counter (Coulter Electronics, Hialeah, Fla.) or calculated from spectrophotometric measurements made with a Beckman DU spectrophotometer. The optical density was 0.700 for 1×10^9 cells/ml at 541 mμ and for 1.08×10^8 cells/ml at 414 mμ. Hemoglobin-free membranes were prepared by osmotic lysis in 20 mosm Tris/HCl buffer, pH 7.6. The membranes were collected by centrifugation for 15 min at $27{,}000 \times g$ and 4 °C. An average weight of 6.8×10^{-13} gm/ghost was found by weighing lyophilized membranes lyzed from a known amount of red cells.

The membrane protein of HK and LK sheep red cells was extracted by n-butanol (Maddy, 1964). Four volumes of precooled white membranes (2 to 3 mg/ml) were mixed with three volumes of ice-cold n-butanol and kept for 15 min at −2 °C. After contrifugation for 15 min at $27{,}000 \times g$ (2 to 4 °C), the waterphase was removed with a syringe and immediately lyophilized. The amount of protein obtained was usually more than 80% of the total membrane protein.

Iso-immune anti-M antiserum (S 11) was kindly provided by Dr. Ben A. Rasmusen (College of Agriculture, University of Illinois, Urbana). The preparation of this specific antiserum was described by Rasmusen and Hall (1966*b*). Serum complement was inactivated by heating the antiserum for 30 min at 56 °C. Natural antibodies against sheep blood group R were removed by two absorptions of S 11 with one-third volume of packed human type A erythrocytes (20 min at room temperature and 10 min at 4 °C). A second agglutinating antibody of anti-sheep blood group D specificity also present in S 11 (titer 256) was not removed because at the time of the experiments no D-positive, M-negative sheep cells were available. It was shown by Rasmusen, Stormont, and Suzuki (1960) that the M-system is separate from the D-system.

Guinea pig serum complement served as the source of complement throughout all experiments. Guinea pigs (800 gm) were bled by heart puncture. The serum was collected after clotting of the blood in the refrigerator (3 to 5 hr). It was centrifuged twice (0 °C, 10 and 5 min) and stored at −20 °C for not longer than 1 month. As determined with a Forssman-antiserum and sheep red cells, the guinea pig serum prepared in this manner contained 200 to 220 C′ H_{50} units (Mayer, 1961).

The buffer (ionic strength 0.147, pH 7.35) used as diluent in the hemolytic assays was prepared according to Mayer (1961) except that 10 mM NaCl was exchanged for KCl. No gelatin was used.

ATP (Lot 185A) was obtained from P-L Biochemicals Corporation (Milwaukee, Wisc.) and ouabain (Lot 6713-0610) from Sigma Chemical Company (St. Louis, Mo.). Hydrolyzed starch was purchased from the Connaught Medical Research Laboratories

(Toronto, Canada). Other chemicals used were of analytical grade, and solutions were made in deionized water.

The K^+ and Na^+ content of HK and LK sheep red cells was determined with a Perkin-Elmer atomic absorption spectrophotometer.

Hemolytic Assay

Two hemolytic assay systems were used. Qualitative testing of red cells from a variety of sheep for the M-antigen was done in micro-hemolysis U-plates (Cook Engineering Co., San Mateo, Cal.). A 0.025-ml sample of a 1 % cell suspension of the blood to be tested was added to 0.050 ml of each anti-M antiserum dilution; this was followed by addition of 0.025 ml of $^1/_{10}$ diluted guinea pig serum. Controls included: a) diluent and red cells; b) diluent, red cells and guinea pig serum; and c) diluted antiserum, red cells and diluent. The plates were carefully mixed and incubated at 37 °C. Hemolysis was read after 1 and 2 hr of incubation. This test was also employed for determination of hemolytic and agglutinating activities of the fractions of antiserum obtained in the gel filtration experiment.

For kinetic studies, the scaled-down procedure of Mayer (1961) was used. A 0.5-ml portion of antiserum dilution was mixed with 0.5 ml of HK red cells suspended in diluent (5.04×10^7 cells), and the test tubes were kept in an ice bath. Then, 0.25 ml of the diluted guinea pig serum was added, and the samples were immediately transferred into a 37 °C waterbath. The samples were frequently mixed during the 1-hr period of incubation. After exactly 1 hr, 2.5 ml of ice-cold diluent was added, and the samples were centrifuged at 3,000 rpm for 5 min in a GLC-1 Sorvall-centrifuge. The supernatants were poured into separate test tubes, and the degree of hemolysis was determined by reading the optical density at 414 mμ. Controls consisted of cell blank (0.75 ml diluent, 0.5 ml cells), complement plus cell blank (0.5 ml diluent, 0.5 ml cells, 0.25 ml diluted guinea pig serum) and antiserum plus cell blank (0.5 ml diluted antiserum, 0.5 ml cells and 0.25 ml diluent). These controls were analyzed for hemolysis at the beginning and the end of the 60-min period of incubation. The values of optical density found in the antiserum plus cell blank control never exceeded that of the cell blank at time zero. The degree of lysis was expressed in per cent lysis (y) of a completely lyzed sample (0.5 ml cells plus 3.25 ml 0.1 % Na_2CO_3). All values were corrected for the values of the cell blank and that of complement color (0.25 ml of diluted guinea pig serum in 3.75 ml diluent). In the studies of hemolysis as a function of time, guinea pig serum was added in 0.5-min intervals. The reaction was stopped at given time intervals by adding diluent containing 0.01 M EDTA [disodium-(ethylenedinitrilo)-tetraacetate]; this was followed by immediate centrifugation as indicated above. The 50 % hemolysis point was determined by plotting the logarithm of the dilution of the antiserum versus the logarithm of the ratio of cells lyzed (y) to the cells not lyzed ($1-y$). (*See* Rapp, 1953.)

Antibody Absorption

Absorption of anti-M antibody by HK and LK cells and their membranes was carried out as follows. A known number of cells were diluted in geometric order and mixed with equal amounts of various dilutions of antiserum. The membranes were diluted similarly except in 20 mosm Tris/HCl buffer, pH 7.6. Equal volumes of 570 mosm Tris/HCl buffer were added to these membrane dilutions to obtain isosmotic conditions with plasma. Then, various dilutions of antiserum were added. The test tubes were mixed and incubated for 1 hr at 37 °C and for 10 min at 0 °C. The red cell-antibody suspensions were spun at $27{,}750 \times g$ for 15 min at 2 to 4 °C. The clear supernatants were transferred into separate test tubes and further diluted to estimate the dilutions

required to produce 50 % hemolysis. Samples (0.5 ml) of the absorbed or unabsorbed antiserum dilution (control) were used in the hemolytic assay system as described above. Attempts to measure absorption of anti-M antiserum by lyophilized membranes or by the butanol-extracted membrane protein were performed similarly.

The following procedure was adopted to investigate the effect of ATP and ouabain on the absorption of anti-M antibodies by HK membranes. Membranes were diluted as mentioned above. Equal volumes of 80 mM Tris/Cl buffer, pH 7.6, containing 2 mM ATP, 2 mM $MgCl_2$, 20 mM KCl, and 200 mM NaCl, with or without 0.2 mM ouabain, were added in the cold to each test tube of a set of four series of membrane dilutions. At time zero, all samples were placed in a 37 °C waterbath. After 30-min incubation, equal volumes of 1/400, 1/800, 1/1,600 and 1/3,200 diluted antiserum (dilutions made in half of the strength of the above Tris/Cl-ATP-ion buffer) were added to each series, and the samples were incubated for an additional 60 min at 37 °C. Antiserum dilutions were also added to test tubes containing only the Tris-ATP-ion buffer. After incubation, all samples were placed into an ice bath for 10 min and centrifuged at $27{,}700 \times g$ for 15 min. The clear supernatants were transferred into a separate set of test tubes. Dilutions and assay of 0.5 ml of absorbed or nonabsorbed antiserum were done as described above. In all cases, the bivalent cation concentration was adjusted to that optimal for the action of complement in the assay system (0.15 mM Ca^{++}, 0.5 to 1.0 mM Mg^{++}) at the time when test cell suspension and guinea pig serum complement were added.

The estimation of the amount of antibody removed by a given number of cells or membranes in the absorbing system was carried out as follows. One antibody unit (AU) was defined as $n/2$, where n is the number of cells in the hemolytic assay system (usually 5.04×10^7) which undergo 50 % immune-hemolysis in 60 min. The number of AU in a test sample containing antibody was computed by the relation

$$AU = \frac{n}{2}\left(\frac{V}{D_{50}}\right)$$

where D_{50} is the dilution of the test sample required to produce 50 % lysis in 60 min at 37 °C, and V is the volume added to the assay system. S 11 anti-M serum was found to contain 3.1 to 3.9×10^{11} AU/ml. The number of AU absorbed by sheep red cells or their membranes was estimated by determining the D_{50} of S 11 antiserum before and after exposure to a known number of cells (c) or membranes (m). Thus, the number of AU bound per cell or membranes (AU_{abs}) was computed from the equation

$$AU_{abs} = \frac{n\left(\frac{1}{D_{50}^0} - \frac{1}{D_{50}}\right)}{c \text{ or } m}$$

where D_{50}^0 is the dilution of the anti-M serum which was not exposed to absorbing cells or membranes, and D_{50} is the dilution of the same antiserum after exposure to a known number of cells (c) or membranes (m).

Fractionation of sheep anti-M antiserum was done on Sephadex G 200 in 1.0 M NaCl:0.1 M sodium phosphate buffer, pH 7.82, at room temperature. The elution profile was continuously monitored at 254 mμ. Next, 1.8 ml of anti-M antiserum was dialyzed against the column buffer and then applied to a column of the following characteristics: gel bed 34.5×2.5 cm, total bed volume 175 ml, void volume 65 ml, flow rate 18 ml/hr, and collection volume 3.75 ml/test tube. Each test tube was also checked for its optical density at 280 mμ. For determination of hemolysis and agglutinin activity of

the fractions obtained, each test tube was separately dialyzed against the diluent and then further assayed by the qualitive method as indicated above. The eluates in the test tubes were pooled to six fractions, dialyzed against deionized water and lyophilized. These fractions were then tested by immunoelectrophoresis.

Immunoelectrophoresis was carried out according to the micromodification of Scheidegger (1955), using 0.05 ionic strength veronal acetate buffer, pH 8.2, and 1 % agar (buffered) for the slides. Electrophoresis was run for 85 min at a voltage gradient of 6 V/cm. Immediately after electrophoresis, rabbit anti-sheep plasma protein antiserum (Lot 707 F, Behringwerke, Marburg, Germany) was placed into the antibody troughs, and diffusion was allowed to proceed for 20 to 24 hr.

For electrophoretic analysis of butanol-extracted protein of HK and LK membranes, the procedure of Azen, Nazhat and Smithies (1966) was followed. The starch gel (500 ml) contained 76 gm starch, 8 M urea, 0.07 M 2-mercaptoethanol and 0.012 M barium lactate. Prior to application, the lyophilized samples were solubilized according to Lauf and Poulik (1968). The gels were run for 24 hr at room temperature at a voltage gradient of 4.5 V/cm and stained with amido black.

Results

Hemolysis Characteristics of Homozygous HK and LK Red Cells

Fig. 1 shows the degree of hemolysis of HK sheep red cells at various dilutions of anti-M antiserum in presence of 1/10 diluted guinea pig serum after 1-hr incubation at 37 °C. At a 1/100 dilution of anti-M antiserum, all HK 2562 red cells were lyzed whereas the erythrocytes of LK 2582 did not lyze and were assumed to be M-negative. The alkali-metal ion composition of these cells as well as of other sheep red cells used in the experiments (*see* Discussion) was similar to that described by Tosteson (1966).

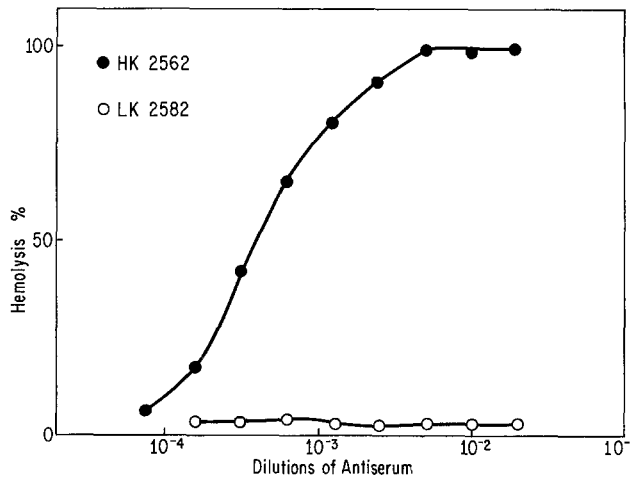

Fig. 1. Hemolysis of 5.04×10^7 HK (2562) and LK (2582) sheep red cells at various dilutions of S 11 anti-M antiserum

Fig. 2 illustrates the kinetics of hemolysis of HK cells by anti-M antiserum in the limited complement system (Fig. 2a) and in the limited antibody system (Fig. 2b). It can be seen that 100% hemolysis was obtained at a

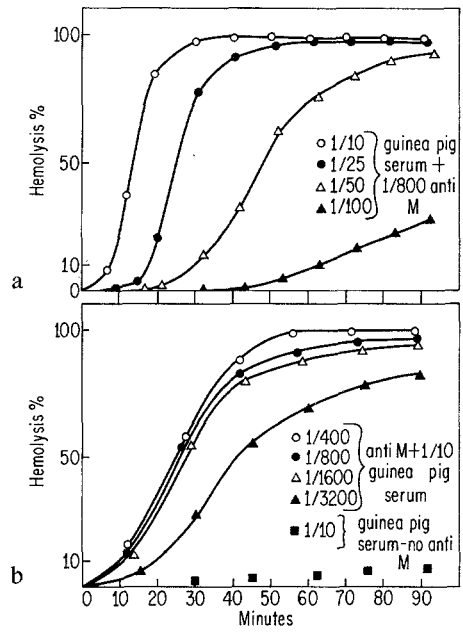

Fig. 2a and b. Immune hemolysis of HK 2562 red cells as a function of time. (a) Hemolysis by 1/800 diluted anti-M serum in presence of four dilutions of guinea pig serum complement. (b) Hemolysis by four dilutions of anti-M in presence of 1/10 diluted guinea pig serum complement

dilution of 1/800 anti-M antiserum after an incubation period of 40 min when 1/10 diluted guinea pig serum was used. In the presence of 1/10 diluted guinea pig serum, a dilution of 1/400 antiserum produced 100% lysis after 60-min incubation at 37 °C, and dilutions of 1/800 and 1/1,600 of the antiserum caused 90% lysis. The slopes of the hemolysis curves of these three antiserum dilutions are almost identical. It should be noted that in the limited complement as well as in the limited antibody system all curves approach 100% hemolysis, indicating that all cells can be lyzed ultimately when the incubation is carried on for a sufficient period of time. When HK cells were exposed to guinea pig serum (complement) alone, less than 10% hemolysis was observed after 60 min of incubation. It was also found that some LK sheep red cells lyzed to a greater extent in the presence of guinea pig serum alone.

Binding of Anti-M Antibody by HK and LK Sheep Red Cells and Membranes

Fig. 3 presents the hemolytic assay system used to determine the amount of antibody absorbed in a log-log plot of the ratio of cells lyzed (y) to cells not lyzed ($1-y$) on the ordinate as a function of the dilution of five different samples of S-11 anti-M antiserum. It can be seen that high antiserum concentrations were necessary to produce 50% lysis of the test cells [$(y/1-y)=1$] when increasing amounts of HK red cells were added to absorb the

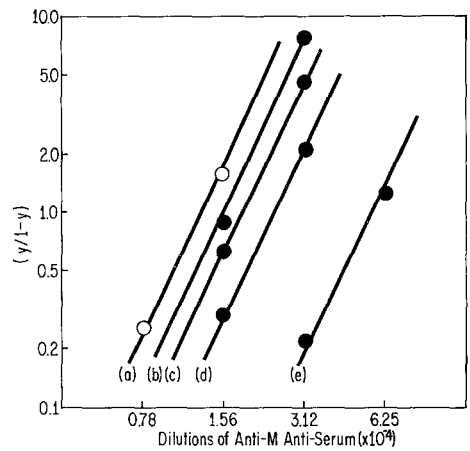

Fig. 3a–e. Determination of D_{50}. The ratio of cells lyzed (y) to cells not lyzed ($1-y$) is plotted on the ordinate as a function of dilution of five different samples of S 11 anti-M antiserum. These samples were previously absorbed with the following numbers of red cells expressed as 10^7 cells/ml: (a) zero, (b) 3.12, (c) 6.25, (d) 12.5 and (e) 25. The D_{50} is defined as the dilution at which the number of cells lyzed is equal to the number of cells not yet lyzed [$(y/1-y)=1$]

antibody. The slopes of the curves are identical. The figure shows that absorption studies detecting amounts of remaining antibody lower than a dilution of 1.3×10^{-4} cannot be undertaken using this assay system because at least two suitable points of hemolysis, one above and one below 50% hemolysis [$(y/1-y)=1$], are necessary. Fig. 4 shows the per cent antibody absorbed by various numbers of HK and LK sheep red cells when the initial concentration of anti-M in this absorption experiment was 2.4×10^8 AU/ml. The number of AU absorbed by the number of cells used can be seen on the right ordinate of the graph (Fig. 4). The uptake of anti-M antibody by HK cells and their membranes was also studied as a function of time. We found that most of the antibody was absorbed within the first 5 min after the cells or membranes were mixed with antiserum. Only a small

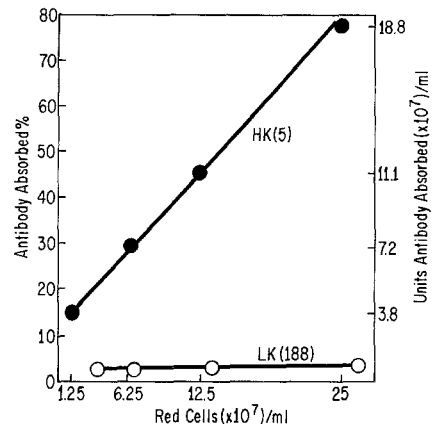

Fig. 4. Absorption of 1/1,600 diluted anti-M antiserum (initial concentration of the diluted serum 2.4×10^8 AU/ml) with increasing numbers of HK (5) and M-negative LK (188) red cells

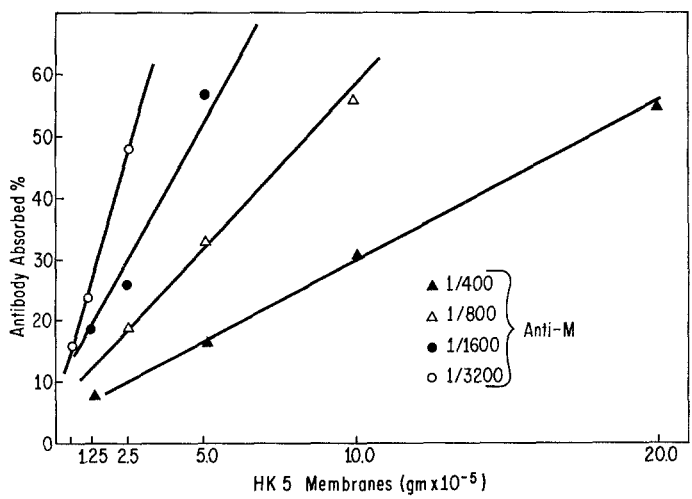

Fig. 5. Absorption of four geometric dilutions of anti-M serum by increasing amounts of HK (5) hemoglobin-free membranes

increase in antibody absorption was noted when the samples were incubated for longer than 60 min at 37 °C (up to 6 hr). It was also found that incubation for 1 hr at 37 °C followed by incubation for 10 hr at 4 °C as well as the presence of 1/20 diluted guinea pig serum complement did not alter the amount of antibody absorbed per membrane.

The results of an experiment in which increasing amounts of membranes isolated from M-positive cells were added to four geometric dilutions of S 11 antiserum (1/400, 1/800, 1/1,600 and 1/3,200) are depicted in Fig. 5.

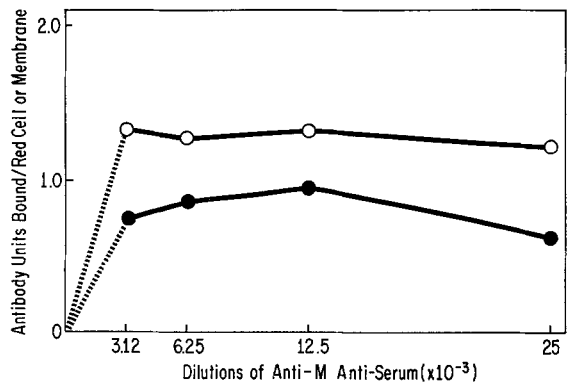

Fig. 6. The number of AU bound per red cell (solid circle) or hemoglobin-free membrane (open circle) at four different dilutions of anti-M antiserum

Plotting the per cent of anti-M absorbed versus the amount of membranes in the absorbing system results in four straight lines with slopes increasing in proportion to the initial dilution of the antiserum. A similar relationship was also obtained when red cells rather than membranes were used. The data from these absorption experiments were used to compute the number of AU bound per HK red cell or hemoglobin-free membrane at each dilution of anti-M used (Fig. 6). It can be seen that about 0.8 to 1.0 AU is bound per HK red cell and about 1.2 to 1.3 AU per hemoglobin-free membrane. It should be noted that even at the highest dilution of anti-M (3.12×10^{-3}, corresponding to 1.21×10^{8} AU/ml), maximum absorption occurred. No further antibody was taken up when higher concentrations of anti-M were used (dilution 2.5×10^{-2} corresponding to 9.7×10^{8} AU/ml). Experimental points for absorption of anti-M antibody at dilutions higher than 3.12×10^{-3} could not be obtained at the present time because of the limits of the assay system.

The Nature of the M-Antigen in HK Sheep Red Cells

Lyophilized HK membranes (5 mg/ml absorbing system) did not bind anti-M antibody under these experimental conditions. Furthermore, when n-butanol-extracted protein of HK membranes (2.5 mg/ml) was tested, no M-antibody inhibitory activity was found. Despite this fact, an attempt was made to characterize the electrophoretic behavior of sheep red cell membrane proteins solubilized by n-butanol. Fig. 7 depicts the urea-starch gel electrophoretic pattern of such membrane protein preparations obtained from homozygous HK cells (Fig. 7: 1, 3, 5, 7) and homozygous LK cells (Fig. 7: 2, 4, 6). A multitude of zones can be observed in all samples migrat-

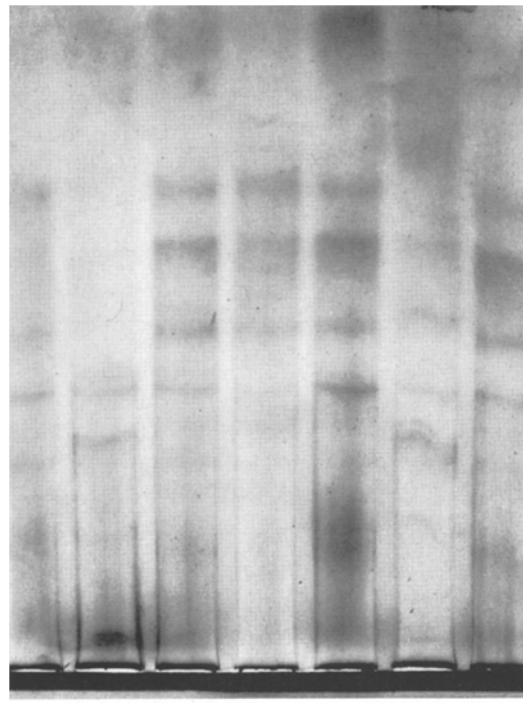

Fig. 7. Urea-starch gel electrophoresis of n-butanol-extracted membrane proteins from M-positive HK sheep red cells (*1*, *3*, *5*, *7*) and M-negative LK sheep red cells (*2 4*, *6*, *8*). The migration is to the cathode

ing to the cathode. No migration occurred to the anode. The patterns do not reveal differences in the proteins (or their subunits) of HK and LK sheep red cell membranes. This might be due to technical difficulties, because, in spite of apparently complete solubilization of the samples prior to application, a substantial amount of protein applied was found to be precipitated at the origin of the gel. It was also noted that the proteins of the sheep red cell membranes did not stain as well as the protein extracted from the human red cell membrane.

Because of the possible relation between the M-antigen and the Na^+-plus-K^+-stimulated ATPase, it was of interest to study the effect of ATP and ouabain on the absorption of the M-antibody using freshly prepared membranes. Fig. 8 presents three binding curves of such an experiment. Curve (a) shows the binding of antibody per membrane at four dilutions of antiserum without ATP and ions in the absorbing system. The number of AU bound per membrane (average 1.7) in the absence of ATP and ions was

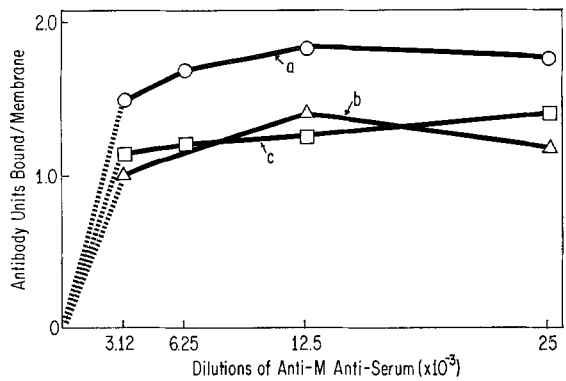

Fig. 8. The number of AU bound per membrane (HK 5) in the presence and absence of ATP and ouabain. (*a*) No ATP and ions in absorbing assay, (*b*) ATP, Mg^{++}, Na^{+}, K^{+}, and (*c*) ATP, Mg^{+}, Na^{+}, K^{+} and ouabain present 30 min prior to addition of four dilutions of anti-M serum

slightly higher than the number of AU bound per membrane when the absorption was done in presence of 1 mM ATP, 1 mM Mg^{++}, 100 mM Na^{+}, 10 mM K^{+} and 30 mM Tris/Cl, pH 7.6, in the absence (curve b) and presence (curve c) of ouabain (10^{-4} M). Thus, these data show clearly that ATP ions and ouabain do not produce a substantial change in the maximum number of M-antibody molecules bound per membrane when the concentration of M-antibody in the system is high. Furthermore, no effect of these agents on the affinity of membranes for antibody was detected, although this may have been because of the relative insensitivity of the assay system.

The Nature of the Anti-M Antibody

Fig. 9 shows the elution diagram of the anti-M antiserum (S 11) on Sephadex G 200. As seen in the lower part of the diagram, three major peaks (I, II & III) of material which absorbs light of wave length 254 mμ were observed. The void volume was carefully tested by blue dextran (molecular weight approx. 10^6). The hemolytic activity was found to be comparatively high in peak I and substantially lower in peak II. Agglutinating activity of the anti-D antibody present in the S 11 anti-M antiserum was detected mainly in peak I. The test tubes were combined to six fractions (CF-a to CF-f lower part of the diagram) and analyzed by immunoelectrophoresis as seen in Fig. 10. The immunoprecipitin lines of rabbit anti-sheep plasma protein antiserum with sheep anti-M antiserum and its fractions obtained by gel filtration were compared. The precipitin line of sheep β_2 M globulin (indicated by arrow) and probably of an α_2 macro-

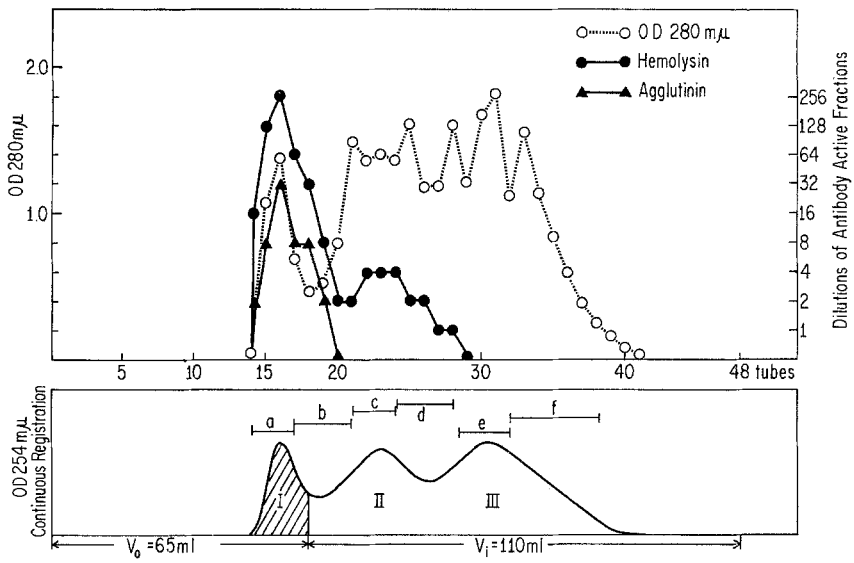

Fig. 9. Gel filtration profile and antibody activities of S 11 iso-immune anti-M serum after elution on Sephadex G 200 in 1 M NaCl: 0.1 M phosphate buffer pH 7.82. The shaded area indicates the appearance of blue dextran, V_0 the void volume and V_i the internal resin volume. The optical density of each test tube was measured separately at 280 mμ (open circles). The titer of the hemolytic anti-M antibody activity (solid circles) and of the anti-D agglutinin (solid triangles) are indicated at the right-hand scale of the diagram. Segments $(a-f)$ indicate the pooled column fractions CFa – f

globulin were only present in CF-a. These proteins are known to have a molecular weight of up to 10^6. Fractions CF-b, c and d did not contain the β_2 M line; the predominant precipitin line in these fractions was that of the 7 S γ-globulin with its characteristic gull-wing appearance as described by Silverstein, Thorbecke, Kraner and Lukes (1963). Fractions CF-e and f exhibited only the precipitin arcs of some α_1-globulins and albumin.

Discussion

In our attempt to find M-positive HK and M-negative LK sheep among crossbreeds of Suffolk, Rambouillet and Hampshire, we analyzed the red cells of 53 sheep for K^+ and Na^+ and for the presence of the M-antigen as seen in Table 1. The table confirms the original findings of Rasmusen and

Table 1. *Correlation between K^+ content and M-antigen in sheep red cells*

Sheep	M-positive	M-negative
HK	11	—
LK	24	19

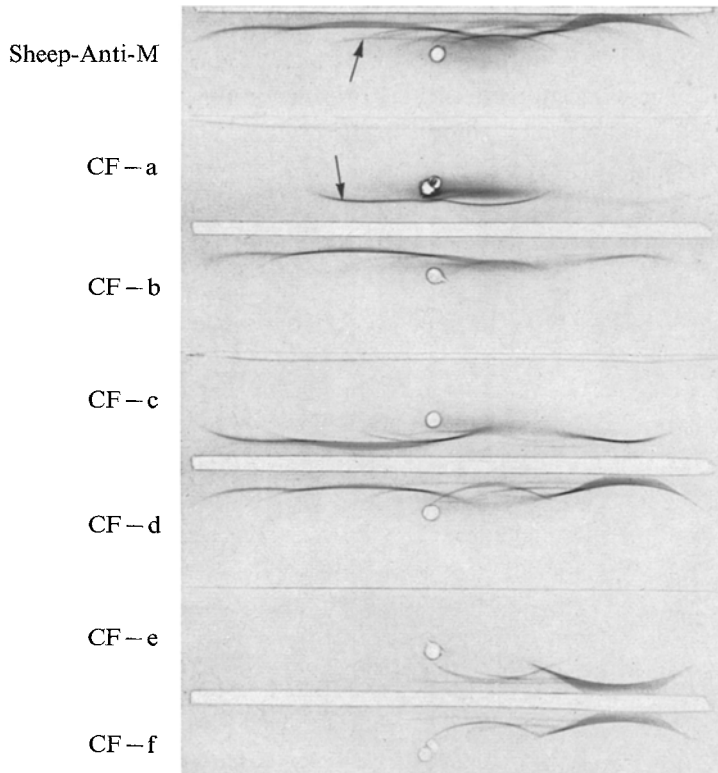

Fig. 10. Immunoelectrophoretic analysis of S 11 iso-immune anti-M antiserum and its subfractions (CF a – f) obtained by gel filtration. The anode is to the right. The precipitin arcs were developed with rabbit anti-sheep plasma protein antiserum. Arrow indicates the position of the β_2 M globulin

Hall (1966*a*, *b*) that HK sheep are always M-positive. According to these authors (1966*b*), all M-positive LK red cells can be classified as heterozygous and can be ascribed to the genotype $Ka^L\ ka^h = Mm$, whereas all M-negative red cells are homozygous for LK character ($Ka^L\ Ka^L = mm$). The fact that 56% of the LK animals examined were M-positive is also in agreement with the results of Rasmusen and Hall (1966*b*), since populations which they studied included some with higher and some with lower incidences of M-antigen in the LK-population. Evans and Phillipson (1957) observed that red cells of heterozygous LK sheep have slightly higher K values than red cells from homozygous LK sheep. The (K)/(Na) ratio of M-positive LK sheep, therefore, should be slightly higher than that of M-negative LK sheep. The mean value for the (K)/(Na) ratios of M-positive LK sheep is 0.187 ($\pm$0.074 SD, $\pm$0.015 SE), whereas the mean value for the (K)/(Na) ratios of M-negative LK sheep was found to

be 0.139 (±0.057 SD, ±0.013 SE). Thus, the mean value for the (K)/(Na) ratio is significantly higher in animals heterozygous for the M-antigen than in M-negative LK sheep. However, the magnitude of the standard deviation indicates that the variability among individuals is so large as to preclude the possibility of distinguishing between homozygous and heterozygous states on the basis of the (K)/(Na) ratio. This point is further supported by the data in Table 2 which shows the (K)/(Na) ratio of six M-positive and six M-negative LK sheep. It is apparent that the (K)/(Na) ratio can be as low in M-positive as in M-negative LK sheep red cells.

Table 2. *The (K)/(Na) ratio in six M-positive and six M-negative LK sheep bloods*

Sheep	(K)/(Na)	M-antigen
10	0.05	+
8	0.09	+
21	0.10	+
7	0.13	+
31	0.13	+
43	0.14	+
3	0.04	−
11	0.08	−
33	0.08	−
35	0.08	−
17	0.10	−
29	0.10	−

At sufficient concentrations, the antiserum used produced lysis of all M-positive red cells, whether from HK or LK sheep. This finding indicates that the M-character is expressed on all red blood cells of M-positive animals and is not restricted to a part of the erythrocyte population. The absorption studies show that the antibody has a high affinity for the M-antigen, since the number of antibody units bound per HK red cell or HK membrane remains constant even when high dilutions of antiserum are used in the absorption experiment. Thus, increasing the concentration of antibody does not favor the binding of more antibody units as one would expect in the case of an antibody with a low association constant. The assay system used was not sufficiently sensitive to permit a precise measurement of the association constant for the reaction of M-antigen with its homologous antibody. It should be noted that the ratio of the amount of antibody absorbed per cell or membrane to the amount of antibody necessary for lysis is about unity. The presence of guinea pig serum complement in the absorbing system did not substantially enhance binding of anti-M antibody

by HK membranes. These experiments were carried out because it has been shown by Rosse, Borsos and Rapp (1968) that the fixation of cold reacting human anti-blood group-I antibodies is enhanced in the presence of C'1a, the first subcomponent of complement.

Rasmusen and Hall (1966*a*) have observed that the inheritance of the M-antigen is apparently associated with the inheritance of the HK character in sheep red cells. This finding makes it important to define the nature of the relation between the Na^+-K^+-transport process and the M-antigen in these cells. Active transport of cations across the sheep red cell membranes involves both a ouabain-sensitive active Na^+-K^+-transport system and passive leakage of these ions. Both the pump and the leak for K^+ and Na^+ are different in HK and LK sheep red cells (Tosteson & Hoffman, 1960). The pump operates about four times faster in HK than in LK cells. The observation of Tosteson (1963) that the Na^+-plus-K^+-stimulated and ouabain-sensitive ATPase is also about four times more active in membranes isolated from HK rather than from LK cells suggested that the S-ATPase and active transport of cations across sheep red cell membranes are closely associated functions. Since both the $Na^+ - K^+$ pump and the S-ATPase can be inhibited by 10^{-4} M ouabain (Schatzman, 1953; Glynn, 1957; Post, Merritt, Kinsolving & Albright, 1960), it was of considerable interest to study the effect of ouabain on the binding of anti-M antibody to HK membranes. Our results did not reveal any effect of ATP on the capacity of HK membranes to bind M-antibody in the presence or absence of 10^{-4} M ouabain. Studies are in progress to see if the enzymatic activity of the S-ATPase as well as the active cation transport are impaired by the reaction of the M-antigen with its homologous antibody. The findings of Brewer, Eaton, Beck, Feitler and Shreffler (1968), however, would indicate that the M-antibody does not interfere with the enzymatic activity of S-ATPase in HK sheep red cell membranes. It is also necessary to explore the relation between the M-antigen and the leakage of K^+ and Na^+ in HK and LK sheep red cells.

Little is known about the immunochemical nature of the species-specific sheep red cell antigens. The M-antigen appears to be very labile to lyophilization and extraction by n-butanol. By contrast, Maddy (1968) showed that butanol extraction of the protein from cattle red cell membranes did not impair the majority of the antigenic activities. Only one antigen of sheep red cells, i.e., the D-antigen, is detected by agglutinating rather than by hemolyzing antibodies (Rasmusen et al., 1960). In contrast to the M-antigen, the D-antigen did survive lyophilization and butanol extraction. The M-antigen activity seems, therefore, to require a relatively

intact structure of the membrane matrix, an observation also made for the enzymatic function of the S-ATPase in sheep red cells (Tosteson, 1966).

Nelson (1967) and Reed (*see* Tosteson, Cook & Blount, 1965) found that HK and LK sheep red cell membranes apparently do not differ significantly in their lipid composition. Differences in their membrane protein constituents have not yet been studied in detail because of the slow development of suitable methods to solubilize membrane protein. However, one of the methods to ascertain differences in the proteins of such membranes is the use of electrophoresis in depolymerizing gels (Poulik & Lauf, 1965; Azen et al., 1966). Application of this method to HK and LK sheep red cell membranes did not reveal a significant difference in the protein pattern of the two genetic types.

The immunoelectrophoretic analysis of whole anti-M antiserum gave precipitin lines similar to those observed by Silverstein et al. (1963) and Chordi and Kagan (1964) in normal adult sheep sera. The association of anti-M antibody with both high and low molecular weight proteins of sheep serum points to the structural heterogeneity of the antibody. Heterogeneity of the anti-M antibody is also suggested by the findings of Rasmusen et al. (1960) that anti-M antisera cross-react with cattle red cells of type S_2 and U. The major anti-M antibody activity may be present in the β_2 M globulin clearly visible in the first fraction of the eluate obtained by gel filtration of anti-M antiserum. Its hemolytic activity is independent of the agglutinating activity of the anti-D antibody also found in this fraction, since M-positive D-negative red cells were lyzed to a similar extent. Elution experiments are being carried out to clarify which class of antibody is bound by HK membranes.

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References

Azen, E. A., R. A. Nazhat, and O. Smithies. 1966. Acidic buffer systems for urea-starch gel electrophoresis. *J. Lab. Clin. Med.* **67**:650.

Brewer, G. J., J. W. Eaton, C. C. Beck, L. Feitler, and D. C. Shreffler. 1968. Sodium-potassium stimulated ATPase activity of mammalian hemolysates: Clinical observations and dominance of ATPase deficiency in the potassium polymorphism of sheep. *J. Lab. Clin. Med.* **71**:744.

Chordi, A., and I. G. Kagan. 1964. Analysis of normal sheep serum by immunoelectrophoresis. *J. Immunol.* **93**:439.

Evans, J. V., and A. T. Phillipson. 1957. Electrolyte concentration in the erythrocytes of the goat and ox. *J. Physiol.* **139**:87.

Glynn, I. M. 1957. The action of cardiac glycosides on sodium and potassium movements in human red cells. *J. Physiol.* **136**:148.

Lauf, P. K. 1969. Binding of iso-immune anti-M by HK and LK sheep red cell membranes. *Fed. Proc.* **28**:315.

—, and M. D. Poulik. 1968. Solubilization and structural integrity of the human red cell membrane. *Brit. J. Hematol.* **15**:191.

Maddy, A. H. 1964. The solubilization of the protein of the ox erythrocyte ghost. *Biochim. Biophys. Acta* **88**:448.

— 1968. Some problems relating to the chemical composition of membranes. *In* Symposium on the Molecular Basis of Membrane Function. Symposia of the Society of General Physiologists. *In press.*

Mayer, M. M. 1961. *In* Experimental Immunochemistry. E. A. Kabat, editor. p. 154. Chas. C., Thomas, Springfield, Ill.

Nelson, G. J. 1967. Studies on the lipids of sheep red blood cells. I. Lipid composition in low and high potassium red cells. *Lipids* **2**:64.

Post, R. L., C. R. Merritt, C. R. Kinsolving, and C. D. Albright. 1960. Membrane adenosine triphosphatase as a participant in active transport of sodium and potassium in the human erythrocyte. *J. Biol. Chem.* **235**:1791.

Poulik, M. D., and P. K. Lauf. 1965. Heterogeneity of water-soluble structural components of human red cell membrane. *Nature* **208**:874.

Rapp, H. J. 1953. Purification and immunochemical characterization of the heat stable alcohol — soluble hemolytic antibody inhibitor of the sheep erythrocytes. Ph. D. Thesis. The John Hopkins University, Baltimore, Md.

Rasmusen, B. A., and J. G. Hall. 1966*a*. Association between potassium concentration and serological type of sheep red blood cells. *Science* **151**:1551.

— — 1966*b*. An investigation into the association between potassium levels and blood types in sheep and goats. *In* X. Congres Europeen sur les groupes sanguins et le polymorphisme biochimique des animaux. p. 453. Paris.

— C. Stormont, and Y. Suzuki. 1960. Blood groups in sheep. III. The A, C, D and M systems. *Genetics* **45**:1595.

Rosse, W. F., T. Borsos, and H. J. Rapp. 1968. Cold-reacting antibodies: The enhancement of antibody fixation by the first component of complement (C'la). *J. Immunol.* **100**:259.

Schatzmann, H. J. 1953. Herzglykoside als Hemmstoffe für den aktiven Kalium- und Natriumtransport durch die Erythrozytenmembran. *Helv. Physiol. Acta* **11**:346.

Scheidegger, J. J. 1955. Une micro-methode de l'immunoelectrophorese. *Intern. Arch. Allergy Appl. Immunol.* **7**:103.

Silverstein, A. M., G. J. Thorbecke, K. L. Kraner, and R. J. Lukes. 1963. Fetal response to antigenic stimulus III. γ Globulin production in normal and stimulated fetal lambs. *J. Immunol.* **91**:384.

Tosteson, D. C. 1963. Active transport, genetics and cellular evolution. *Fed. Proc.* **22**:19.

— 1966. Energy sources in ionic movements. *In* The Myocardial Cell. H. L. Conn and S. A. Briller, editors. p. 111. U. of Pa. Press, Philadelphia.

— P. Cook, and R. Blount. 1965. Separation of adenosine triphosphatase of HK and LK sheep red cell membranes by density gradient centrifugation. *J. Gen. Physiol.* **48**:1125.

—, and J. F. Hoffman. 1960. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. *J. Gen. Physiol.* **44**:169.