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ON THE STRUCTURE OF AGGLUTINATED SHEEP RED BLOOD CELL MEMBRANES

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SUMMARY

Specimens of isolated sheep red blood cell membranes are prepared by an agglutination technique in which membranes are stacked in regular arrays. X-ray diffraction patterns are recorded from such specimens which show meridional and equatorial diffraction phenomena. The meridional reflections correspond to single lamellar repeat periods of 160–186 Å. It is concluded that two asymmetric membranes are contained in the elementary period. Lipid phases with preferentially oriented hydrocarbon chains are part of the membrane structure. The stacking of the membranes is also demonstrated in the electron microscope. The X-ray scattering curve of intracellular hemoglobin of intact sheep red blood cells is recorded to a spacing of about 8 $Å^{-1}$. The broad diffraction rings of this scattering curve are replaced by a series of rather sharp rings, when the red blood cells are agglutinated and placed in a hypertonic medium. Both the presence of a functioning membrane and the agglutination appear to be essential for the full expression of this phenomenon.

INTRODUCTION

The adult red blood cell consists of a membrane and a cell interior which may be regarded with good approximation as a concentrated hemoglobin solution. The protein and lipid composition of the membrane is quite well known and the location of some components in the membrane has been deducted from biochemical studies (e.g. ref. 1). It is established that lipid phases exist and that proteins in general are asymmetrically distributed in the membrane [2]. Membrane and cell specimens which are suitable for direct structural studies by physical methods can be prepared from red blood cells. Wilkins et al. [3] have shown that X-ray diffraction data can be recorded from random dispersions of red blood cell membranes. However, specimens with a higher degree of order have advantages for diffraction studies. Model experiments show that a few stacked membranes suffice to give sampled diffraction data [4]. It may be guessed that agglutination will produce stacked membrane specimens of higher water content than the oriented specimens of Finean et al. [5, 6]. Suspensions of agglutinated red blood cell membranes are expected to contain both regions of stacked

membranes which are randomly oriented on a macroscopic scale and regions of a more disordered structure. Contributions from the ordered regions will dominate the pattern of diffracted X-rays and an equivalent of Debye-Scherrer powder patterns should be observed. Very weak patterns of this type can be recorded experimentally. It was found, however, that membrane pellets can be prepared by agglutination techniques which contain such unexpected large regions of stacked membranes that a lamellar type diffraction pattern is recorded in which meridional and equatorial diffraction phenomena can be separated.

The diffraction pattern of intact red blood cells is dominated by the scattering of intracellular hemoglobin. Dervichian et al. [7, 8] have recorded the scattering curve of intracellular hemoglobin to spacings of about 50 $Å^{-1}$. Diffraction maxima at higher angles have been reported [9]. A secondary maximum at 62 $Å^{-1}$ of the intensity distribution curve is due to interparticle interference of the closely packed hemoglobin molecules [10]. In the present experiments the scattering curve of intracellular hemoglobin was recorded to an angle of about 8 $Å^{-1}$ in order to obtain information on the structure of the red blood cell from the state of intracellular hemoglobin.

MATERIALS AND METHODS

Blood of Grison mountain sheep was obtained under sterile conditions from Graeub AG, Berne. A group of 2–3 animals of the same family was selected for each series of experiments and the individual donor animal from this group was identified for each blood sample. The blood was collected in an equal volume of Alsever's solution [11, 12] and stored at 4 °C. It was used within at most 4 days (usually within 2 days) after bleeding.

To prepare red blood cell suspensions the cells were sedimented at about $450 \times g$ (15 min, 4 °C) and then washed twice in about 5 volume parts of Alsever's solution with 25 % sheep serum. After each centrifugation step the white top layer was removed carefully from the packed red blood cells. For experiments with intact cells the middle portion of the cell pellet was used exclusively after the final washing. Isolated membranes were obtained by careful osmotic lysis of the washed and packed cells in 5–7 volume parts of distilled water at 0–4 °C. After lysis the membranes were pelleted at 12 000–14 000 $\times g$ (20 min, 4 °C) and washed repeatedly (usually 3 washings) in Tris · HCl buffer (10^{-2} M, pH 7.4) with 10^{-4} M EDTA and 5 % sheep serum. Usually a two-layered membrane pellet was obtained after the final washing; the top and pearly white part of the pellet was used exclusively. Buffer solutions containing mercury (to be called $HgCl_2$-solution) were prepared by adding $HgCl_2$ from a concentrated stock solution to the same buffer immediately before use. After standing, these solutions became turbid. All solutions were sterile by Millipore filtration. All preparation steps were done on a laminar air flow bench and centrifugations were done under sterile conditions.

Phytohemagglutinin M was purchased from Difco Laboratories, dialysed against dilute buffer and lyophilised. The processed sample of the agglutinin was stored at 4 °C and solutions were made up immediately before use. Agglutination titers were determined with a Takatsy microtitrator kit [13] using 2 % washed sheep red blood cells. If necessary, solutions were dialysed against isotonic buffer prior to titration. Specimens of agglutinated cells were prepared by incubating suspensions of

about 25 % washed red blood cells with 10–20 mg phytohemagglutinin M per ml cell suspension (15 min at 0–4 °C and 30 min at 25 °C). Agglutinated membrane pellets were prepared by incubating suspensions of washed membranes with phytohemagglutinin M (15 min at 0–4 °C and 30 min at 25 °C), followed by centrifugation at $2.8 \cdot 10^5 \times g$ (15 h at 4 °C). 0.1–0.2 mg phytohemagglutinin M per mg of final pellet was used for the incubation. Hypertonic media were prepared by increasing the buffer concentration or by adding NaCl to the buffer. Osmolarities were measured with a Knauer osmometer. Sheep hemoglobin solutions were prepared by osmotic lysis of red blood cells. The solutions were then concentrated with an Amicon ultrafiltration cell. The cell was pressurized with pure nitrogen gas. The concentrated solutions were equilibrated with air under sterile conditions.

Protein was determined by the method of Lowry et al. [14]. Hemoglobin concentrations were measured spectrophotometrically as cyan-methemoglobin [15]. Hematocrit values were determined in calibrated micro-hematocrit tubes (inner diameter 0.56 mm) with a Clay Adams Autocrit centrifuge. All reagents were analytical grade. Water was distilled once in a glass and twice in a quartz apparatus.

Sodium dodecyl sulphate-gel electrophoresis was done following the procedures of Bretscher [16]. 7.5 % polyacrylamide gels were used; the gel buffer contained 0.2 % sodium dodecyl sulphate. If necessary, the membrane pellets were dispersed in gel buffer by 2–3 sonication steps of 20 s duration (Branson sonifier, power level 1). At least 5 mg sodium dodecyl sulphate per mg protein, phenyl methyl sulfonyl fluoride in *n*-propanol to a final concentration of $2 \cdot 10^{-3}$ M and β-mercaptoethanol to a final concentration of 2 % was added to the protein samples. The samples were then placed in a water bath at 60 °C for at least 5 min. Gels were stained with Coomassie brilliant blue and with periodic acid/Schiff's reagent [17].

Specimens for electron microscopy were fixed in buffered 3 % glutaraldehyde (2 h, 4 °C) and in 1 % osmium in water (30 min, 4 °C). They were then dehydrated, embedded in Epon, thin-sectioned and stained with lead citrate and uranyl acetate.

X-ray diffraction experiments were done with an Elliott GX 6 rotating anode generator with 100 μm cup (Ni-filtered Cu-K-α radiation) and a Franks' camera with double mirror optics, guard slits and a thermostated specimen holder. The camera was evacuated during exposures. The specimens were kept either in a small cell with Melinex windows or in a sealed glass capillary of 0.5 mm diameter and 10^{-2} mm wall thickness. All specimens could be oriented in a plane perpendicular to the X-ray beam, and the glass capillaries could be rotated around their long axis. Some experiments were done with a sample holder where capillaries were mounted on a goniometer and could be kept oscillating through a predetermined angular range during the exposure. Exposure times were 24–48 h in most experiments. The specimens were usually kept at 4.5 ± 0.1 °C during exposure. The sample holder could be sealed with additional Melinex windows against the camera vacuum and could be flushed with water vapour saturated helium in order to eliminate any risk of unwanted drying of the specimen. Diffraction patterns were recorded on Ilford Industrial G X-ray film. Densitometer traces were obtained with a Joyce-Loebl MK III B microdensitometer.

RESULTS

The density of the agglutinated membrane pellets in different experiments varies within a range of about 0.08–0.15 mg total protein per mg pellet. Membrane

pellets which are prepared in the same way except that the agglutination step is omitted, have densities of about 0.04–0.08 mg total protein per mg pellet. On sodium dodecyl sulphate gels the phytohemagglutinin M-preparation shows several bands. Corresponding bands are found on sodium dodecyl sulphate gels of the agglutinated membrane pellets. The intensities of the phytohemagglutinin M-bands of the gels from membrane pellets were compared by eye with the band intensities of known amounts of phytohemagglutinin M. Thus it can be estimated that the agglutinated membrane pellets contain approximately 10–20 % phytohemagglutinin M relative to the total protein content. The amount of phytohemagglutinin M bound on the membranes can also be roughly estimated from the agglutination titer in the supernatant of the agglutinated membrane pellet and from a standard titer; a value of the same order of magnitude is obtained as from the evaluation of the sodium dodecyl sulphate gels.

When agglutinated membrane pellets are placed between crossed polarizers and viewed with a stereoscopic microscope, distinct and brightly birefringent areas are seen, which occupy about 20–30 % of the pellet. X-ray diffraction patterns from the birefringent parts of the pellets show a clear separation into meridional and equatorial diffraction phenomena (Fig. 1). This demonstrates that the membranes are not randomly oriented in that part of the pellet. In Fig. 1 relatively sharp and strong reflections (to be called meridional reflections) are recorded at spacings of 62.0 $Å^{-1}$, 46.5 $Å^{-1}$ and 16.9 $Å^{-1}$. These reflections spread off the meridian to some extent and thus appear as arcs. A broad 10.5 $Å^{-1}$ diffraction ring, an equatorially accentuated 4.6 $Å^{-1}$ ring, and a 3.3 $Å^{-1}$ water band are also recorded in Fig. 1. The ratio of the radially integrated intensities of the 4.6 $Å^{-1}$ ring at the equator and the meridian is about 2 · 1.

The exact spacings and the relative intensities of the meridional reflections vary from experiment to experiment, although the three reflections are reproducibly found at approximately the same spacings as in Fig. 1. In exposures with longer specimen-to-film distances, additional meridional reflections at lower diffraction angles are observed. These reflections are weak and partially masked by diffuse scattering around the beam stop, but they can be recognised in those patterns where the strong meridional reflections are located at slightly higher angles than in Fig. 1. The spacings of the meridional reflections in those patterns are 160 $Å^{-1}$, 80 $Å^{-1}$, 53 $Å^{-1}$ and 40 $Å^{-1}$. The diffuse scattering around the beam stop is prominent even under conditions where the background radiation of the camera is negligible.

The ordered regions in the membrane pellet which have to be postulated from the diffraction pattern (Fig. 1) can be visualised in the electron microscope (Fig. 2). This electron micrograph stems from the same specimen which was used for the diffraction pattern in Fig. 1; the specimen was removed from the diffraction camera and processed for electron microscopy after X-ray exposures of a total of 72 h. Ordered arrays of 10–20 stacked membranes are seen. The single membrane period in the stacking direction in the micrograph is approximately 100–120 Å; it cannot be measured more accurately, because the specimen has disintegrated to some extent and the orientation of the cutting plane to the planes of the membranes is not known. It can be estimated from the size of the stacked regions that membrane areas of several μm^2 are involved. Other regions of the specimen show a more disordered or even amorphous structure. It cannot be decided whether stacked membranes are cut at an oblique angle or whether the structure is truly amorphous in these regions. From the

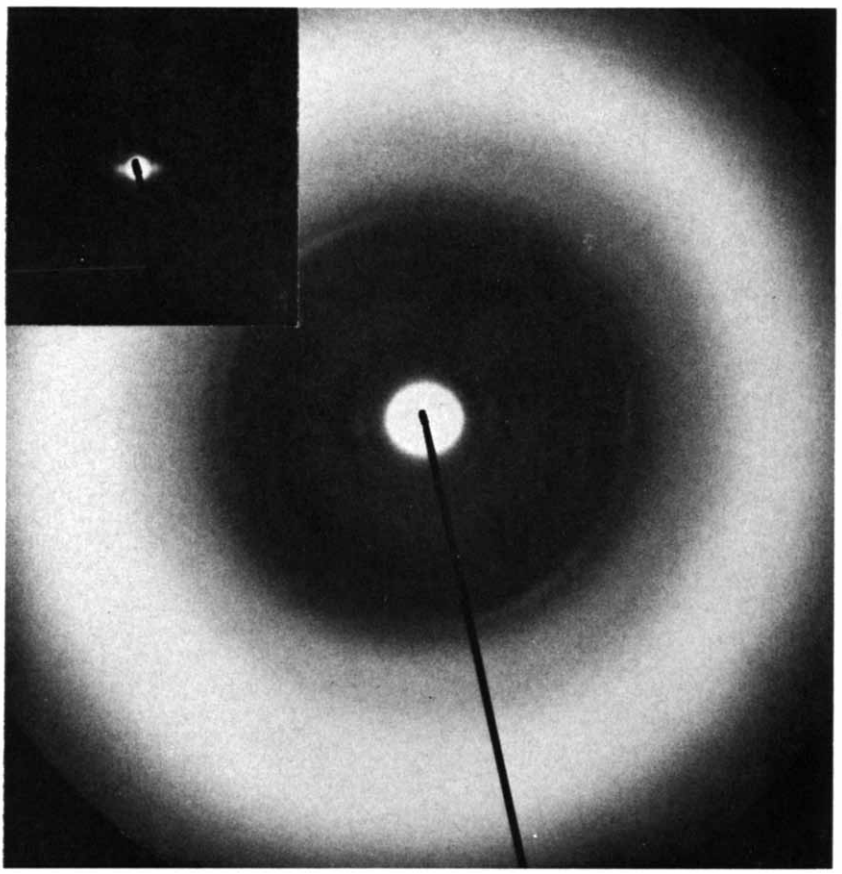

Fig. 1. Diffraction pattern of an agglutinated sheepred blood cell membrane pellet. Meridional reflections at $(62.0\ \text{Å})^{-1}$, $(46.5\ \text{Å})^{-1}$ and $(16.9\ \text{Å})^{-1}$, diffuse rings at $(10.5\ \text{Å})^{-1}$ and $(3.3\ \text{Å})^{-1}$, and an equatorially accentuated $(4.6\ \text{Å})^{-1}$ ring are recorded. (The additional oriented diffraction rings are derived from a set of Melinex windows. Specimen-to-film distance 65.5 mm. Print magnification 1.46 ×. The insert shows the center of the pattern at lower exposure.)

relative proportions of the ordered and disordered regions it must be concluded that the pellet contains few and rather large stacked arrays, whose orientations are unrelated.

The sequence of the membrane surfaces facing each other in the stacked array is inside-inside-outside-outside. This can be demonstrated in electron micrographs of membrane pellets which were prepared in the presence of $5 \cdot 10^{-4}$ M $HgCl_2$ (Fig. 2 insert). Every second intermembrane space has a high electronoptical contrast which must be due to an accumulation of the heavy metal. The contrast is higher than expected from the molarity of the $HgCl_2$ solution; some preferential binding of mercury at the membrane surface or to proteins associated with the membrane must have occurred. The complete membrane of a collapsed ghost could not be traced in the micrographs, but loops of membranes were observed which probably correspond to the rim of collapsed ghosts. These loops were inserted into the dense intermembrane spaces where they created a bifurcation in the stacking order. The inside of the loops

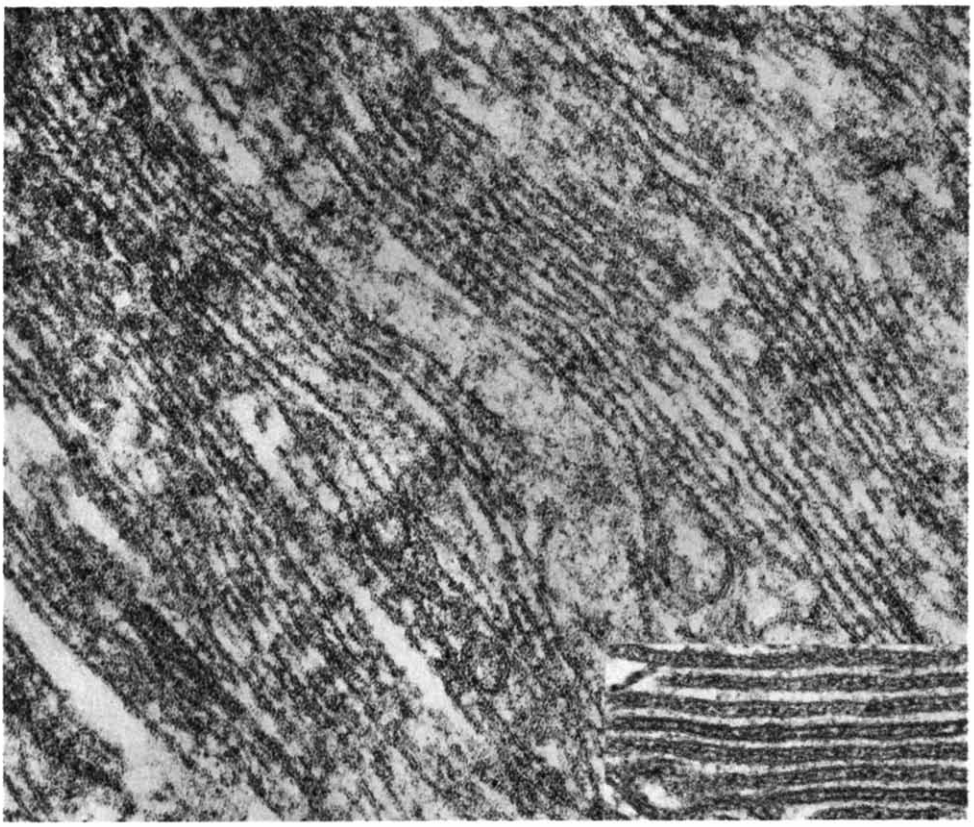

Fig. 2. Electron micrograph of an agglutinated sheep red blood cell membrane pellet. The specimen was processed for electron microscopy after the X-ray exposure. Total magnification 126000 . The insert shows a pellet of agglutinated membranes prepared in the presence of $HgCl_2$ (see text for details).

corresponds to the low density intermembrane space; it is likely, therefore, that the dense intermembrane space is bounded by the extracellular faces of adjacent membranes. A difference in the contrast on the two sides of the membrane can be observed also in control experiments, but the magnitude of the difference is much larger and the effect is reproducible in membrane specimens prepared with $HgCl_2$. Diffraction patterns were also recorded from agglutinated membrane pellets prepared with 10^{-4} M $HgCl_2$ (Fig. 3). Relatively strong meridional reflections at about 60 $Å^{-1}$ and 45 $Å^{-1}$ and several weaker meridional reflections at higher and lower angles are recorded.

A different type of diffraction pattern is observed from agglutinated membrane pellets, if the specimens are kept for 10–14 days or are allowed to dry slowly [5, 6]. In the final stage a series of meridional reflections is recorded with spacings of 59.5 $Å^{-1}$, 29.8 $Å^{-1}$, 19.7 $Å^{-1}$, 14.9 $Å^{-1}$ and 11.9 $Å^{-1}$. The 10.5 $Å^{-1}$ ring and an equatorially accentuated 4.6 $Å^{-1}$ ring are recorded also, but the 3.3 $Å^{-1}$ water band is practically absent.

Diffraction patterns of intact red blood cells in isotonic medium show five broad rings (Fig. 4). Their maxima are located at about 63 $Å^{-1}$, 34 $Å^{-1}$, 19 $Å^{-1}$,

13 $Å^{-1}$ and 10 $Å^{-1}$. There is a diffuse scattering around the origin; the scattering intensity decreases continuously from the center outwards and the 63 $Å^{-1}$ ring forms a secondary maximum of this curve [7–10]. Diffraction patterns of hemoglobin solutions from 4.8 g hemoglobin/100 ml to 45 g/100 ml are analogous to the pattern in Fig. 4, except that the aspect of the first ring depends on the hemoglobin concentration [18].

Fig. 3. Diffraction pattern of an agglutinated sheep red blood cell membrane pellet prepared with 10^{-4} M $HgCl_2$. The meridional reflections at (60 Å)$^{-1}$ and (45 Å)$^{-1}$ are shown. (Specimen-to-film distance 108.7 mm. Print magnification 1.5 ×.)

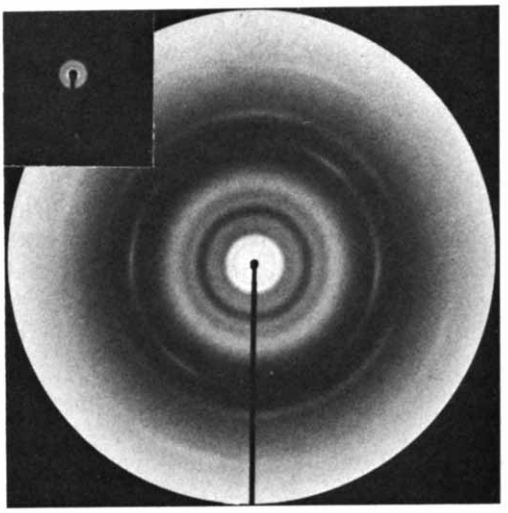

Fig. 4. Diffraction pattern of intact sheep red blood cells in isotonic medium. The maxima of the broad rings are at (63 Å)$^{-1}$, (34 Å)$^{-1}$, (19 Å)$^{-1}$, (13 Å)$^{-1}$ and (10 Å)$^{-1}$. (The additional oriented diffraction rings are derived from a set of Melinex windows. Specimen-to-film distance 69.0 mm. Contact print of original pattern. The insert shows center at lower exposure.)

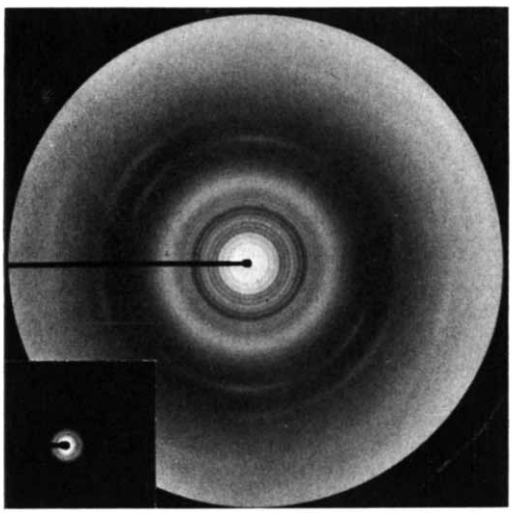

Fig. 5. Diffraction pattern of agglutinated sheep red blood cells in hypertonic medium. The three innermost rings are located at $(85\ Å)^{-1}$, $(61\ Å)^{-1}$ and $(51\ Å)^{-1}$. Their estimated relative intensities are very weak/strong/strong. (The additional oriented diffraction rings are derived from a set of Melinex windows. Specimen-to-film distance 69.0 mm. Contact print of original pattern. The insert shows the center at lower exposure.)

A different type of diffraction pattern is obtained from sheep red blood cells, if they are agglutinated and placed in a hypertonic medium of 750 mosM (Fig. 5). A series of rather sharp diffraction rings is recorded in the same angular range where the five broad rings of Fig. 4 are located. The estimated relative intensities of the sharp rings follow the continuous scattering curve in Fig. 4. The spacings of the first few rings are about 61 $Å^{-1}$, 51 $Å^{-1}$, 36 $Å^{-1}$ and 34 $Å^{-1}$. A very weak ring at 85 $Å^{-1}$ is observed also; it is masked by a diffuse scattering around the origin. The angular width of the sharp rings cannot be measured accurately, since they are poorly resolved in the densitometer traces. However, a lower limit for the width can be determined, if a linear interpolation of the minima between rings is taken as baseline. The halfwidth of the rings then becomes 0.0047 radians. Both patterns in Figs. 4 and 5 represent the same type of weak scattering. The specimens had the same cell density and the same dimensions in both experiments. Exposure times are comparable and the intensity of the Melinex rings may be taken as an internal radiation standard. The sharp rings in Fig. 5 are not added to, but replace the broad rings of Fig. 4. This can be most clearly seen in the region of the second broad ring. Therefore, practically all hemoglobin molecules experience the same state in Fig. 5.

A rough estimate of the red blood cell volume can be obtained from hematocrit values. The hematocrit value of erythrocyte suspensions of equal cell density in isotonic medium, in isotonic medium after agglutination and in hypertonic medium after agglutination (750 mosM) were determined as 21.7 ± 0.6, 21.7 ± 0.6 and 15.1 ± 0.6 %. From these values an intracellular hemoglobin concentration of approximately 46–47 g/100 ml is calculated for the cells in Fig. 5. In diffraction patterns of sheep hemoglobin solutions of about 47 g/100 ml a few weak and poorly resolved sharp rings started to appear in addition to the five broad rings of solutions at lower concen-

tration. At 47 g/100 ml the solutions were highly viscous, but without visible gel formation. At still higher concentration the sheep hemoglobin solutions solidified without forming a proper gel. Thin slices of this hydrated, solid hemoglobin produced a solution-type diffraction pattern with additional rings of different type and spacing from those in Fig. 5.

The agglutinated red blood cells in hypertonic medium were examined in the light microscope. Practically all cells are seen to maintain a smooth and biconcave shape under the conditions under which the pattern in Fig. 5 was recorded. No peculiar shapes of cells are observed and no birefringence can be detected. Standard blood smears stained after May Grünwaldt-Giemsa were inconspicuous except that a relatively high percentage of crenated forms was found.

The red blood cells of certain individual animals from the selected group of sheep reproducibly gave good membrane pellets and cell specimens which showed a clear transition from broad to sharp diffraction rings, whereas cells of other animals of the same group gave poor results. The cells of one sheep yielded good membrane pellets and good cell specimens. A second sheep was a good source for cell specimens, but its membranes disintegrated into small vesicles in the course of the preparation. Preliminary experiments with human red blood cells show that stacked membrane pellets can be prepared in an analogous way, but only diffuse diffraction bands equivalent to those in Fig. 4 are recorded from cell specimens under the same conditions where sheep cells produce the pattern in Fig. 5.

DISCUSSION

The separation into meridional and equatorial diffraction phenomena and the sampled character of the meridional diffraction are the prominent features of the pattern in Fig. 1. They require a preferentially oriented structure of well-ordered periodicity. The stacked membranes in Fig. 2 represent such a structure. The main difference to the oriented specimens studied by Finean et al. [5, 6] is the higher total water content of the agglutinated membrane pellet.

The three strong meridional reflections of Fig. 1 at 62.0 $Å^{-1}$, 46.5 $Å^{-1}$ and 16.9 $Å^{-1}$ are interpreted as third, fourth and eleventh order reflections of a single lamellar repeat period of 186 Å. In other experiments the strong meridional reflections have slightly different spacings, but they fall always on the reciprocal lattice points of a single repeat period in the range from 160 to 186 Å. The variations of the periods must be due mainly to the variable water content of the intermembrane spaces, since smaller periods are obtained by a careful reduction of the water content of the specimens. Even with fully hydrated specimens the periods are not constant. Differences in the sedimentation behaviour of the membranes of the different animals can partly explain this observation which may be related also to the variable density of the pellets. First and second order reflections are recorded in those experiments where the period is at or near 160 Å. They establish the single period despite their weakness. The probable reason for their weakness is that the unit cell transform has small values at those sampling points.

The periods of the membranes in the stacking direction from the electron micrograph (Fig. 2) need not match the elementary period calculated from the meridional reflections in the diffraction pattern, because it is not known to what extent

the specimens swell or shrink in the course of the electron microscopic preparation in which the specimens are handled and exposed to a series of solutions of different osmolarities and to organic solvents. Moreover, the observed periods in the electron micrographs of different specimens vary over a significantly larger range than the respective periods from the diffraction experiments. However, if the periods from electron microscopy and from diffraction experiment are compared, it becomes likely that two membranes are contained in the 186 Å period of Fig. 1. It must then be assumed that the density of the pellet is not uniform, because the theoretical density of a two-membrane structure in 186 Å is higher than the measured average density of about 0.12 mg protein/mg pellet. This assumption agrees with the observation that only parts of the pellets are birefringent.

Symmetry considerations provide good evidence that two membranes are contained in the elementary period of membrane pellets prepared in solutions containing mercury (Fig. 3). It is noted that these considerations do not depend on the assignment of the intermembrane space with high electron optical contrast to the extracellular side of the membrane. Only every second intermembrane space has a high contrast in the electron microscopic image (Fig. 2 insert) and the bound heavy metal should make the electron density profile of the single membrane asymmetric regardless of its native structure. The observed meridional reflections in Fig. 3 all fit a 180 Å-period. If one membrane was contained in 180 Å, the true elementary period would have to be 360 Å. Theoretically, the observed reflections could represent the even order interferences of a 360 Å-period. It would then have to be assumed, however, that all odd order interferences are absent. This appears unlikely, rather it is concluded that the unit cell in Fig. 3 has a 180 Å-period and contains two asymmetric membranes. By analogy it is likely that the 186 Å-period from Fig. 1 also contains two membranes which must, therefore, be asymmetric.

The 10.5 $Å^{-1}$ ring has been observed previously by Finean et al. [5, 6]. It was thought to be due to a protein component of the membrane. The equatorially accentuated 4.6 $Å^{-1}$ ring must be interpreted as a diffraction from hydrocarbon chains of membrane lipids. Lipid phases must exist in the membranes where the chain axes have some preferential orientation perpendicular to the membrane plane which is analogous to the arrangement found in pure lipid systems [19, 20]. The observed ratio of the equatorial and meridional intensities of the 4.6 $Å^{-1}$ ring is probably a lower limit for the true ratio; it needs correction for the contributions of membranes not included in the stacked array and for disorientation of membranes within the stacked array.

A scattering curve proportional to the absolute square of a single membrane transform has been recorded from dispersions of rat red blood cell membranes by Wilkins et al. [3]. The transforms of a single membrane and of a two-membrane unit are different, but the strong regions of the transform of the two-membrane unit must be strong regions of the single membrane transform, if the membranes are identical. The 62.0 $Å^{-1}$ and 46.5 $Å^{-1}$ reflections of Fig. 1 fall in a region where the single membrane transform has large values [3]. However, this transform has also large values in the range of 40 $Å^{-1}$ to 30 $Å^{-1}$ where reflections are absent in Fig. 1; it is unlikely that this is due only to the differences of one-membrane and two-membrane unit transforms. It must be concluded rather that the structures of the two membranes are not identical.

The sampled meridional diffraction in Fig. 1 must stem from membranes which

are linked into an ordered array by the agglutinin. It is not known if and to what extent a lateral separation of components in the plane of the membrane has occurred after the agglutination. Electron microscopic studies of freeze-etched red blood cell membranes by Tillack et al. [21] provide no evidence for an aggregation of agglutinin receptors under the experimental conditions of the present experiments and the aspect of the 4.6 $Å^{-1}$ ring in Fig. 1 makes a lipid phase transition unlikely. The 186 Å-period excludes that a pure membrane-lipid phase is the source of the pattern, although lipid phases are a part of the stacked membranes. Changes in the membrane structure are observed as a function of age and water content of the specimens. Lipid multilayer type diffraction patterns with lamellar periods of about 60 Å are recorded from agglutinated membrane specimens after having aged for 10–14 days or upon drying. This demonstrates that membrane components can separate and pure lipid phases can be formed [5, 6, 22]. The large periods of 160–186 Å are the best evidence that no significant separation has occurred in fresh and fully hydrated specimens. The membrane areas involved in the agglutinated regions are so large that the diffraction patterns should contain information on the average membrane structure, especially since no unusual inhomogeneity of the membranes is observed in electron micrographs. The electron micrograph in Fig. 2 provides a rough measure for the specimen deterioration due to 72 h of X-ray exposure and handling. Specimens which are fixed in the fresh state are better preserved, but it does not appear unrealistic to study the native structure of membranes within the first 48–72 h.

The five broad diffraction rings in Fig. 4 represent the solution scattering curve of intracellular hemoglobin. Minor differences in the aspect of the first ring of intracellular hemoglobin and of hemoglobin solutions of the same concentration were discussed by Fournet [18]. In the normal red blood cell the individual hemoglobin molecule has complete freedom of rotation [10]. The diameter of the smallest sphere into which a hemoglobin molecule in the cell can be inscribed is about 75 Å [10], a value which agrees with the inter-particle distance calculated from the position of the first diffraction ring of the scattering curve [7, 8, 10]. The likely interpretation of the sharp rings from agglutinated cells in hypertonic medium is that the osmotic gradient induces a transfer of water into the extracellular space and, as a consequence, the packing of the hemoglobin molecules acquires a higher degree of order than in the normal cell. It must be concluded that the position of molecules becomes correlated within small groups of molecules such that interference of the scattered X-rays occurs. These groups are randomly oriented and Debye-Scherrer type diffraction rings are produced; the rings cannot be indexed from the available data [23]. The groups of molecules cannot be large; a characteristic length of at most 300 Å is calculated from the halfwidth of the rings. The intracellular hemoglobin concentration in Fig. 5 calculated from the relative hematocrit values is about 46–47 g/100 ml. At this concentration, the sheep hemoglobin solutions are highly viscous, but macroscopically fluid. Attempts to find conditions under which the broad rings from hemoglobin solutions are completely replaced by sharp rings in an analogous way as with intracellular hemoglobin, were unsuccessful. However, the observation that a few weak sharp rings are recorded in addition to the five broad rings from hemoglobin solutions of 47 g/100 ml provides further evidence that the sharp rings originate from macroscopically fluid solutions.

The cells were kept in contact with medium in all experiments and the cell

membranes must have been able to maintain the osmotic gradient during the 24–48 h required for exposure. Only a few weak and sharp rings superimposed on the five broad rings were observed with cells exposed to the same osmotic gradient without agglutination. Furthermore, cells of the same blood sample which produce patterns with well resolved sharp rings under the normal conditions defined above, show only few and weak sharp rings superimposed on broad rings when they are deprived of glucose and ATP. Therefore, the membranes must have retained some of their native properties after the agglutination, and both the presence of the membrane and the agglutination appear as important factors for the expression of the sharp rings. An aggregation of phytohemagglutinin receptors in the plane of the membrane is not known to occur under the conditions under which the present experiments were done [21].

The electron density profile of the agglutinated membrane can be calculated, if the phases of the meridional reflections are determined. This may be achieved either by treating the patterns from different specimens with periods of 160–186 Å as a set of swelling experiments or by evaluating patterns from specimens with different heavy metal content (Fig. 2 insert). The biochemical structure of the erythrocyte membrane with regards to the distribution of the different protein and lipid constituents is asymmetric [2, 24]. Even without calculating the electron density profile by Fourier methods it must be concluded from the present data that the density profile of the single membrane is also asymmetric.

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