THE BIREFRINGENCE OF THE HUMAN RED CELL GHOSTS*

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Schmitt, Bear, and Ponder (1936, 1938) observed that the rabbit red cell ghost, prepared by freezing and thawing, is slightly birefringent. Extraction of the lipids and suspension of the ghosts in media of varying refractive indices led to the conclusion that a positive intrinsic birefringence, due to the radial orientation of lipid, is nearly balanced by a negative form birefringence due to the tangential orientation of proteins. The net birefringence is very small, and, looking back on the experiments, the ghosts were not as Hb-free as was thought; in 1938, the possibility of the complexing of Hb with lipoproteins had not even been suggested.

Mitchison (1950, 1953) and Swarm and Mitchison (1950) have described a technique, excellent in itself, for making quantitative measurements of the birefringence of the human red cell ghost, prepared by adding red cells to glycerol. Their results led them to the conclusion that there is a birefringent region, about 5000 Å thick, at the red cell surface, and Mitchison has proposed a structure for the mammalian red cell or ghost in which bundles of protein, arranged like Chinese crackers, occupy a layer about 5000 Å thick. Such a layer would make up about half the volume of the red cell. Mitchison does not think that the birefringence measured by his method is dependent on the admitted presence of Hb in the surface ultrastructure of his ghosts, but the reasons given (cf. Perutz and Mitchison, 1950) have been criticised by Der- vichian (1955).

The complexing of Hb with the lipoproteins of the red cell ultrastructure has now been demonstrated experimentally (Bessis, Bricka, Breton-Gorius, and Tabuis, 1954; Ponder, Ponder, and Barreto, 1955). This paper is concerned with the question as to how much of the birefringence of the red cell ghost is due to its containing complexed Hb. Starting with the observation (Ponder, 1952) that there is a large variation in the amount of residual Hb contained in ghosts prepared in glycerol, by freezing and thawing, and by many other methods, we propose to show that only those ghosts which contain enough Hb to render them visible with the ordinary microscope show birefringence of the type described by Mitchison, while ghosts which contain so little Hb that they are invisible with the ordinary microscope, although

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clearly visible with phase contrast, do not exhibit measurable birefringence. It is particularly easy to demonstrate this in the case of the ghosts prepared by adding red cells to glycerol, for in this case the number of ghosts which contain enough Hb to make them visible with the ordinary microscope, as well as to be birefringent under the polarizing microscope, is a function of time.

Fig. 1. Human red cells in glycerol. Ordinate, per cent of initial number of objects (cells plus ghosts) visible with the ordinary microscope and showing birefringence: abscissa, time in minutes. Dotted line at top indicates that all the initial number of objects (cells plus ghosts) are visible with phase contrast. Line through circles, fresh blood; line through crosses, blood stored for 24 days at 4°C.

EXPERIMENTAL

The results obtained are best described by giving an account of experiments.

Experiment 1.—The unwashed red cells of 0.1 ml. of fresh human blood were added to 10 ml. of glycerol. Immediately after mixing, which takes about a minute, the number of cells plus ghosts was measured, at the same magnification, on a standard counting chamber, (a) with the ordinary microscope, and (b) with a phase contrast microscope. The number of red cells plus ghosts visible with phase optics is denoted by 100 on the ordinate of Fig. 1; this number does not change with time.

1 High dry 4 mm. objective, × 40, N.A. 0.65, × 5 eyepiece used in conjunction with a Spencer brightline counting chamber.
2 Zeiss-Winkler microscope fitted with a long working distance (8 mm.) phase condenser, 4 mm. phase objective × 40, N.A. 0.65, × 5 eyepiece, used in conjunction with a Spencer brightline counting chamber.
because all the cells and ghosts can be seen. The number of red cells plus ghosts seen, as a function of time, is accordingly represented by the horizontal dotted line at 100 on the ordinate. The number of cells plus ghosts visible with the ordinary microscope is shown in the full line passing through circles, the number being expressed as a percentage of the number of cells plus ghosts seen with phase contrast. It decreases rapidly as a function of time; after 10 minutes, 43 per cent of the cells plus ghosts are visible with the ordinary microscope, while after 30 minutes only 9 per cent are visible, although the remaining 91 per cent are easily visible with phase contrast.

Experiment 2.—The same type of experiment was repeated, a polarization microscope\(^8\) (convertible into an ordinary microscope by pushing the analyzer out of the light path) being used in conjunction with a phase microscope at the same magnification.\(^4\) Slide and coverslip preparations were used instead of preparations on a counting chamber, and the number of cells plus ghosts (a) visible with the ordinary microscope, (b) showing birefringence, and (c) visible with the phase contrast microscope, were counted as nearly simultaneously as possible, in ten fields and with the three optical arrangements. As before, the number of visible objects decreased as a function of time when they were observed with the ordinary microscope, but insertion of the analyzer and compensator into the light path showed that all the objects visible with the ordinary microscope were slightly birefringent. The same cells, however, when observed with the ordinary microscope (i.e., with the analyzer removed from the light path), also showed residual \(\mathrm{Hb}\). The condition necessary for the ghost showing birefringence accordingly seems to be that the ghost contain enough residual \(\mathrm{Hb}\) to enable it to be seen with the ordinary microscope; if the residual \(\mathrm{Hb}\) is so small that it does not enable the ghost to be seen with the ordinary microscope, no birefringence is observed in glycerol (refractive index 1.47), although the ghost may be clearly visible with phase contrast.

The birefringent ghosts were photographed, and pictures similar to those shown by Mitchison were obtained. Densitometry measurements made along a radial line would certainly give results similar to his, but the measurements were not made

\(^8\) Leitz polarizing microscope with double thickness polaroids; \(\times 90\) achromatic 2 mm. oil immersion objective, N.A. 1.3 reduced to N.A. 1.0 with an internal stop, \(\times 10\) compensating eyepiece with cross-hairs; three-element condenser stopped down to N. A. 0.3 and oil-immersed to the slide; \(\lambda/30\) compensator with verniers; zirconarc light source used with Köhler illumination and a narrow iris; super XX film developed with DK 60a, exposure time about 30 seconds. Each object was centered for examination and photography. Great care was taken with the alignment of the optical system, and a vertical light train consisting of the zirconarc below a supporting table with an aperture in it, the polarizing microscope above, and a Leica camera mounted on a Leitz aristophot support, gave excellent results. This arrangement allows observations to be made in a darkened room with the dark-adapted eye. The zirconarc requires to be cooled by a cooling unit placed on the floor below it.

\(^4\) For examining ghosts between slide and coverslip, we have used a conventional Bausch and Lomb phase microscope with an oil immersion objective, \(\times 90\), N.A. 1.3, and an \(\times 10\) compensating eyepiece.

\(^4\) This is simply the polarizing microscope described in footnote 3, with the analyzer and compensator removed from the light path.
because their interpretation would be qualitatively quite different from Mitchison's, since he supposes that residual Hb has no effect on the birefringence, whereas we conclude that its presence is essential for birefringence to be observed. A number of observations, using compensators, of the retardation produced by partially hemoglobinised ghosts gave values between 4A and 7A, which are of the same order as those found by Mitchison.

Experiment 3.—So as to bring these observations into relation with those of Schmitt, Bear, and Ponder in 1936 and 1938, similar experiments were carried out on human red cell ghosts prepared by freezing and thawing and suspended in saline (refractive index, 1.33). These ghosts are partially hemoglobinised to a greater or lesser extent, but again it is apparent that birefringence can be observed only when the ghost is sufficiently hemoglobinised to be visible with the ordinary microscope. If the ghost is so little hemoglobinised as to be visible only with phase contrast, no birefringence is observed. The only difference between this result and that obtained in Experiments 1 and 2 is that the partial hemoglobinisation of ghosts prepared by freezing and thawing is not a function of time.

Experiment 4.—This is a combination of Experiments 1 and 2, but the red cells added to the glycerol had been drawn into acid-citrate-dextrose fluid and kept at 4°C. for 24 days. The number of red cells plus ghosts visible with phase optics is still described by the horizontal dotted line in Fig. 1, but the number of cells plus ghosts seen with the ordinary microscope is shown by the full line passing through crosses. Again, all the cells visible with the ordinary microscope show a small retardation of from 3 to 6 A. Storage of the red cells at 4°C. for 24 days apparently leads to a less firm association of Hb with other components of the surface ultrastructure.

DISCUSSION

The completely Hb-free human red cell ghost, clearly seen with phase optics, has no measurable birefringence, and is not even visible when examined with the polarization microscope, regardless of the setting of the compensator. Schmitt, Bear, and Ponder's measurements of the rabbit red cell ghost, made by freezing and thawing, were almost certainly affected by the ghosts being partially hemoglobinised. At the same time, Schmitt, Bear, and Ponder found that the form birefringence was increased by extracting the lipids, and that the form birefringence was decreased by placing the ghost in media of different refractive index. Their picture of the orientation of the surface ultrastructure as being one in which a birefringence of form, due to tangentially arranged protein, is balanced by an intrinsic birefringence due to radially arranged lipid is still a qualitatively acceptable one.

When the ghost contains residual Hb, the birefringence is small but measurable, and it must be concluded that the residual Hb contributes to the retardation, directly or indirectly. In view of the recent developments concerned with Hb-lipoprotein complexes (Bessis, Bricka, Breton-Gorius, and Tabuis, 1954; Ponder, Ponder, and Barreto, 1955) this is understandable. It should be noticed, however, that the ghost produced by adding red cells to glycerol,
and examined in glycerol (refractive index, 1.47) shows no birefringence if it is completely Hb-free; on the simple hypothesis used by Schmitt, Bear, and Ponder, this would be explained on the basis of the form birefringence and the intrinsic birefringence cancelling each other. But the same thing is true for completely Hb-free freezing and thawing ghosts examined in saline (refractive index 1.33), a point which needs to be explained. The most obvious explanation is that the two kinds of ghost are different in structure, together with the possibility that, when the two types of ghost are not completely Hb-free, the same amount of retardation may correspond to a greater amount of partial hemoglobinisation in the case of one type of ghost than in the case of the other. In view of this complexity, nothing more than the simplest kind of qualitative statement is justified.

**SUMMARY**

The type of birefringence described by Mitchison, which extends some 0.5 μ in from the surface of the human red cell ghost in glycerol and which shows a maximum retardation of about 7 Å, is only found in ghosts which are sufficiently well hemoglobinised to be seen with the ordinary microscope. Ghosts from which all hemoglobin has been lost are not visible with the ordinary microscope and are not birefringent, although they are clearly visible with phase contrast. About 90 per cent of the ghosts in glycerol preparations are of the latter type, the exact percentage being a function of time. Mitchison’s measurements of birefringence, although reproducible, accordingly apply only to ghosts in which some hemoglobin still remains complexed with the lipoprotein layers of the red cell ultrastructure, and do not enable one to draw

4 The results obtained by Mitchison for red cell ghosts in water (i.e., for ghosts similar to those in Schmitt, Bear, and Ponder’s experiments) are different from those obtained after glycerol hemolysis. This might be used as additional evidence that the watery ghost and the glycerol ghost are essentially different, or that the degree to which the two kinds of ghost are hemoglobinised is different. There is no point in discussing these findings further, once attention has been called to an as yet unexplained difference.

5 This is a convenient place to call attention to the fact that the birefringence of red cell ghosts may depend on the way in which they are prepared and treated. Freeze-drying associated with 1 per cent osmic acid fixation results in a remarkable degree of birefringence (Fig. 4 in the paper by Williams, 1952); this is due, however, to the osmic acid fixation, and has no direct bearing on the problem of orientation in the red cell ultrastructure. A second instance is the marked birefringence which results from the treatment of normal human red cells and their ghosts with 3 per cent sodium metabisulfite. This reagent is often used to bring about sickling in the red cells of sickle cell anemia; these sickled cells are birefringent, but a quantitative measure of their birefringence cannot be arrived at unless the contribution of sodium metabisulfite to the birefringence is known and allowed for.
conclusions as to the thickness and orientation of the lipoprotein surface layers.

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