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Red Blood Cells, Phase Contrast, Interference Contrast Microscopy and Microspectrophotometry

N. V. B. Marsden

From the Department of Physiology and Medical Biophysics, Biomedical Center, Uppsala University, Uppsala, Sweden

ABSTRACT

Following Teorell's (1) observation that the ghosts of hypotonically hemolysed erythrocytes reseal, it was shown that during the time they are permeable to hemoglobin, foreign macromolecules (dextran) can enter and that the hemolysed cell can achieve a final colloid-osmotic equilibrial state containing dextran and some residual Hb. In this way dextran reduces the hemoglobin loss in hypotonic hemolysis. Some hemoglobin loss is, however, inevitable, as it begins with a non-diffusive bulk outflow, sometimes observable as a jet, during which time a diffusive influx of the colloid-osmotic "balancer", dextran, is not possible. Finally, as expected from a process which is for the most part diffusive, transmembrane macromolecular transport is bidirectional; during hemolysis smaller molecules escape to a greater extent than larger ones.

When I arrived In Uppsala in the mid 1950's, Teorell had already changed the direction of his interests. Instead of gastric secretion, he had begun to address yet another problem with clinical implications and one which might be considered to have a simpler cellular basis; in hemolytic diseases the erythrocytes lose their hemoglobin (Hb). Already in 1952, using hypotonic hemolysis as his chosen system and thereby concentrating on a single cell model, he showed quantitatively that hypotonically hemolysed erythrocyte ghosts were not simply ruptured sacs but that for some time permselectivity was maintained and that the ghosts behaved as perfect osmometers (1). Although other workers had postulated that the hemolysed erythrocyte was not simply a ruptured sac, this was, as far as I know, the first quantitative demonstration that, even after becoming leaky to macromolecules, the erythrocyte membrane had functionally resealed, even if only temporarily.

In the introduction to this paper Teorell succinctly explains his aims: "in view of the seemingly very complicated permeability processes in intact erythrocytes, it was thought to be advantageous to attempt some investigations on hemolysed

erythrocytes, the so called 'ghosts'. It was believed that these cells, probably devoid of many of the "living" constituents, could serve as somewhat simplified models capable of shedding some light on the more elementary properties of cell membranes".

The work I shall describe was a direct consequence of Teorell's observations on hypotonic ghosts and is really an extension of his hypotonic ghost model to study the interactions of these ghosts with macromolecules, in particular the polyglucoside dextran, a substance of high clinical significance.

From his findings and our knowledge we knew that hypotonically hemolysing erythrocytes were transiently permeable to macromolecules (the escape of Hb) for several seconds in the case of each cell, a time period which should allow considerable transmembrane migration, even bidirectional, of macromolecules. Had we adequately contemplated and comprehended the implications of such a state, all our subsequent findings, except jet emission, could have been anticipated.

The work I shall describe was done in collaboration with two colleagues, firstly Martin Zade-Oppen and later Göran Östling.

Teorell was always aware of the latest useful instrumental advances and for his studies had acquired one of the first microscopes equipped with phase contrast, an optical system enabling the visualization of very weak phase objects such as Hb-free erythrocyte ghosts; for his work on phase contrast, F. Zernike was awarded the physics Nobel Prize in 1953.

When erythrocytes are hemolysed in a hypotonic electrolyte solution, two classes of cell are visible under phase contrast: 1) bright unhemolysed cells each with a bright halo, and 2) pale gray, nearly Hb-free ghosts without a halo or with only a faint one (Fig. 1a). In this case hemolysis is an essentially all-or-none phenomenon, the fraction of ghosts is, allowing for small variations in cell mass, the same as the fraction of Hb released from the cells and is therefore a satisfactory measure of the degree of hemolysis (2,3), which in this case can be defined as the fraction of cells which have hemolysed.

In order to investigate this process further, we planned to estimate the residual material content of the ghosts by measuring their refractive indices (RI) by phase contrast. For this purpose we intended to use the polyglucose dextran as a reference of known RI. Initially, trying to make a short cut, we hemolysed the erythrocytes in a hypotonic electrolyte solution containing a known dextran concentration and thus of known RI, arguing that as the osmolal concentration of the dextran was very low, it would not significantly alter the degree of hemolysis, which in this case would be high due to a low electrolyte concentration.

However, although it should not have been, the result was unexpected. Instead of two clearly distinguishable classes of cells as in Fig, 1a, the cells now showed great variation, in that the ghosts did not appear to constitute a homogeneous class as in

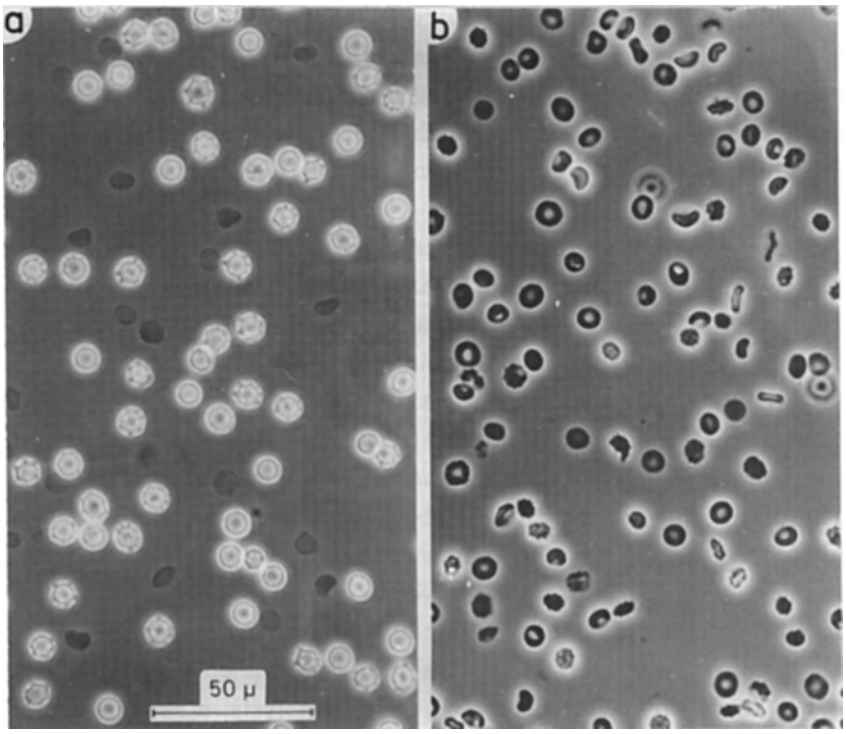

Fig. 1a. Phase contrast appearance of erythrocytes hemolysed in a dextran-free hypotonic electrolyte solution. The bright cells, some coarsely crenated, each with a distinct halo, are unhemolysed, whereas the ghosts are dark gray, without haloes or with only faint ones. Although they appear dark in this picture, they are described as pale gray in the text, a description which is more accurate in comparison with the background intensity when observed under the microscope. The degree of hemolysis was about 60%.

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Fig 1b. Phase contrast appearance of erythrocytes hemolysed in a dextran-containing hypotonic electrolyte solution. In this picture there are no typical unhemolysed cells as in Fig. 1. These cells appear to be all altered and are, in fact, hemolysed cells containing different amounts of Hb. At this osmolality (electrolyte = 25 mM and dextran [M_W = 250,000] 38.4 g/l), all the cells have lost some Hb and most have a contrast varying between the two classes shown in Fig. 1.

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Fig. 1a, but now there were no longer any bright haloed cells as in Fig. 1a; all the cells appeared to belong to another, quite different heterogeneous population as shown in Fig. 1b. This was interpreted as meaning that all, or very nearly all, the erythrocytes

had lost part of their Hb but there were very few cells which had the appearance of the nearly Hb-free pale gray ghosts shown in Fig. 1a. The explanation for this is is that if erythrocytes are hemolysed in the presence of dextran, dextran molecules up to a size of about 300 kDa enter the cells as Hb escapes (4). The hemolysing cell thus contains Hb and dextran, both of which contribute to the internal colloid osmotic pressure.

Teorell (1) had shown that hemolysing erythrocytes are able to reseal, and the most plausible explanation for the dextran effect is that the hemolysing cells containing a mixture of Hb and dextran are able to reach colloid osmotic equilibrium with the external solution, and at some point resealing presumably occurs in the absence of a swelling pressure and with some Hb still retained.

A not inconsiderable amount of dextran enters the hemolysing erythrocyte. In the observations reported in Ref. No. 4 the residual Hb was about 50% and the number of dextran molecules in the erythrocytes was about one-third of the number of residual Hb molecules. Further, the solvated volume of the intracellular dextran was about 25% of the mean volume of the unhemolysed erythrocytes, and the intracellular dextran concentration about one-third of that outside.

We had now come to an interesting point and posed the question as to whether dextran had in some way reduced the degree of hemolysis, a seemingly unlikely possibility, but in those days when blood conservation was of central interest, one of some significance.

However, when the experiment was repeated in another way so that the erythrocytes were first hemolysed in a hypotonic solution containing only electrolytes, and dextran was added afterwards, there were now again two clearly distinguishable classes as in Fig. 1a: unhemolysed erythrocytes and ghosts. In other words when the cells were hemolysed in the presence of dextran, the pattern of hemolysis had been perturbed.

Even in an electrolyte-free dextran solution, some Hb is retained. It should be noted that this effect requires the dextran to have a minimal molecular mass of about 2 kDa (5). As far as the isoamylose (dextran) series is concerned, it appears that the smallest effective size is probably the eleven glucose residue oligosaccharide (1.8 kDa). Thus, smaller sugars such as the trisaccharide raffinose (5), or the tetrasaccharide stachyose, are without effect, although once the erythrocytes are resealed post-hemolytically, a smaller species, e.g. sucrose, is locked within the ghost.

Fig. 2 illustrates diagrammatically hemolysis in a pure hypotonic electrolyte solution compared with that when dextran is present (6). In the region BA, hemolysis is incomplete and related to the osmolality, whereas in CB, hemolysis is maximal and constant. The effect of dextran is thus to reduce the maximal loss of Hb.

The conclusion to be drawn from this figure is that dextran does not affect the fractional number of cells hemolysed but only the fractional amount of Hb released.

Thus in the presence of dextran all the cells in BC are hemolysed, but only part of their Hb has been lost. So when an erythrocyte population is partially hemolysed in a

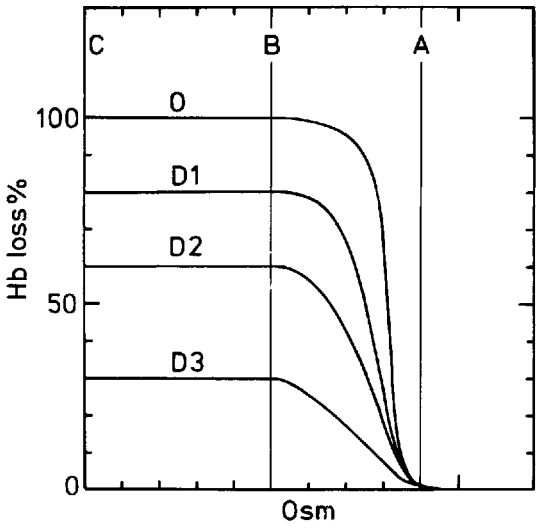

Fig. 2. Diagram showing the pattern of Hb loss from erythrocytes as a function of the osmolality (Osm) of the suspending solution in the absence (0) and presence of dextran, D_1, D_2 and D_3. The latter refer to an ascending concentration of dextran. Reproduced with the permission of Upsala J. Med. Sci.

hypotonic dextran solution, although only a fraction of the total Hb has been released all the cells (region BC) may be hemolysed.

In as much as dextran reduces the Hb loss in hypotonic hemolysis, it is an inhibitor. However, if the hemolysis curves in the presence and absence of dextran are compared as shown in Fig. 2, the conclusion is that dextran does not reduce the number of erythrocytes which have undergone hemolysis, although the Hb loss is reduced.

This means that dextran does not offer any cellular protection against hypotonic hemolysis; it merely changes the pattern from an essentially all-or-none phenomenon in a hypotonic polymer-free solution to a partial liberation in a dextran-containing hypotonic solution (2).

This inevitably introduces an ambiguity into the definition of the degree of hemolysis, since in the presence of an external polymer the deviation from an "all-or-none" Hb loss means that the fraction of the cells that have hemolysed no longer even closely reflects the fraction of Hb released.

As a way round this difficulty, Östling (7) suggested that in the presence of dextran, hypotonic hemolysis could be said to result in "fractional mass hemolysis" and "fractional number hemolysis", each term having a different value; the two terms indicating the fraction of Hb lost and the fractional number of cells that had lost some Hb, respectively. As judged from the curves shown in Fig. 2, dextran reduces the fractional mass hemolysis but leaves the fractional number hemolysis essentially

unchanged. If, as usual, osmotic fragility is measured in a polymer-free hypotonic electrolyte solution, there is, of course, no ambiguity.

Although erythrocytes that have been hemolysed hypotonically in the presence of dextran are, at the termination of their exposure to hypotonicity, apparently intact, in the sense that their membranes are once more impermeable to small molecules and probably, at least temporarily, retain some ionic permselectivity (1), it is quite likely that the membranes are not restored to their original state and thus they may be mechanically more fragile than normal, as is my impression. Notwithstanding this, these cells still have a potential experimental usefulness, even though they may have a shorter life in the "agitated" conditions of the circulation. Dextran, for example, can be made fluorescent and erythrocytes labelled during hypotonic hemolysis in the presence of fluorescent dextran have been used in micropuncture studies on renal nephrons (H. R. Ulfendahl, personal communication).

Later, other microinterferometric and microspectrophotometric studies confirmed that erythrocytes hemolysed hypotonically in the presence of dextran appeared to constitute a single population of cells clustered around an average Hb content lower than normal. In the region BC of Fig. 2 such cells would be described by Östling (7) as having a "number hemolysis" of 100% and a lower "fractional mass hemolysis".

Cinéphotomicrographic studies were also performed with phase contrast and microinterferometry to follow the process of Hb escape from individual hemolysing erythrocytes. For this purpose it was of great advantage to be able to induce hemolysis at will. This would have been somewhat cumbersome with hypotonic hemolysis, as it would have required a device for altering the tonicity while the erythrocytes were in a chamber under the microscope. A much simpler possibility seemed to be to use so-called photodynamic hemolysis in which erythrocytes are first sensitized by exposure for a short time in the dark to an isotonic electrolyte solution containing the dye Rose Bengal (about 10^{-4} M). Then later when they are illuminated under the microscope, hemolysis begins in a few seconds and continues until all the cells in the field are transformed into ghosts.

In this case it was observed that hemolysis began dramatically with the emission of Hb in a rapidly escaping jet-like cloud (8, 9) (Fig. 3). That this cloud was a jet was demonstrated by the recoil of the emitting cell and also because an adjacent cell, onto which a jet impinged, moved away.

The jet phenomenon indicates that hemolysis begins with a non-diffusive bulk outflow of Hb. This is in agreement with Heedman´s report from microinterferometry (10) that initially, Hb escapes with a high velocity.

Although photodynamic and hypotonic hemolysis are different, the existence of jets is well established in the latter; in an elegant study with a video-camera, Zade-Oppen (personal communication) followed the jet-induced movements of hypotonically hemolysing erythrocytes. Further, Kochen (11), in a very short but highly informative

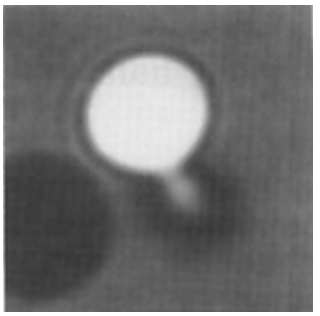

Fig. 3. An erythrocyte emitting a jet containing Hb during photodynamic hemolysis (Interference Contrast). The appearance of the jet depends on the fringe setting of the microscope, and the amount of material is greatest in the lighter region. Reproduced with the permission of Upsala J Med Sci.

note, described how he visualized jets from hypotonically hemolysing erythrocytes using bright-field microscopy combined with an extracellular protein precipitant.

Although the propellant power of these jets is probably rather poor (M. Holwill, personal communication), they do provide an example of how small (intracellular) bodies might be moved from one intracellular site to another.

Since hemolysis begins with a bulk non-diffusive outflow of Hb, dextran, which can conceivably only enter by diffusion, will be excluded until the jet outflow has ceased. Östling (7) only observed the entry of dextran after about 20% of the Hb had escaped. It was thus not possible to reduce the Hb loss below this approximate value, and means that in the presence of hypotonic dextran there is always some hemolysis. He also noted molecular sieving; smaller species entered more rapidly than larger ones. In this respect there is, as might be expected, symmetry as regards transport in both directions across the membrane, since during hemolysis smaller molecules escape to a greater extent than larger ones (Östling et al. (12)). In this case, whereas Hb escaped to a lesser extent than did smaller species, two other molecules, lactate dehydrogenase and catalase, escaped to a lesser extent than the smaller Hb.

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