SHAPE AND SHAPE TRANSFORMATIONS OF HEATED HUMAN RED CELLS

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(With One Text-figure)

The action of temperatures above 48°C. on the red cell is usually regarded as a classical example of the irreversible effects of injury, the lysis which follows being attributed to a surface membrane becoming permeable because its lipid components are melted or because its protein components become denatured. Recently it has been found that the effects are considerably more complex, and increasing emphasis is being placed on the role which fragmentation plays in the phenomenon of heat haemolysis. Ham, Shen, Fleming & Castle (1948) have pointed out that the fragmentation which precedes haemolysis results in the cells of the system becoming more and more spherical, so that the extent to which each cell can swell before its surface is stretched becomes less and less, and the tonicity in which it haemolyses becomes greater and greater; when there is sufficient fragmentation, indeed, human red cells will haemolyse even in an isotonic medium such as plasma. The tonicity-volume relations of cells which have lost one or more of these fragments during heating to temperatures in the neighbourhood of 48°C., however, are substantially the same as those of intact cells (Ponder, 1948a), and both cells and fragments show substantially the same resistance to haemolysis by saponin and by digitonin; these observations point to the osmotic properties and the properties which determine the resistance to lysins being shared by cell and fragment, and to the unit structure upon the integrity of which these properties depend being smaller than the intact red cell. The possibility that this is so suggests that something may be learned by examining the shape of the red cells and fragments more closely, since the ability to maintain shape and to undergo shape transformations are also properties which are usually thought of as associated with the red cell as a unit structure.

MATERIAL AND SPECIAL APPARATUS

The source of the red cells with which this paper is concerned is the heparinized blood of healthy donors, but the same methods can be used for studying the fragmentation of red cells from abnormal blood.

The cells are heated in one of three ways: in a small flat-bottomed flask immersed in a thermostatically controlled water bath, in a glass heating spiral immersed in the same water bath, or in an electrically heated chamber which fits into the moving stage of the microscope. The heating spiral is used to bring the cells to 48–52°C.
almost immediately and to keep them at the desired temperature for a known time. The chamber is used for the direct observation of individual cells before, during, and after heating.

The spiral is made of a piece of glass tubing about 2 mm. in internal diameter and about 200 mm. long, turned into a spiral of about 5 turns. It is mounted in a glass tube, open at the top, in order to protect it; the tube is immersed in a constant-temperature water bath, its lower end being brought outside the bath. It is filled through a small funnel attached to its upper end. The water bath can be set at any temperature in the neighbourhood of 50°C.

The double-walled heating chamber is rectangular in shape, measuring 11.5 x 6.5 x 2.5 cm. It is open above and below, and has inner walls of metal separated from its outer walls of Transite by 2 cm. A heating element, drawing about 1 amp. on the 110 V. circuit, is mounted between the outer and inner walls, and the current passing through it can be regulated by a variable resistance. The inner metal walls are perforated by small holes. The slide carrying the preparation forms the floor of the central space, which measures 7.7 x 2.7 x 2.3 cm., being kept just clear of the stage of the microscope by a narrow metal shelf projecting inwards from the lower edges of the inner metal walls. The central space is large enough to allow a high dry or an oil immersion objective to be focused on the cells of the preparation; considerable movement of the preparation is possible, and the slide is so close to the upper surface of the condenser as to allow critical illumination to be used if necessary (e.g., for photomicrography). Since the chamber is carried on the moving stage, the cells of successive fields can be inspected in the usual way. When the objective is in position and focused, the open top of the chamber can be covered with two pieces of mica, one on each side of the objective; more usually only one side is covered, the other being left open so that the cover-glass can be pressed on with a dissecting needle.

The temperature of the central space is indicated on a thermometer which projects into it. The chamber is heated by turning on the current for a minute or two. The rate of heating of the chamber is controlled with the resistance, and the observer soon becomes so familiar with the heating characteristics of the apparatus that he is able to control the temperature to within a degree or so. When it is desirable to know the temperature of the preparation more exactly, the latter is mounted on a slide which has a shallow well near one end; a single thermocouple projects into this well, which is filled with saline and covered with a cover-glass exactly as in the case of the preparation itself. Connected to a suitable galvanometer, this thermocouple measures the temperature in the well, which is presumably very nearly the same as that in the preparation.

The extent of fragmentation is measured by counting the objects seen on a haemacytometer chamber. The illumination used should be sufficiently high to prevent ghosts from being seen and included in the count. Care must be taken to allow the smaller fragments to settle on to the floor of the chamber and the precautions to be taken are, in general, the same as those taken in the counting of platelets. When

* This heating chamber is made by Mr Paul Cutajar of the New York University machine shop.
there is extensive fragmentation, the cell suspension is diluted to a greater extent than when there is not. \( N \) is the ratio of the number of cells plus fragments in the heated system to the number of cells in the unheated system.

**SHAPE AND SHAPE TRANSFORMATIONS AFTER HEATING**

(1) *Washed red cells in saline at 48°C.*

When heated to 48°C in the heating spiral for between 2 and 8 min.*, and when examined soon afterwards between plastic surfaces under the microscope (high dry and oil immersion objectives) in the usual manner at room temperature,† washed human red cells in saline appear as a mixture of spheres, crenated spheroids, and flat cells with a distorted outline (Fig. 1 A); there are also a few fragments, but \( N \) does not usually exceed 1.5. The outline of the flat cells, which can be shown to be more or less typical disks by turning them up on edge, may present every kind of irregularity from simple scalloping to disruption into a fringe bordered with minute irregularly shaped fragments. Many of them show a clear central area which looks like a hole about 0.5 μ in diameter, and from which folds run radially towards a broken and fragmented region of the cell margin (Fig. 1 B, C, D); this radially arranged defect can be clearly seen in the cell when it is on edge. Judging by the way in which it behaves during disk-sphere transformations, the clear central area is probably a dimple in the material which forms the biconcavity.

The disk-sphere transformation between *slide and cover-glass* is typical except in so far as the cells are not perfect disks to begin with; some of them are spheres or spheroids, and so the shape transformation is not as striking as in preparations of unheated red cells. Perfect spheres are not as readily formed as in preparations containing unheated red cells; this shape transformation is notoriously difficult to control quantitatively, but the heating seems to result in an increased resistance to sphering. Reversal of the shape transformation occurs on the addition of plasma or of human serum albumin, the disks which reappear having clear central areas, radial defects, and scalloped edges, etc., just as they had before being sphered. The major irregularities on the cell surface, like the major irregularities on poikilocytes and on crenated red cells, are incorporated into the sphere as the disk becomes spherical, and probably reappear at the same sites on the surface when the sphere turns into the disk (cf. Furchgott, 1940).

The shape change produced by *lecithin*, as observed in preparations of heated red cells in saline between plastic surfaces, are very similar to the disk-sphere transformations between slide and cover-glass. As in the case of the latter, the shape

* The fragmentation and other changes which occur in red cells after exposure to heat vary in the case of the cells of different healthy individuals, and vary very noticeably in the case of the abnormal red cells of certain types of anaemia. The specification 'heating to 48°C. for a min. , etc., is not to be taken too literally; the changes described usually occur when the cells are heated to this temperature for this time, but in the case of the red cells of another healthy individual, they may not occur until the heating has continued for a longer time or until the temperature has been raised to 48.5°C.

† No spontaneous shape change takes place when the cells cool. When plasma is run under the cover-glass to cool them quickly, an increased number of distorted disks appear; this is part of the shape change, from sphere to distorted disk, described in § (2).
changes produced by lecithin (distearyl lecithin sol in isotonic saline) do not occur as rapidly as in preparations containing unheated cells, but after a few minutes almost all the cells become perfect spheres. Addition of plasma, of serum albumin and of a number of other 'reversing substances' results in the reappearance of the same irregularly shaped disks, some with clear central areas, as are seen before the addition of the lecithin.

The addition of saponin is followed by crenation; this soon disappears, and the cells become smooth spheres which later develop the peculiar appearance of the prolytic sphere and haemolysis. The behaviour of the clear central area and of the irregularities at the margins of the discoidal cells of the preparation can be better investigated by producing haemolysis with rose bengal (about $10^{-6}$ M). The cells of preparations between plastic surfaces become spherical soon after the dye is added, and the process can be hastened, so far as any individual red cell is concerned, by
racking up the condenser of the microscope so as to increase the illumination incident upon the cell. As the disks lose their irregular outline and become more spherical, the larger of the irregularities may persist for some time before becoming rounded off and incorporated into the smooth sphere; the process resembles the transformation of a poikilocyte into a sphere (Ponder, 1942a). By selecting a cell with a clear central area, waiting until it is almost a sphere and then rolling it over so as to be able to inspect it from all directions, the clear central area can be seen to persist as a dimple on the cell surface until it ultimately becomes invisible in the perfect sphere. The clear central areas seen in the reformed disks after the addition of plasma to the preparation are presumably the same as the original clear central areas and as the dimples which disappear when the sphere is formed.

(2) Washed red cells in saline at 50°C.

When heated to 50°C. for 2 min. in the heating spiral, and when examined between plastic surfaces soon afterwards at room temperature, most of the cells appear as spheres surrounded by a large number of spherical fragments ($N=2\cdot0$ to $3\cdot0$) of varying diameters (1·0 to 0·1 µ) (Fig. 1E). At least the larger of these fragments contain Hb; the preparation also contains ghosts of various sizes, the result of the 15 to 30% haemolysis which occurs in these heated systems. The few remaining disks are irregular in outline, some with clear central areas, some with radial defects, and some with scalloped margins. The occasional 'refractile body' (see below) turns out to be a fragment attached to the cell surface. The disks look peculiarly flat, and the 'rim', which normally looks distinctly thick and rounded, seems to be almost absent. On turning the disks on edge, however, it is clear that they are still biconcave, although they look thinner than unheated red cells do.

The shape transformation between slide and cover-glass and the transformation produced by lecithin are difficult to demonstrate because the preparations contain so few disks, and also because they occur very much more slowly than in preparations containing unheated red cells. The addition of 1% serum albumin, however, produces a remarkable effect which was first observed during an attempt to show that these disk-sphere transformations are reversible. Almost immediately after the serum albumin is added, many of the cells assume bizarre discoidal shapes; some of these disks have a clear central area, radial folds, and scalloping at the margins, but most of them are even more irregular in outline, 'pseudopods', veil-like fringes terminating in groups of minute granules, and other curiously shaped extensions appearing over as much as half the cell surface (Fig. 1F). Some spheres remain unchanged, and some turn into cup-shaped forms, but the preparation after the addition of the serum albumin would certainly be described as a preparation of bizarre and distorted disks rather than as one of spheres with an occasional disk, which it was before. Even the shape of the smaller particles is altered by the addition of the serum albumin; these small particles become oblong or irregular in shape instead of being substantially spherical as they were before.

The spherical cells observed before the addition of the serum albumin must accordingly be regarded as being the spherical end-product of a reversible disk-
sphere transformation, the discoidal product being the bizarre disk. The effect of heating to 50°C is probably first to produce irregularly shaped disks similar to those seen after heating to 48°C but deformed to an even greater extent; these deformed (and also fragmented) disks then become spherical by an incorporation of their irregularities into the sphere (cf. the disk-sphere transformation of poikilocytes, Ponder, 1942a) so that they are seen as spheres even between plastic surfaces. The addition of serum albumin reverses this shape transformation, the sphere becoming the irregularly shaped disk once more, and it should be noticed that the shape of the fragments is affected in much the same way as the shape of the cells from which they were derived. Since the bizarre disks reappear on the addition of serum albumin (a well-known antisphering substance), it is likely that the spheres are the result of the heated cells having lost a material necessary for the maintenance of the (deformed) discoidal form. An anti-sphering substance, which converts the spherical forms of a preparation of unheated cells between slide and cover-glass back into disks, can be demonstrated to be present in considerable amounts in the supernatant fluid of a suspension of cells heated to 50°C for 2 min. It is presumably derived from the heated cells.

*Saponin and rose bengal produce sphering of the discoidal forms of the heated preparation, and all the cells and fragments haemolysed as spheres. There is no relation between the size of the cells or parts of cells and their resistance to lysis.*

(3) Washed cells in saline at 52°C.

When heated to 52°C for 2 min. in the heating spiral and when examined between plastic surfaces shortly afterwards at room temperature, nearly all the cells appear as spheres surrounded by many Hb-bearing spherical fragments of varying sizes \(N = 3 \text{ to } 5\). Some of the particles are so small that one cannot be sure whether they contain Hb or not, and the preparation also contains a considerable number of ghosts of various sizes corresponding to the 30–60% haemolysis which results from heating to this temperature. The preparation also contains a very few deformed disks, and the addition of serum albumin changes a few spheres into distorted disks, although the effect is much less noticeable than after heating to 50°C. The number of disks and of spheres transformable into disks, however, is so small that the slide and cover-glass and the lecithin shape changes can scarcely be demonstrated.

The spherical cells of these preparations appear to be unequally haemoglobinized. This is an appearance which can result from the cells being unequal in diameter and consequently presenting unequal paths for the absorption of light by Hb; it can also be the result of the cell lying above or below the focal plane of the optical system. There are so many cells and particles in the ordinary preparation that it is very difficult to pick out cells of equal diameter and to satisfy one’s self that one is darker than another in all focal planes; the comparison is easily carried out, however, by making a simple modification of a 10× ocular. A circle of black

* I have observed, on several occasions, the fusion of two spherical fragments to form a larger sphere which haemolysed some minutes afterwards. This confirms a neglected observation of Auer (1932).
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Paper, with a central hole and another hole about 2 mm. from it, is cemented on the diaphragm which is built into the ocular in the focal plane of the eye-lens. These holes should be about 0.7 mm. in diameter; the central hole should be exactly centered. A field of the preparation is brought into focus with an ordinary ocular, an oil immersion objective, and a condenser at $\frac{1}{2}$ to $\frac{3}{4}$ cone; the modified ocular is then substituted, and a spherical cell is brought to the centre of the central hole. By rotating the ocular, other cells can be made to appear in the other hole, and each can be compared with the central cell; when the ocular has been rotated through 360° a new cell is brought into the central hole, and the process of comparison is continued.

When this modified ocular is used, there is no difficulty in satisfying one's self that cells of substantially equal diameter are very unequally haemoglobinized. This means that the haemolysis observed in these systems is not all-or-none, and that there are more than two categories of cell—intact cells which have lost none of their Hb, and ghosts with a Hb content the same as that of the surrounding medium. Partial retention of Hb by ghosts has been demonstrated in the case of mammalian ghosts in hypotonic systems in which the ratio of cells to suspension medium is relatively small (<1 to 24, Ponder, 1942b; cf. Ponder, 1948b, in which the retention in Hb by ghosts is discussed in detail), and in the case of chicken ghosts in hypotonic systems (Hunter, Stringer & Weiss, 1940), but in these systems it was shown that the average Hb concentration in the ghost exceeds that in the surrounding medium; in the preparations under consideration, it can be shown that there are differences in the extent to which Hb is retained by the individual cells of the same system. The interest of this observation lies in its implications. The all-or-none loss of Hb from the mammalian red cell is observed in systems sufficiently hypotonic to produce lysis (Saslow, 1928; Parpart, 1931) when the ratio of cells to hypotonic medium is small, and one would expect Hb to be lost in this way from a balloon-like body containing Hb in solution. Both Saslow and Parpart have shown that the 'partial haemolysis' which other investigators observed in hypotonic media is traceable to experimental error, and even when retention of Hb has been demonstrated unequivocally (Ponder, 1942b; Hunter et al. 1940) it has been usual to suppose that the surplus Hb is adsorbed on the material of the cell surface or of an internal network. The unequal haemoglobinization of the spherical red cells of heated preparations cannot be accounted for in these ways, and is difficult to reconcile with the idea of the heated cell as a balloon-like body with Hb in solution. If it is suggested that the heating results in the Hb being 'fixed', it is necessary to specify in what sense it is 'fixed', for it can still leave the cell when haemolysis is brought about by osmotic means (Ponder, 1948a), by saponin, or by digitonin.

Most of the red cells heated to 52° C. for 2 min. contain one or more refractile bodies. These are variable in size (0.05 to 0.5 μ), not always round, and occupy a variety of positions in or on the cell (Fig. 1G). The plane of focus in which they are best defined is rarely that of the cell diameter, which suggests that they are on the cell surface rather than in the interior. They seem to be in Brownian motion and to
change position with respect to each other, but part, if not all, of this movement is probably due to the Brownian movement and rotation of the spherical cell itself. As a result of these changes in position, the bodies sometimes appear centrally placed, sometimes eccentrically placed, and sometimes placed at the very edge of the cell. They almost never appear as protuberances, and if they are associated with the cell surface rather than with the interior, they must extend inwards through the thickness of the surface ultrastructure rather than project from it.

The behaviour of these bodies during haemolysis is best observed by adding rose bengal to preparations containing washed red cells in saline, and accelerating the lysis by increasing the illumination. The refractile bodies disappear from view a fraction of a second before the cell begins to fade. When situated centrally, the refractile body often seems to roll off the cell and disappear; this apparent movement may be due to a sudden rotation of the cell itself. Nothing corresponding to the refractile body is seen in the ghost, except when it is situated at the very edge of the cell. A little roughness of the edge of the ghost may then be seen in a corresponding position.

The number, position, and behaviour of the refractile bodies suggest that they are situated at regions of the cell surface from which fragments have become detached and which are altered in structure as a consequence.

(4) Observations in the heating chamber

Cells in plasma (glass surfaces). As the temperature is raised, isolated disks begin to show either an irregular crenation or an increasing irregularity of their margins. Fragmentation begins so suddenly at about 50°C. that it is difficult to follow, but the process can be described as being of one of three kinds. (1) The discoidal cells become cup-shaped and smaller in diameter; a clear central area then appears in the region of the biconcavities. This area varies in size, is not always regular, and often has a minute haemoglobinized spot in its centre (as in the target cell). Quite suddenly, one or more radial defects run out from it through the material of the rim, and the cell breaks into one or more fragments which at first bear the same spatial relation to each other as they did when they were part of the same cell (Fig. 1H). (2) ‘Pseudopods’, large or small, form at the margin of the discoidal cell, and defects run in from the bases of these through the material of the body of the cell, dividing it into two or more fragments of varying sizes (Fig. 1J, K). (3) The rim of the cell becomes ‘beaded’ (as in the ‘holly wreath’ form of the sickle cell); the beads then tend to round up, and the cell breaks into a number of fragments (Fig. 1L). The last of these fragmentation processes is uncommon; and the relative frequency with which the first and second occur seems to depend principally on whether the discoidal cells of the preparation are, or have a tendency to become, poikilocytes. Fragmentation by the inward extension of defects running from the bases of ‘pseudopods’ is apt to occur when the cells are derived from abnormal blood or when they have been stored for some days; these are conditions which favour the appearance of poikilocytes. Red cells from normal freshly collected heparinized blood usually fragment by the outward extension of a central defect.
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The fragmentation occurs in the cells of rouleaux at the same time and temperature as that at which it occurs in isolated cells. The rouleaux break up into roughly spherical cells and fragments; these, however, tend to remain in alinement, much as they were in the rouleaux, until the cover-glass is pressed on: they are then scattered by being carried at random in the currents in the suspension medium.

Once formed, the fragments tend to round up, but they often retain their position with respect to each other for a long time, and can be seen to be connected by minute threads (Fig. 1 H, I). They haemolyse at about 58°C, some of the fragments derived from the same cell losing their Hb before others do.

Cells in saline between glass surfaces are, at first, spherical. As the temperature is increased, they become more glistening and smooth, but no fragmentation takes place at any time, even when the cells are made to move by pressing on the cover-glass. Ultimately the cells haemolyse, as spheres, at about 58°C. Cells in saline, in preparations between glass surfaces, can be converted into disks by the addition of 1% serum albumin before they are heated. They then behave just like red cells in plasma on being heated, usually fragmenting by an outward extension of a central defect. The process of fragmentation is easier to observe in these preparations than in preparations of red cells in plasma, because of the absence of rouleaux.

Cells in saline between plastic surfaces fragment in much the same way as cells in plasma or in saline plus serum albumin fragment between glass surfaces. Thin plastic surfaces, however, tend to warp with heat.

DISCUSSION

In spite of a good deal of individual variation, it is clear that a number of regularly occurring phenomena are observed when human red cells are heated.

As regards its shape, the unheated red cell possesses shape-maintaining properties which can be modified in a variety of ways. The unheated cell, for example, maintains its normal discoidal shape when in plasma, but can be transformed into a sphere of substantially the same volume; in one of the disk-sphere transformations (slide and cover-glass) this shape change is due to the loss of an antisphering substance from the cell, and the restoration of the substance restores the discoidal shape. In another disk-sphere transformation (lecithin) the discoidal shape is restored by the addition of any one of a number of 'reversing substances'. The shape can be modified without the ability to undergo reversible shape transformations being modified, as, for example, in the case of the typical poikilocyte. This is what occurs in the case of the red cell heated to between 48 and 52°C.; the discoidal cell becomes an extremely irregular poikilocyte, but the irregularly shaped cell is still capable of undergoing reversible shape transformations. The irregularity can be accounted for by supposing that the molecular pattern upon which the discoidal shape depends has undergone an irreversible modification as a result of the heating; the modification, however, cannot amount to a complete destruction of the pattern, for the cell is able to pass from one metastable form, the poikilocyte, to another metastable form, the sphere, and back again. This capacity for undergoing transition from one metastable form to another becomes less as the cell is heated for longer times or to higher
temperatures, but fragmentation of the cell does not destroy it; on the contrary, spherical fragments change into irregularly shaped fragments on the addition of anti-sphering substances just as the spherical cells of the same preparation change into poikilocytes. This means that the properties upon which the shape transformations depend are not necessarily dependent on the integrity of the red cell as a unit.

The effect of heat on red cell shape is complicated by the loss of an anti-sphering substance from the cells; this substance can be found in the suspension medium and can be replaced by adding 1% serum albumin. Its loss, with the sphering which results, disguises the extent of the poikilocytosis which accompanies the fragmentation and which can be appreciated only after the addition of serum albumin and the reconversion of the spheres into irregularly discoidal forms. As already remarked, the addition of anti-sphering substance results in not only the cells, but also many of the smaller fragments, becoming irregularly shaped objects.

The loss of anti-sphering substance takes place at about the same temperature (49 to 51°C.) as that at which large amounts of potassium are lost by the cell and exchanged for some of the sodium of the suspension medium (Ponder, 1948a). At a slightly higher temperature, Hb is lost from the cells and fragments. It is not lost in an all-or-none fashion, spherical cells of many different degrees of haemoglobinization being readily distinguishable. Taken together with the loss of anti-sphering substance, the loss of potassium, and the shape changes preceding fragmentation, this step-wise loss of Hb suggests that the phenomena observed in the heated red cell are part of a process of disintegration of a plastic Hb-bearing 'solid' rather than manifestations of the breakdown of a balloon-like structure in which Hb is held in solution.

SUMMARY

This paper describes the shape changes and shape transformations observed in human red cells heated for 2 min. to 48, 50 and 52°C.

The first change observed is an irregularity of the cell; this is followed by sphering, but the spherical forms can be turned into irregularly shaped disks again by the addition of serum albumin. The sphering is accordingly part of a reversible disk-sphere transformation due to the loss of a (recoverable) anti-sphering substance from the cells. At a slightly higher temperature (50°C.) fragmentation of the cells occurs with the production of fragments which are also capable of undergoing something equivalent to a disk-sphere transformation. The properties upon which the shape transformations depend are therefore not necessarily dependent on the integrity of the red cell as a unit.

The fragments derived from the cell may be unequally haemoglobinized, and the Hb is usually lost in a step-wise, as opposed to an all-or-none, manner. Taken together with the loss of anti-sphering substance and an accompanying loss of potassium from the cells, the shape changes preceding fragmentation and the step-wise loss of Hb suggest that the phenomena observed in the heated red cell are part of a process of disintegration of a plastic Hb-bearing 'solid'.
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REFERENCES