Effects Produced by Trypsin on Certain Properties of the Human Red Cell

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The observation that human red cells treated with trypsin and suspended in saline agglutinate in the presence of incomplete Rh antibodies (Morton and Pickles), and the probability that this is due to effects on components of the red cell surface, lead one to ask whether this is an isolated phenomenon or whether trypsinization affects red cells in other ways. This question is of additional interest because there is no known instance in which the more general properties of the red cell surface are affected by proteolytic enzymes (cf. Ballantine and Purpart, who found that trypsin produces no effect on permeability). It can be answered by examining a variety of properties associated with the normal red cell and seeing how these are affected by trypsin and the related enzyme mexacain, a papain-like enzyme for which no activator is required. This paper is concerned with the results of such observations, which will be reported with remarks as to technic and with other comments.

Experimental Observations

Unless otherwise stated, these observations refer to the red cells of heparinized human blood, treated with trypsin in the following way. A stock solution is prepared by dissolving 200 mg. of Armour crystalline trypsin in 10 ml. of N/20 HCl. One part of the stock solution is added to 3 parts of a phosphate buffer at pH 7.4 to make the working solution, of which 0.1 ml. is used for every ml. of a suspension of washed red cells with a volume concentration of 0.02. If the cell suspension has a higher volume concentration, a proportionally greater volume of working solution is used. After the addition of the trypsin to the cell suspension, the mixture is kept at 37 C. for varying lengths of time (usually fifteen minutes to two hours); the cells are then thrown down, washed twice in 1 per cent NaCl or in a NaCl-buffer, and resuspended in a known volume concentration which is determined by the type of observation to be made.

When mexacain is used, the procedure is similar except that the enzyme is dissolved in NaCl-buffer at pH 6.0 in a concentration of 100 mg./100 ml.*

1. Volume and Density

The volume of trypsinized red cells is slightly larger than that of untreated cells, the increase observed after trypsinization for one hour varying from 8 to 12 per cent. This volume increase can be easily detected either by measurements made in Hamburger hematocrit tubes or by measurement of the Hb concentrations in equal volumes of untreated and trypsinized cells respectively. It is accompanied by a corresponding decrease in density, which is reflected in a decrease in the sedimentation rate of trypsinized cells in saline. It is a function of

* The trypsin used is Armour crystalline trypsin. I would like to thank Dr. Maria Refugio Baleazar of Mexico City for the sample of mexacain. Since Armour trypsin contains about 50 per cent of MgSO₄, suitable controls should be set up in which the cells are treated with MgSO₄ only.
time, developing at the rate of about 5 per cent per hour until a total volume increase of about 10 per cent is reached; longer periods of trypsinization do not seem to result in further volume increases.

2. Volume-Tonicity Relations in Hypotonic Systems

Two red cell suspensions of volume concentration 0.067, one of trypsinized cells and the other of untreated cells, are prepared in 1 per cent NaCl. Of these, 0.5 ml. volumes are added to 2 ml. of NaCl of tonicity varying from 1.0 to 0.2, 1 per cent NaCl being considered as having a tonicity of 1.0. The decrease in tonicity is by steps of \( T = 0.1 \) in the range in which there is no hemolysis, and by steps of \( T = 0.02 \) below \( T = 0.4 \). The hypotonic systems are allowed to stand at 25°C. for one hour; measured volumes are then transferred to calibrated Hamburger hematocrit tubes and spun for thirty minutes at a speed which gives a centrifugal force of about \( 2 \times 10^4 \) G (about 10,000 r.p.m.). From the lengths of the columns of packed cells in the capillaries of the hematocrit tubes one can express the ratio of \( V/V_0 \) for the cells in each hypotonic system, \( V \) being the cell volume in the hypotonic system and \( V_0 \), put equal to unity, being the volume of the cells in the system of tonicity \( T = 1.0 \). The number of cells which hemolyze in any tonicity, expressed as a fraction \( p \) of the whole, is obtained from a colorimetric determination of the Hb concentrations in the fluids in the cups of the hematocrit tubes after the spinning is ended. The fractional increase in volume of the average intact red cell in any tonicity is then \( V/(1 - p) \).

If the fractional volume \( V/(1 - p) \) is plotted against a special function of the tonicity, \( f(T, a) \), which is nearly the same as \( (1/T - 1) \) for systems containing red cells in small volume concentration, a straight line with a slope of \( W \) is obtained if the red cell gains water in accordance with the van’t Hoff-Mariotte law, i.e., if the cells are “perfect osmometers;” here \( W \) is the fraction of the cell volume occupied by water (about 0.7). The usual experimental result, however, differs from the expected one in two ways. The first is that the tonicity-volume relation starts off as a straight line with a slope, not of \( W \), but of \( RW \), where \( R \) is a constant the meaning of which is not entirely clear, but which usually has a value between 0.75 and 0.9. The second is that when tonicities are reached in which there is some hemolysis, the experimental values of \( V/(1 - p) \) are no longer linear with \( f(T,a) \), but are at first greater and then smaller (fig. 1, curve marked O). The best explanation for this peculiar tonicity-volume relation is that it is partly due to the columns of packed cells containing a number of semirigid ghosts, and partly due to the intact cells in the lowest tonicities behaving as very imperfect osmometers, i.e., as having a very small value of \( R \).

The type of tonicity-volume relation obtained with red cells treated with trypsin or mexacain is shown in figure 1 by the curves marked \( T \) and \( M \) respectively. The tonicity-volume relation departs from linearity more than does that for untreated red cells (curve marked O). This result resembles that obtained for human red cells treated with resorcinol or with colloidal silicic acid, and the best explanation is that the ghosts of the cells in these systems are unusually rigid or “volume-occupying.”
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3. Osmotic and Mechanical Fragilities

As will be seen from figure 1, in which the amount of hemolysis in each tonicity is indicated as a value of $p$ marked beside the experimental points, the resistance to hypotonic hemolysis of red cells treated with trypsin or mexacain is slightly less than that of untreated cells. The mechanical fragility of trypsinized cells, as measured by the method of Shen, Castle and Fleming, is slightly increased. The increased tendency of trypsinized cells, as compared with untreated cells, to hemolyze is also shown in other ways, e.g., there is more hemolysis in isotonic NaCl when trypsinized cells are kept at 37°C for twelve to twenty-four hours, or at lower temperatures for still longer times; this difference is observed even when the systems are sterile.

4. Ghosts of Trypsinized Red Cells

A good yield of ghosts of trypsinized or of untreated human red cells can be obtained by the following procedure, not hitherto described. The washed trypsinized cells are made up to a volume concentration of about 0.07 in 1 per cent NaCl and are kept at 37°C for six to eight hours. The cells are then packed at about 4000 r.p.m. for thirty minutes, and 0.2 ml. of the packed mass is added to 25 ml. of water. The mixture is allowed to stand for twenty-four hours at 4°C. Cells which have not been treated with trypsin are treated similarly.

Fluffy ghosts appear as a sedimented layer beneath the Hb-containing fluid, a measured amount of which is carefully decanted; the remaining fluid with the
layer of ghosts is transferred to centrifuge tubes, and is spun at about 4000 r.p.m. for 30 minutes to give a column of ghosts. The ghosts of the trypsinized cells are translucent and almost white, while those of the untreated cells are opaque and contain considerable quantities of Hb. The ghosts of both trypsinized and untreated cells are discoidal and biconcave; they are very difficult to see under the microscope unless their visibility is increased by adding a trace of a dye (rose bengal). They may be crenated, distorted in outline, and may even appear to be about to fragment; like most ghosts, they tend to fragment spontaneously if allowed to stand at room temperatures or at higher temperatures than this. Their volume, measured by the combination of a hematocrit and a conductivity method is about half that of the intact red cell, but no significant difference has been found in isotonic media between the volume of the ghost of the untreated cell and that of the ghost of the trypsinized cell.

5. Electrophoretic Mobility

The electrophoretic mobility of human red cells and their ghosts is remarkably constant, and several different forms of lysis result in ghosts which have the same mobility as that of the cells from which they were derived (Abramson, Furchgott and Ponder). It is therefore surprising to find that trypsinization results in a decrease in mobility.

The measurements were made at the stationary levels in a vertical cell of a more convenient design (fig. 2) than that described by Abramson, Gorin and Moyer, although essentially the same as regards operation. The tubes leading to the flat rectangular chamber C end in three-way stopcocks which can be turned either so as to allow the tubes and chamber to be filled by suction from above at A and emptied by gravity, or so as to connect them with the horizontal non-polarizable electrodes E₁ and E₂. The electrodes, which are separated by plaster of Paris plugs from the stopcocks, are filled with saturated CuSO₄ into which rods of Cu dip through tightly applied vaccine bottle stoppers. The tubes leading from the chamber are fused at two points to a frame F of glass rod, which in its turn is rigidly attached to the stage of a microscope, tilted so that its stage is vertical. A circular rotating stage with centering screws is a convenience. The double right angle bend near the lower electrode enables the apparatus to be mounted with the center of the chamber in the optical axis. The chamber is filled, emptied, or cleaned without removal from the stage, and the CuSO₄ can be renewed as required without the apparatus being disturbed.* The electrical circuit is the same as that described by Abramson, Gorin, and Moyer (180 volts from B batteries, a potentiometer, a milliammeter and a reversing switch), and the optical system consists of an 8 mm. objective and a 20× eyepiece fitted with a chessboard micrometer scale. It is important to eliminate heat effects by placing a large water tank in front of the microscope lamp, which is best situated several meters away.

When human red cells are trypsinized for one hour, the normal mobility of the red cell of 1.3 μ/sec./volt/cm. obtained in NaCl-buffer at pH 7.2 and at

* This apparatus is made by E. Machlett & Son, New York City.
25 C. is replaced by a mobility of about 30 per cent smaller. This cannot be interpreted as the result of a complete coating of the red cell with trypsin, for the pH-mobility curves show clearly that the isoelectric point of the trypsin-treated cells is at a pH less than 3.5 and therefore not much above that of the untreated cells; it is certainly not that of trypsin (pH between 7 and 8). Since the normal red cell surface is dominated by strongly acidic groups, trypsinization may result in the appearance of ionized groups of a less acidic character. This could be due to the breaking of bonds during a general “loosening-up” process which may now be suggested as occurring when trypsin acts on the protein-containing red cell ultrastructure. It might also be due to the incorporation of trypsin with its high isoelectric point into relatively small parts of the surface. Additional support for the occurrence of such a “loosening-up” process, which would be of particular interest in view of the current ideas regarding the orderly arrangements of molecules in the red cell surface and interior, can be derived from the increased volume of the trypsinized red cell, from its unusually anomalous behavior in hypotonic media, and from its voluminous and relatively Hb-free ghost.

Fig. 2.—Vertical electrophoresis cell. C, flat chamber; F, frame of glass rod. The chamber is filled by suction at A and empties at B. E₁ and E₂, electrodes separated from stopcocks by plugs of plaster of Paris.
6. Color Changes

The red cells of suspensions which have been trypsinized for an hour or more and then washed become slowly and progressively darker on standing at 37 C. or at 25 C. This color change occurs in sterile systems, and can be demonstrated spectrophotometrically in hemolyzed systems in which 1 volume of the suspension is added to 25 volumes of water. The system prepared from the trypsinized cells transmits about 4 per cent more at 5000 A and about 4 per cent less at 6300 A than does the system prepared from untreated cells. The color change is not accompanied by hemolysis, and is apparently due to an alteration in the intracellular Hb, perhaps in the Hb most readily accessible to the action of trypsin, i.e., the Hb situated near, or even in, the cell surface ultrastructure.

7. Negative Results

Treatment with trypsin or mexacain does not produce any consistent alteration in red cell shape or changes in the shape transformations which occur when the cells are placed between slide and coverglass, or when they are treated with lecithin or with hypolytic concentrations of saponin, the bile salts, or the anionic detergents. The heat fragility is unaffected. Trypsinized red cells exchange K for Na at the same slow rate as untreated red cells do. No change in resistance to lysis by saponin or by the bile salts is observed after trypsinization, and lysis by saponin or by sodium taurocholate is as much inhibited by the supernatant fluid of trypsinized red cell suspensions as it is by the supernatant fluid of suspensions of untreated cells, i.e., trypsinization does not result in any unusual liberation of inhibitory material from the cell.

Summary

Human red cells treated with trypsin in such a way as to become agglutinable in the presence of incomplete antibodies are affected in certain other respects. Their volume is slightly increased, their ghosts are unusually rigid or "volume-occupying," their osmotic and mechanical fragilities are slightly increased and their electrophoretic mobility is reduced. These changes are probably due to effects on the protein components of the red cell surface ultrastructure. Similar effects are also produced by the related enzyme mexacain.

REFERENCES

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