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RELEASE OF HEMOGLOBIN AND POTASSIUM FROM HUMAN RED BLOOD CELLS TREATED WITH TRITON X-100 UNDER THE CRITICAL MICELLAR CONCENTRATION

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Abstract

The action of detergents is thought to be connected primarily with micelle formation. However, detergent monomers can also effect biological systems. It was found in this study that human red blood cells can be disintegrated with Triton X-100 non-ionic detergent at a concentration of 0.007%, lower than the critical micellar concentration (CMC). The time dependent release of hemoglobin and potassium was detected at 37°C and both were sigmoid in character. Although potassium was released faster than hemoglobin, a cooperative relationship between potassium and hemoglobin within the intact red blood cell is suggested by this observation.

Key words: Hemoglobin, Potassium, Red Blood Cells, Triton X-100.

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Introduction

The use of non-ionic detergents in cell permeabilization experiments has become common in the last few years [10]. Their ability to remove membrane lipids and proteins (i.e. to permeabilize) is generally attributed to micelle formation which occurs only above the critical micellar concentration (CMC) [6, 11]. However, below this concentration, detergent monomers can attach to biological membranes which might influence the local molecular organization [11, 13, 28].

Triton X-100 is one of the most widely used non-ionic detergents. Its CMC is 0.24mM (0.015%) [6] and is commonly used far above this concentration (0.1-0.5%) which effectively mobilizes not only the surface-localized structures but also the majority of internal proteins and lipids [14, 19, 25]. The remaining structures are referred to as the detergent resistant cytoskeleton [22]. In the case of human red blood cells (RBC) the detergent resistant framework comprises only a few percent of the total protein content [4]. The majority of RBC proteins can easily be removed, being almost equal to the hemoglobin content.

In this paper we report a sequential decomposition of human red blood cells which can be achieved at a concentration of Triton X-100 below the critical micellar concentration (i.e. 0.007% and 37°C). In this case the time required for the release of the total amount of hemoglobin is about 2 hours as opposed to seconds for concentrations above CMC. The release of hemoglobin and potassium may both be described by sigmoid shape curves, suggesting cooperative processes with potassium release being the faster of the two. These release kinetics are consistent with the idea that hemoglobin and potassium are associated to structures within the intact red blood cells [7]. Thus, an intricate interaction between hemoglobin, potassium and the detergent-resistant frame (ghost) within the red cells is suspected. The possible co-compartmentalization of these major components will be the major subject of this paper.

Materials and Methods

Purification of RBC: Blood samples were collected from healthy adult patients of both sexes. Clotting was inhibited with heparin. All chemicals were obtained from Reanal (Hungary, Budapest) at analytical grade. The freshly drawn samples were processed within an hour. Plasma was separated by

centrifugation for 10 minutes at 1000g (Sorvall RC-5, rotor HB-4). The plasma was drawn off and the cells were washed once with buffered physiological salt solution (PSS) consisting of 145 mM NaCl and 10 mM Tris-HCl pH 7.4. Cells were pelleted at 1000g for 10 minutes.

Experimental procedure: 100 microliter RBC, containing about 10^9 cells, were incubated in 5 ml of medium (PSS) at 37°C. The incubation medium contained Triton X-100 at a final concentration of 0.007% in PSS. In the case of controls no detergent was added. The incubation consisted of 15 minute cycles of 10 minutes of incubation plus 5 minutes of centrifugation at 2000g in a Hettich EBA 3S table centrifuge. After each pelleting the supernatant was removed carefully and the cells were resuspended in fresh medium. The incubation was continued until the supernatant was no longer colored, i.e., no more hemoglobin was released. The supernatants were collected separately. At the end of incubation the remaining pellet was treated with 1% Triton X-100 to liberate the remaining detergent-mobile proteins.

Determination of hemoglobin and potassium: The hemoglobin was determined photometrically at 542 nm. Potassium was measured by flame photometry with a digital flame photometer (OMSZOV, Hungary).

Results

Human red blood cells incubated in a physiological salt solution maintain their integrity, as far as their hemoglobin and potassium contents are concerned, for at least two hours. Even after repeated centrifugation the release of hemoglobin and potassium from the untreated (control) red blood cells did not exceed 6-8% up to the longest incubation time (Fig. 1.). On the other hand, red blood cells treated with 0.007% Triton X-100 in the same solution lost both potassium and hemoglobin, sequentially. The release kinetics of potassium and hemoglobin are described by curves of similar characteristics. In the case of hemoglobin the 50% release occurs at 75 minutes while half of the potassium of red cells was lost before 60 minutes of incubation. The two curves converge at 120 minutes where the release of both components is essentially 100% (Figure 1). The points in Figure 1 are the means of 8 determinations.

If red blood cells were incubated in the detergent containing solution without changing the incubation medium for two hours, not more than 10% of hemoglobin and 40% of potassium ions was lost (data not shown). When the concentration of Triton X-100 was raised above the CMC to 0.1% the release of hemoglobin and potassium was achieved within seconds.

Discussion

We have investigated the release kinetics of hemoglobin and potassium from human red blood cells exposed to Triton X-100 non-ionic detergent under the critical micellar concentration at 37°C. The release kinetics of both hemoglobin and potassium from red blood cells treated with Triton X-100 at 0.007% concentration showed a sigmoid character; however, there was a slight time shift with a half-time of release of less than 60 and more than 75 minutes for potassium and hemoglobin, respectively. The time required for a complete release of hemoglobin and potassium under these conditions was about 120 minutes.

In preliminary experiments, when ATP release was followed with the chemiluminescence method of Koszegi [16], we found that the release of intracellular ATP coincided with hemoglobin release (unpublished observation). The so-called hemolytic activity of non-ionic detergents is well known and used in experimental and laboratory practice [1, 3, 16]. In the case of Triton X-100, the commonly used concentration is between 0.1-0.5%; this concentration range is rather effective in solubilizing different cells, cell organelles and macromolecules. This range is far above the CMC and there is a good agreement that the detergent action is connected to micelle formation [19].

Although it has been well documented that proteins and lipids can interact with detergent molecules [5, 9, 18, 19, 27], the action of detergents under the CMC is more uncertain [11]. In a more complex system, like the living cell, the interaction between single detergent molecules and structures on the cell surface and interior are multiple. Thus, it is not possible to determine the primary site of cell decomposition or to fully know the mechanism of detergent induced disintegration of cells. However, the periodical change of incubation medium that contained the detergent must result in an increasing amount of "cell-bound" detergent molecules that is responsible for the advancing loss of cell components. If red cells were incubated in detergent containing medium without changing it periodically, the degree of cell decomposition was much lower.

The time-dependence of the release of potassium showed a parallel with the hemoglobin release. Though the prolytic loss of potassium was observed previously by Ponder and others [23, 24], the mechanism of the phenomenon has not been fully understood yet. This observation however, cannot be understood on the basis of a destroyed membrane-barrier function, but the compartmentalization of potassium and hemoglobin would explain the parallel of hemoglobin and potassium release. If so, this supports the idea that within the living cell potassium may be selectively adsorbed to cellular proteins [15, 20:chapter 8].

The observation that the release kinetics may be described by sigmoidal shaped curves is suggestive of cooperative interactions between cellular constituents. The skeletal architecture of red blood cells is established and is highly organized, particularly in the marginal region of the cells where protein interactions are the subject of intensive investigation [2, 8, 12, 21]. Our study shows an overall response of red cells to the disruption of multiple protein (and lipid) interactions which involves membrane skeleton-cytoskeleton and hemoglobin-cytoskeleton connections as well [8, 17, 26].

In conclusion, our experiments provide indirect evidence of a conceivable potassium-hemoglobin compartmentalization. The nature of this interaction though remains to be identified. As ATP is released simultaneously with hemoglobin (preliminary data), one may suppose a direct connection between ATP and hemoglobin which may provide an enhanced stability of protein association.

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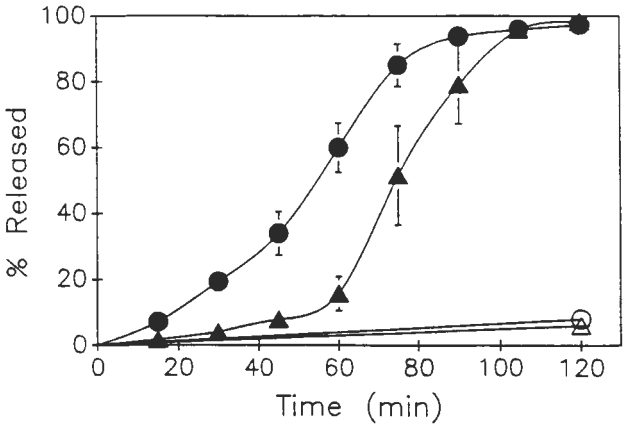

Figure 1. Release of hemoglobin (closed triangles) and potassium (closed circles) from red blood cells treated with Triton X-100 non-ionic detergent at 0.007% concentration at 37°C. The release of hemoglobin (open triangle) and potassium (open circle) from untreated cells is also shown. Each point represents the mean value of 8 separate experiments.

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Discussion with Reviewers

W. Negendank: How do you know that the sigmoid release kinetics of potassium and hemoglobin are characteristics of most of the individual cells in the preparation, as implicitly assumed in your conclusion. Is it possible that only a few cells are affected at earlier times, and more cells are affected at later times? Do you have any data that might address this issue?

J.S. Clegg: The sigmoidal release of K^+ and hemoglobin (Hb) is interpreted exclusively as cooperative interactions of K^+-Hb and these with other cell structures. Have you considered any other interpretations? Could different sensitivities to Triton of cells within the cell population be a consideration?

Authors: The red cell population is not homogenous, there are older and younger cells and their resistance to hemolytic agents or osmotic insult is not the same. There is no question that this circumstance must be involved in our results as well. According to our light microscopic studies on cells, at different time intervals of treatment, there are hemoglobin-free ghosts and still "intact" cells. This is seen with 15 to 30 minutes but the majority of red cells shows a sequential loss of hemoglobin i.e., their "redness" decreases continuously with time. In a similar type of experiment (described above) electrophoretic analysis was made of the different fractions. These results showed the accumulation of certain proteins in certain time periods, which suggests a rather uniform behavior of cell disintegration. Please also refer to Ponder's work (ref. 24) who, in studying the volume changes of red cell, came to a similar conclusion.

W. Negendank: How do you know that the release of potassium and hemoglobin (Figure 1) is not caused by an effect of the detergent on plasma membrane, with small "holes" permitting potassium release earlier, and larger "holes" permitting hemoglobin release later?

I.L. Cameron: It appears to me that the observed sigmoidal shaped release kinetic curves of K^+ and of hemoglobin obtained from the Triton X-100 treated erythrocytes has an alternate explanation to the cytomatrix binding one that you offer in your report. How, for example, can you rule out the possibility that the Triton X-100 treatment slowly makes small "holes and pores" in the plasma membrane? These holes would allow initial escape of the smaller K^+ but as these holes eventually grow larger would allow escape of larger hemoglobin molecules? It seems to me that with this explanation of your data the plasma membrane, not sorption of K^+ or hemoglobin in the cytoplasm, becomes the main mechanism for maintenance of the K^+ and hemoglobin gradients.

Authors: A "hole" big enough for the leakage of hemoglobin is more than suitable for potassium to pass through. It could be possible that initially small "holes" permit only the electrolytes to leave but as the "hole"-size reached a certain magnitude, no distinction according to size can be supposed. In other words, if potassium ions were freely dissolved in the cell, considering the diffusion coefficient of K^+ in solution, one should observe complete equilibrium with the surrounding medium by the time hemoglobin started to leak from the cells.

J.S. Clegg: With regard to Figure 1, and your experimental procedures: you say you collect the supernatants separately but Figure 1 indicates that you add the values of the separate K^+ and Hb measurements. Please clarify.

Authors: At fifteen minute intervals the samples were centrifuged, the supernatants decanted, and the potassium and hemoglobin concentrations were determined. (Knowing the volume, the amount of potassium and hemoglobin released during that fifteen minute segment was determined). Also at each fifteen minute interval, the pelleted cells were resuspended in fresh medium containing the detergent. Thus, at the end of the complete experiment, we were able to determine the percentage of the total at each fifteen minute interval. This procedure was performed on eight separate blood samples; and, the average value (for the eight samples at each fifteen minute interval) is plotted in figure 1.

J.S. Clegg : You refer to preliminary unpublished data on ATP release and suggest that ATP-Hb interactions may be involved. Do you know of any other data that suggest such interactions might occur?

Authors: We are not aware of any studies of potassium adsorption directly to purified hemoglobin. We do wish to mention two sources of information which indicate that ATP is involved in the binding (adsorption) of potassium to hemoglobin in particular, and proteins in general. First, the potassium content of "red blood cell ghosts" appears to be directly proportional to the protein (and hence, hemoglobin) content of the preparation (for a review, see reference 20, pages 128-131). Second, the role of ATP and even its non-hydrolyzable analogues in mediating protein assembly is beginning to be discovered. On one hand, its importance is described in nuclear processes [e.g., Mastrangelo IA, et al. (1989): ATP-dependent assembly of double hexamers of SV40 T antigen at the viral origin of DNA replication. Nature 338, 658-662]; on the other hand, dynamic protein associations between cytoskeletal elements are also dependent on ATP or its non-hydrolyzable analogues [Mandelkow EM, et al. (1988): Dynamics of the microtubule oscillator: role of nucleotides and tubulin-MAP interactions. EMBO J. 7, 357-365].

P.B. Bell: This paper reports that potassium and hemoglobin are released with similar kinetics when red blood cells are treated with Triton X-100 at a concentration below its critical micellar concentration. This result is said: (1) **to suggest** a cooperative process; (2) to support the **possibilty** that potassium **may** be selectrively adsorbed to hemoglobin; and (3) to provide evidence for **possible** hemoglobin-cytoskeletal and hemoglobin-hemoglobin interactions. The emphasis is mine but the words are the authors'. Therefore, as this paper is written, the authors provide jumping-off point for a lot of speculation about possibilities. But could the results not suggest some other explanation? Could the similar behavior of potassium and hemoglobin be merely coincidental?

R.L. Post: The authors note that the release kinetics was sigmoid. They relate this sigmodicity vaguely to "cooperative interaction between cellular constituents", adsorption of potassium to cellular proteins hemoglobin-cytoskeletal interactions, and a multiple protein interaction. No supporting evidence is offered. The kinetics was sigmoid with respect to

time, not with respect to concentration. Sigmodicity with respect to time, not concentration, does not require cooperativity; a precursor-product relationship is sufficient.

Authors: The reviewers' points are well taken. It is correct that we are suggesting that the interaction between potassium and hemoglobin is cooperative. This postulation evolves primarily from a concept and three sets of data. The concept is that all cells come from a unicellular origin--the fertilized egg--thus, the red blood should exhibit phenomena similar to other cells. The data are: (1) the cooperative uptake of potassium has been demonstrated in three smooth muscle types (arterial, intestinal, and uterine), skeletal muscle, and lymphocytes (for a review on this subject, please see chapter 11, pages 319-376, reference 20). (2) It is reported (Ling et al., Physiol. Chem. & Phys. & Med. NMR 16: 381, 1984) that the potassium concentration is directly proportional to the protein concentration of red blood cell ghosts. (3) The selective adsorption of sodium to hemoglobin as well as the selectivity decreasing in the order sodium, lithium, potassium, rubidium, and cesium has been demonstrated (Ling and Zhang, Physiol. Chem. & Phys. & Med. NMR 16: 221, 1984).

Lacking direct evidence for the cooperative uptake of potassium in the red blood cell, we can only agree with the reviewers that other explanations are possible.