# Nonhemoglobin Proteins in Young and Old Red Blood Cells[1] (35253)

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Little is known concerning the chemical basis for the diminution of enzyme activities in erythrocytes as a function of red blood cell age (1–4). Suggestions to account for this decrease include mixed disulfide formation between enzymes and oxidized glutathione (5), depletion of essential cofactors (3, 6, 7), a variety of other reactions or interactions between enzymes and small molecules present in red cells (8), the accumulation of inhibitors and catabolism of enzymes (9).

In this paper we report that the quantity of nonhemoglobin proteins released on hemolysis under identical conditions of young red blood cells is greater than that from old cells, while no particular differences in the acrylamide gel electrophoresis patterns of the nonhemoglobin proteins from these two sources are discerned. Possibilities that can account for these findings are discussed.

*Methods.* Erythrocytes of different ages can be obtained by centrifugation of blood with the younger cells having a lower specific gravity than the older ones. The procedure for this separation has been described (7). Each lysate was assayed for aspartate aminotransferase activity as previously reported (7). The assay is based on the method of Karmen (10). Enzyme activity is defined in terms of arbitrary units: 1 unit = Δ 0.001 A unit/min per ml of enzyme solution (hemolysate) at 340 nm. Specific enzyme activity is expressed as enzyme units/hemoglobin absorbance at 540 nm.

The lysates corresponding to one young and one old fraction were adjusted to contain an equal amount of hemoglobin in an identical volume. The preparation of nonhemoglobin proteins from the lysates with or without stroma was then carried out. The procedures for the isolation of nonhemoglobin proteins on DEAE-cellulose from erythrocytes described by Hennessey *et al.* (11) and Schrago and Falcone (12) were partially combined and slightly modified. Protein content was determined by the Lowry procedure (13).

The pooled eluate was lyophilized until the volume was reduced to about 10 ml or less. The concentrate was dialyzed against water. The dialyzate was lyophilized further until the volume was about 1 ml or less. It was then centrifuged (16,000*g* for 30 min) and the clear yellowish supernatant was reassayed for protein.

The concentrated nonhemoglobin proteins were separated by acrylamide-gel disc electrophoresis. Between 145 and 170 μg of protein in 15 to 30 μl were used in each disc electrophoresis. The electrophoresis gel was 7% acrylamide and the concentrating gel, 2.5% acrylamide. Electrophoresis was carried out at 4° in Tris–glycine buffer (0.37 *M*, pH 9.5) with a current of 2.5 mA/tube during 15 to 30 min. The gels were stained in 0.1% amido black in 7% acetic acid for 2 hr and were destained by electrophoresis in 7% acetic acid, at 2.5 mA/tube, 1.5 hr.

*Results and Discussion.* Table I presents the results of experiments with blood from seven different individuals. Aspartate aminotransferase activity and the amount of nonhemoglobin proteins recovered from the top (young) or bottom (old) 10–15% layers of centrifuged blood are shown. The specific en-

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TABLE I. Recovery of Nonhemoglobin Proteins and Aspartate Aminotransferase Activity from Young and Old Human Erythrocytes.[a]

| | Young | Old | Ratio (Y/O) |
|---|---|---|---|
| Aspartate aminotransferase activity | | | |
| Specific enzyme activity | 14.83 | 5.60 | 2.65 |
| Standard error of the mean | 0.92 | 0.36 | |
| No. of determinations (individuals) | 38(12) | 36(12) | |
| Recovery of nonhemoglobin proteins | | | |
| A. Without stroma | | | |
| Nonhemoglobin proteins (mg) / 100 hemoglobin OD (540 nm) | 5.00 | 3.91 | 1.28 |
| Standard error of the mean | 0.21 | 0.22 | |
| No. of determinations (individuals) | 11(4) | 11(4) | |
| B. With stroma | | | |
| Nonhemoglobin proteins (mg) / 100 hemoglobin OD (540 nm) | 7.42 | 6.05 | 1.23 |
| Standard error of the mean | 0.16 | 0.08 | |
| No. of determinations (individuals) | 17(4) | 17(4) | |

[a] See text for details.

zyme activity is higher in lysates derived from young red cells as compared to those obtained from older erythrocytes. These enzyme activities are an indicator of the efficiency of our fractionation procedure (7). The quantitative recovery of nonhemoglobin proteins from these cells (Table I) is in line with that reported by Hennessey *et al.* (11) for unfractionated human blood. However, the amount of nonhemoglobin proteins recovered from the young red cell fractions is about 25% greater than that derived from the older cells (Table IA, B).

These results differ from the change in hemoglobin content reported in red cells as a function of age (14). The mean hemoglobin content per cell is slightly higher (about 4%) in older erythrocytes than in the top (young) layer of centrifuged cells. Some hemoglobin synthesis is apparently still taking place in the reticulocytes in the peripheral blood. The values in Table I, which are related to hemoglobin, might, therefore, have to be corrected by the above-indicated 4% when recoveries of young and old nonhemoglobin proteins are compared.

While catabolism of nonhemoglobin proteins in aging erythrocytes is a possibility that could explain our results, it would have to be catabolism of only these proteins and not of hemoglobin. Also conceivable is the interaction of proteins with the cell membrane as a function of red cell age which results in differential extraction of nonhemoglobin proteins from young and old cells. To investigate this the quantity of nonhemoglobin proteins obtained from lysates from which the stroma had not been removed (Table IB) was measured. The nonhemoglobin proteins recovered from both old and young fractions increases appreciably, about 1.5-fold, in this case (compared to Table IA), indicating that much nonhemoglobin protein is not released on simply lysing the cells. The ratio of nonhemoglobin proteins from young and old red cells remains, however, virtually unchanged. It is possible that very precise conditions, as yet not worked out, are needed with respect to ionic strength and pH for the complete extraction of nonhemoglobin proteins from red cell ghosts. This would be analogous to the detailed conditions specified by Dodge and co-workers (15) for the virtually complete extraction of hemoglobin from unfractionated red cells.

In Fig. 1 we present the acrylamide-gel disc electrophoretic patterns obtained for nonhemoglobin proteins derived from young

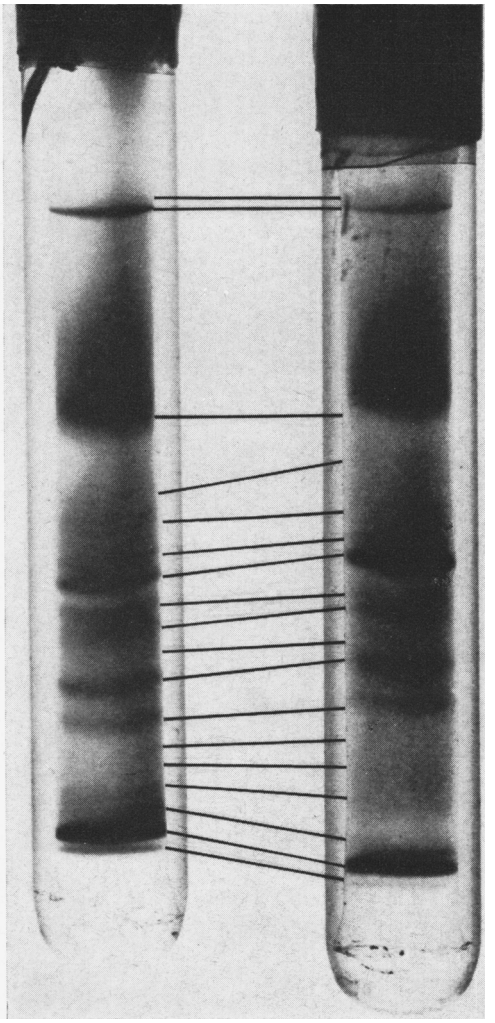

FIG. 1. Acrylamide-gel disc electrophoresis patterns of nonhemoglobin proteins from young (left) and old (right) erythrocytes. For details of procedure and discussion see text.

and from old red cells. The nonhemoglobin protein preparations used were those without stroma. During concentration of nonhemoglobin proteins for electrophoresis by lyophilization (or other methods) there is always some protein which comes irreversibly out of solution and is lost. The soluble young or old nonhemoglobin proteins show no obvious differences in electrophoretic pattern. Each fraction has between 16 and 19 bands. Thus the qualitative protein content of young and old red cells appears unchanged. Acrylamide-gel electrophoresis of nonhemoglobin proteins from unfractionated human erythrocytes yields about 16 bands (16). Consequently, the agreement between the number of bands found in unfractionated erythrocytes by Todorow (16) and those found in young and old red cells by ourselves is good.

*Summary.* When young and old human red blood cells are lysed, more nonhemoglobin proteins are released from the young than from the old cells, reflecting the changed intracellular environment. Qualitatively, as judged by acrylamide-gel electrophoresis, there are no differences between the nonhemoglobin proteins from these two sources. It is shown for one enzyme (and known for many) that the enzyme activity associated with young cells is appreciably higher than that found in older cells. The lower quantity of nonhemoglobin proteins recovered from older cells may well be related to this phenomenon. Possibilities accounting for the smaller quantity of nonhemoglobin proteins recovered from older cells include protein catabolism in older cells or, more likely, an age-dependent interaction between nonhemoglobin proteins and the cell membrane.

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