Re-evaluation of the structural integrity of red-cell glycoproteins during aging *in vivo* and nutrient deprivation

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Results presented in this paper show that removal of white-cell contaminations from human red blood cells by filtration through cellulose [Beutler, West & Blume (1976) *J. Lab. Clin. Med.* **88**, 328–333] is a necessity whenever red cells are incubated at elevated temperatures or haemolysed after density separation. Omission of this precaution results in proteolysis of sialoglycoproteins in membranes from less-dense (young), but not dense (old), subpopulations. This proteolytic damage occurs during haemolysis of the cytoplasmic domain of glycophorin. A different type of proteolysis occurs if white-cell-contaminated red cells are incubated in the absence of glucose at elevated temperatures. Red cells release sialoglycopeptides. This process is stimulated by Ca^{2+} ions and is accompanied by the release of vesicles that differ from spectrin-free vesicles [Lutz, Liu & Palek (1977) *J. Cell Biol.* **73**, 548–560]. This sialoglycopeptide release is dependent on white-cell contamination and is not required for the release of spectrin-free vesicles.

INTRODUCTION

Red cells were reported to lose sialic acid by a neuraminidase-like activity and/or sialoglycopeptides by a proteolytic activity during aging *in vivo* as well as during nutrient deprivation (Danon *et al.*, 1972; Cohen *et al.*, 1976; Luner *et al.*, 1977; Brovelli *et al.*, 1977; Aminoff *et al.*, 1981; Gattegno *et al.*, 1976, 1981; for reviews, see Clark & Shohet, 1985; Vacha, 1983). Others have, however, shown that red-cell senescence does not correlate with desialylation of sialoglycoproteins and suggested that the uncontrolled proteolysis which occurred during density separation of red cells might be responsible for the divergent findings (Lutz & Fehr, 1979). A detailed study was not performed. We therefore investigated the integrity of sialoglycoproteins from nutrient-deprived and from density-separated red cells. Nutrient deprivation or ATP-depletion of human red blood cells, also called ‘*in vitro* aging’, has been carried out under a variety of conditions either to study the osmotic fragility, morphology and deformability of ATP-depleted red cells (Nakao *et al.*, 1960; Féo & Leblond, 1974; Féo & Mohandas, 1977; Sheetz & Singer, 1977; Birchmeier & Singer, 1977) or to investigate the release of spectrin-free vesicles which follows ATP depletion from echinocytic red cells (Lutz *et al.*, 1977; Müller *et al.*, 1981). These vesicles are sealed and contain haemoglobin. They retain integral, but not peripheral, membrane proteins. The mechanism by which vesicles are released is as yet unclear. It does not involve extracellular Ca^{2+} ions, though Ca^{2+} and ionophore A23187 stimulate the release of similarly structured vesicles (Allan *et al.*, 1976). It might involve S–S bond formation, because glutathione decreases during ATP depletion (Palek *et al.*, 1978; Lutz, 1978). Likewise, it could involve proteolysis in confined areas.

Some authors indeed reported a release of soluble sialoglycopeptides concomitant with vesicle release (Pessina & Skiftas, 1983). Hence it appeared as if vesicle release occurred parallel with a partial degradation of integral membrane proteins.

We investigated this possibility by assessing the release both of vesicles and of soluble sialoglycopeptides from ATP-depleting red cells. We report here that the vesicle release is independent of the release of sialoglycopeptides. Sialoglycopeptide, but not vesicle, release was suppressed by omitting Ca^{2+} ions and by filtering red cells through cellulose (Beutler *et al.*, 1976) to remove white cells. Likewise, filtration of red cells before density separation eliminated sialoglycoprotein degradation in membranes from density-separated red cells.

EXPERIMENTAL

Isolation of red cells from whole blood

Fresh human blood was collected in 3.8% (w/v) sodium citrate (as anticoagulant). Erythrocytes were washed three times with PBS and the buffy coat was removed by centrifugation at 4 °C at 1700 g_{max} for 5 min or, alternatively, once-washed cells were passed through a mixture of α - and microcrystalline-cellulose as described by Beutler *et al.* (1976) and then washed three times in PBS.

Nutrient deprivation

Method 1. Washed and packed human red blood cells were suspended in 1 vol. of KRp, pH 7.4 (Brovelli *et al.*, 1977), containing 0.5 mM-EDTA (or 1 mM- CaCl_2) and ampicillin (50 $\mu\text{g}/\text{ml}$) and placed in dialysis bags. The bags were mounted in containers filled with the same buffer used to prepare the cell suspension and incubated in a water bath at 37 °C under gentle agitation. The pH

Abbreviations used: AChase, acetylcholinesterase; PBS, phosphate-buffered saline (145 mM-NaCl/5 mM-phosphate, pH 7.4); KRp, Krebs–Ringer phosphate buffer; PMSF, phenylmethanesulphonyl fluoride; WGA, wheat-germ agglutinin; PAS, period acid/Schiff reagent.

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of the medium surrounding the bags was adjusted to pH 7.4 every 6–8 h. Nutrient deprivation was stopped after 24 h and the contents of the bags were centrifuged for 10 min at 1700 g_{max} . The cells were recovered as a pellet and the supernatants were collected and filtered through a Gelman membrane filter with a pore size of 0.8 μm . The filtrates were centrifuged for 50 min at 12500 g_{max} at 4 °C to collect released vesicles. The supernatants were saved for further analyses.

The cells recovered from the pellet after the first centrifugation were washed and lysed in 10 vol. of 0.5 mM-Tris/HCl buffer (containing 0.2 mM-PMSF and 1 mM-EDTA as proteinase inhibitors), to prepare ghost membranes by the method of Marchesi & Palade (1967). Control cells which were not nutrient-deprived were stored in suspension at 4 °C until nutrient-deprived cells were ready for membrane isolation. Ghosts were washed twice with 0.5 mM-Tris/HCl buffer, then once with the same buffer containing 0.5 M-NaCl to remove haemoglobin, and finally with 0.5 mM-Tris/HCl buffer.

Method 2. Alternatively cells were nutrient-deprived in the absence of phosphate at 20% haematocrit, as

outlined elsewhere (Lutz *et al.*, 1977; Müller *et al.*, 1981). Released vesicles were purified as outlined and the supernatants after sedimentation were saved for further analyses. Ghosts were obtained from nutrient-deprived and washed cells by the method of Dodge *et al.* (1963) as modified by Lutz *et al.* (1984).

Separation of erythrocytes of different cell age

Erythrocytes of different cell age were prepared from human blood as previously described (Brovelli *et al.*, 1983) by a modified version of Murphy's (1973) procedure (method A). Erythrocytes filtered through a cotton column, or not filtered, were washed twice, suspended in autologous plasma (haematocrit 60%) and centrifuged for 2 h at 1000 g_{max} at 20 °C. Top and bottom cells ($\frac{1}{8}$ of the total cell population respectively) were collected and washed twice with PBS. Alternatively cells of different age were obtained by self-forming Percoll gradients as outlined elsewhere (Lutz & Fehr, 1979) (method B). The creatine content of fractionated red cells (Griffiths, 1964; Fehr & Knob, 1979) served as a cell-age parameter.

Fig. 1. Membranous and soluble constituents released from nutrient-deprived red cells

A 20 ml portion of packed cells were nutrient-deprived according to method 1 or 2 (*). The suspensions were incubated for 24 h at 37 °C. Red cells were then collected by centrifugation. The extent of sialoglycopeptide release was assessed by determining the sialic acid (■) and protein (□) content in the non-dialysable supernatant as outlined in the text. Sialic acid recoveries were 85 and 100% for filtered red cells incubated in the presence of Ca²⁺ and EDTA respectively. Recoveries were variable for unfiltered red cells and ranged from 44 to 81% for samples incubated in the presence of Ca²⁺ and from 81 to 100% for samples incubated in the presence of EDTA; this variability was due to generation of dialysable sialoglycopeptides, primarily when Ca²⁺ ions were present. Since the peptides released into the supernatant originated from sialoglycoproteins and other proteins, sialoglycopeptide release was estimated on the basis of sialic acid data. The heterogeneity of the released material and generation of dialysable peptides can explain the variability in the sialic-acid-to-protein ratio in the non-dialysable supernatant. The dialysable fraction was not routinely determined. The amount of non-dialysable material is given as a percentage of the total sialic acid and protein content in 20 ml of red cells. Values are means $\pm$ 1 s.d. of results from three independent experiments. Vesicle release was quantified from the AChase activity in the supernatant and the total activity in 20 ml of cells. These percentages are given where recoveries were 87% or more. In samples from unfiltered and from filtered cells incubated with Ca²⁺, recoveries were 51 and 75% respectively because of proteolytic damage and AChase inactivation. Thus vesicle release was also estimated from the volume of pelleted material indicated by + (50–70 μl) to +++++.

Characterization of membranous and soluble constituents released from nutrient-deprived red cells

The initial supernatant obtained after sedimenting nutrient-deprived red cells was assayed for AChase activity as described by Ellmann *et al.* (1961). The supernatants containing non-dialysable soluble material obtained after pelleting the vesicles were chromatographed on Dowex 2 resin (X8; acetate form) to concentrate the released sialoglycopeptides (Brovelli *et al.*, 1976). The material eluted with 1 M-acetate buffer, pH 4.4, was analysed for sialic acid and protein.

Sialic acid was determined by the Svennerholm (1958) method as previously reported (Brovelli *et al.*, 1976). Protein was determined by the method of Lowry *et al.* (1951), with serum albumin as a standard.

Isolation of glyophorin

Glycophorin was isolated from young and old erythrocytes by affinity chromatography on wheat-germ agglutinin coupled to Sepharose 6B (Pharmacia). The conditions used are those reported by Shapiro & Marchesi (1977) to isolate glycoprotein directly from cells by affinity chromatography on columns containing WGA-Sepharose. Red cells suspended in 1 vol. of PBS were treated with SDS to a final concentration of 1% and then processed as outlined.

SDS/polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out as described by Laemmli (1970). Samples were denatured for 40 min at 37 °C or for 5 min at 100 °C in 10 mM-Tris/HCl buffer, pH 6.8, containing 40 mM-dithiothreitol, 1 mM-EDTA, 1% SDS, 7% saccharose and 0.01% pyronin as tracking dye. Electrophoretic separation was performed on polyacrylamide-gel slabs (14 cm × 13.5 cm; 1.5 mm thickness) with running times of about 5 h at 40 mA. The protein content of each sample was 70 µg and 250 µg for samples subsequently stained with Coomassie Blue or PAS respectively. The PAS-stained gels were scanned at 546 nm with an Isco 1310 gel scanner.

RESULTS

Red-cell protein integrity during nutrient deprivation

Human red cells, nutrient-deprived in the presence of Ca^{2+} ions, released sialoglycopeptides (Fig. 1; no filtration). The extent of sialoglycopeptide release was measured from the sialic acid content in the non-dialysable supernatant (see also the legend to the Figure with regard to protein release) and showed large variations. These must have come from uncontrolled white-cell contamination of washed red cells, because red-cell filtration through cellulose (filtered red cells) (Beutler *et al.*, 1976) reduced the variability and minimized sialoglycopeptide release. Red-cell filtration inhibited the release by $54 \pm 30\%$ ($P < 0.05$) from cells nutrient-deprived in Ca^{2+} and by $68 \pm 29\%$ ($P < 0.02$) from cells kept in EDTA in all four experiments. Complexation of Ca^{2+} , which is known to activate certain white-cell proteinases (Bocci *et al.*, 1981; Legendre & Jones, 1983; Pontremoli *et al.*, 1985), and erythrocyte calpain (Pontremoli *et al.*, 1980) similarly inhibited sialoglycopeptide release by 77 ± 33 ($P < 0.02$) for unfiltered red cells and by $86 \pm 13\%$ ($P < 0.01$) for

Fig. 2. SDS/polyacrylamide-gel electrophoresis of vesicles released from nutrient-deprived red blood cells which were either filtered through cellulose or not before nutrient deprivation for 24 h in the given buffer.

Vesicles released from 20 ml of cells were resuspended in small volumes of PBS. The amount of vesicle protein which was electrophoresed represented the given fraction of the total amount of vesicles released from 20 ml of cells. The stained polypeptides of human red-blood-cell membrane were labelled as described by Fairbanks *et al.* (1971). The polypeptide of M_r 67000 in (b), lanes 1–3, represents human serum albumin, which was present in trace amounts. Added albumin or ovalbumin adsorbs to vesicles but has no influence on the extent of vesiculation (results not shown). The polypeptide designated '62' in (a) and (b) represents a breakdown product of band 3 protein (Golovtchenko-Matsumoto *et al.*, 1982) and is not identical with the 67000- M_r protein. This is also evident from (a), lane 3, which is repeated in (b), lane 4. (a): Lane 1, vesicles from unfiltered cells incubated by method 1 in a buffer containing 1 mM- Ca^{2+} ; lane 2, vesicles from filtered cells incubated by method 1 in a buffer containing 1 mM- Ca^{2+} ; lane 3, vesicles from filtered cells incubated by method 2 for 36 h in a buffer containing 0.5 mM-EDTA. (b): Lanes 1 and 2, vesicles from unfiltered and filtered cells incubated by method 1 in a buffer containing 0.5 mM-EDTA; lanes 3 and 4, vesicles from cells incubated by method 2, from two independent experiments in which nutrient deprivation lasted for 24 and 36 h, in a buffer containing 0.5 mM-EDTA; lane 5, 80 µg of membrane protein of whole ghosts; lane 6, M_r markers: phosphorylase, 94000; albumin, 67000; catalase, 60000; ovalbumin, 43000; carbonic anhydrase, 30000; trypsin inhibitor, 20000.

filtered red cells ($n = 4$). Furthermore, elimination of Ca^{2+} and omission of phosphate completely suppressed the sialoglycopeptide release (Fig. 1). In contrast with the sialoglycopeptide release, vesicle release from red cells was not inhibited by nutrient deprivation in a medium containing EDTA (Fig. 1). Vesicle release reached 1% (method 1) and 2% (method 2) of the total membrane content as judged from the released AChase activity. Thus vesiculation and sialoglycopeptide release are independent from one another. The release of sialoglyco-

Fig. 3. SDS/polyacrylamide-gel electrophoresis of ghost membranes prepared from unincubated and nutrient-deprived erythrocytes

(a) PAS stain; (b) Coomassie Blue stain. Lanes 1–3, filtered cells were either stored at 4 °C (1) or were nutrient-deprived for 24 h by method 1 in a buffer containing 0.5 mM-EDTA (2) or 1 mM- Ca^{2+} (3). Lanes 4–6, unfiltered cells were either stored at 4 °C (4) or were nutrient-deprived for 24 h by method 1 in a buffer containing 0.5 mM-EDTA (5) or 1 mM- Ca^{2+} (6). Lane 7, M_r markers as given in Fig. 2. The arrows in (a) and (b) denote breakdown products (see the text).

peptides from red cells was highest in the presence of Ca^{2+} , whether cells were filtered or not. Concomitant vesicle release was, however, difficult to assess by determining AChase activity, because the recoveries were low (see the legend to Fig. 1). The low recoveries were due to proteolysis, as evident from the polypeptide patterns of purified vesicles (Fig. 2a, lanes 1 and 2). These vesicles contained no band 3 protein and only traces of PAS-stainable bands (results not shown); instead they contained variable amounts of a 62000- M_r protein. Filtered red cells kept in Ca^{2+} released vesicles that also contained high- M_r polymers (Fig. 2a, lane 2). The use of filtered red cells improved the quality of released vesicles if cells were nutrient-deprived in EDTA (Fig. 2b, lanes 1, 2; see also the legend). Thus vesicles containing band 3 protein as the predominant constituent, with only a few breakdown products and no high- M_r polymers, were obtained from filtered red cells nutrient-deprived in EDTA or in EDTA in the absence of phosphate (Fig. 2b, lanes 2–4). These results support the conclusion drawn from quantitative results (Fig. 1), namely that vesiculation was independent of sialoglycopeptide release.

In contrast with the different patterns of vesicle proteins, the polypeptide patterns of membranes from red cells, nutrient-deprived under the various conditions, showed only minor changes. The content of a few Coomassie Blue-stained polypeptides in the region between band 4.2 and band 5 (see the arrows in Fig. 3) was slightly enhanced in membranes from cells kept in Ca^{2+} ions (Fig. 3b). Membranes from unincubated, but stored, unfiltered, red cells revealed instead an altered pattern in the 4.1 region and the PAS 1 band (Figs. 3a

and 3b, lane 4). Thus membranes from unincubated, but stored, red cells showed instead of a loss, a dramatic change in the PAS staining pattern, which was more pronounced with unfiltered than with filtered red cells (Fig. 3a). This suggests that white-cell-derived proteinases acted differently according to whether they were present during ATP depletion or only during haemolysis.

Sialoglycoprotein integrity during *in vivo* aging

Results summarized in Fig. 4 indicate that ghost membranes prepared from unfiltered red cells, density-separated by a modified Murphy (1973) procedure, contain degraded sialoglycoproteins. The proteolytic breakdown is primarily evident in less-dense 'young' cells. This breakdown did not result in a substantial loss of PAS-stainable material, but generated mainly two polypeptides (fragments I and II) having a higher electrophoretic mobility than PAS 1 (Fig. 4a,i). Generation of these membrane-associated sialic-acid-containing fragments suggested proteolytic cleavage from the cytoplasmic domain. Removal of white-cell contamination by filtration through cellulose greatly improved the results (Fig. 4a,ii). The PAS 1 band was apparent in all density subpopulations. Only the lightest fractions also revealed a degradation product. Proteolytic damage was further decreased when filtered red cells were separated on self-forming Percoll gradients instead of by the modified Murphy (1973) procedure (Fig. 4a,iii).

Hence density separation of unfiltered, but washed, red cells by the widely used Murphy (1973) procedure caused dramatic proteolysis of the sialoglycoproteins, primarily in the less-dense subpopulations, where

Fig. 4. SDS/polyacrylamide-gel electrophoresis of ghost membranes prepared from cells of different density separated by a modified Murphy's (1973) method (Brovelli *et al.*, 1983) or by self-forming Percoll gradients (Lutz & Fehr, 1979)

(a) PAS-stained gels; (b) Coomassie Blue-stained cells. (i) Red cells not filtered through cellulose were separated by a modified Murphy's (1973) method and divided into eight fractions. Lanes 1–5 correspond to the fractions 1, 2, 4, 7 and 8 from the top, with creatine contents of 65, 60, 43, 27 and 24 $\mu\text{g}/10^{10}$ cells respectively. (ii) Red cells filtered through cellulose were separated by a modified Murphy (1973) procedure and divided into eight fractions. Lanes 1–3 correspond to the fractions 1, 4 and 7, with creatine contents of 79, 44 and 32 $\mu\text{g}/10^{10}$ cells respectively. (iii) Red cells were filtered through cellulose and density-separated on self-forming Percoll gradients; five fractions were isolated: lanes 1–3 correspond to fractions 1, 3 and 5, with creatine contents of 136, 68 and 55 $\mu\text{g}/10^{10}$ cells respectively. I and II refer to sialic acid-containing breakdown products of the PAS 1 band.

granulocytes are more likely to band than at high densities. It is important to note that such dramatic changes were not apparent from Coomassie Blue-stained gels of corresponding membranes from the various fractions (Fig. 4*b*,i). Since red-cell filtration eliminated most of the proteolytic breakdown, it was probably not caused by endogenous proteinases, but by exogenous proteinases that gained access to the cytoplasmic domain during haemolysis. To test for this possibility, glycophorin was isolated directly from intact cells by affinity chromatography on WGA after addition of SDS to an iso-osmotic suspension of red cells. Glycophorins thus obtained from red cells separated by the modified Murphy (1973) procedure had the same apparent M_r whether they were obtained from cells filtered through cellulose or not (Fig. 5).

DISCUSSION

In many investigations red cells were obtained from pelleted blood cells by removing the buffy coat and by washing the residual cells in iso-osmotic buffer. Although this isolation procedure is in many cases satisfactory, it

is not in more complicated approaches involving density separation of red cells or incubations of red cells *in vitro* at elevated temperatures, as shown here. Contamination of washed red cells with white cells had been known for many years and triggered Busch & Pelz (1966) and Nakao *et al.* (1973) to establish a protocol to remove white cells from whole blood samples. In 1976, Beutler's group (Beutler *et al.*, 1976) introduced a procedure involving filtration of whole blood through cellulose to increase the efficiency of the removal of white cells, in particular the proteinase-containing granulocytes. Though well known, the procedure is not generally used because it is time-consuming and probably because experiments carried out with or without filtration did not yield marked differences in the polypeptide pattern of membranes thus obtained. The findings presented here show, however, that density separation or incubation of unfiltered red cells resulted in profound changes in the membranes of red cells without markedly altering their Coomassie Blue-stainable polypeptide pattern.

Exoplasmic damage occurred on unfiltered red cells during nutrient deprivation at 37 °C. Damage was evident from a pronounced sialoglycopeptide release,

Fig. 5. SDS/polyacrylamide-gel electrophoresis of glycophorin A isolated from young and old cells by affinity chromatography on WGA coupled to Sepharose

The samples in (a) and (b) correspond to glycophorin A from cells, not filtered and filtered through cellulose respectively, fractionated according to their density by a modified Murphy's (1973) procedure (Brovelli *et al.*, 1983). Lane 1, young cells (fraction 1); lane 2, old cells (fraction 8). Results from two independent experiments are shown. The ratio of the creatine contents (young/old cells) was 2.6 in (a) and 5.9 in (b).

which was 2–3 times higher from unfiltered cells than from filtered ones under comparable incubation conditions. It was highest in the presence of Ca^{2+} ions. Ca^{2+} perturbed the system and enhanced the release of proteolytically damaged vesicles whether filtered or unfiltered red cells were used. We do not think that white-cell-derived proteinases were responsible for the complete degradation of band 3 protein in vesicles, because they are sealed (Lutz *et al.*, 1977). The exogenous proteinases evidently hydrolysed sialoglycoproteins and probably cleaved band 3 into a 62000- M_r fragment (see Fig. 2) as chymotrypsin does (Steck *et al.*, 1976). This proteolytic modification of the exoplasmic surface must have altered the membrane topology of the vesicles in such a way as to activate endogenous Ca^{2+} -dependent proteinases and membrane proteinases (Pontremoli *et al.*, 1984), which are known to degrade band 3 protein and sialoglycoproteins completely (Lorand *et al.*, 1983). Since complete degradation of band 3 occurred with unfiltered red cells in the presence of EDTA buffer, but not with Ca^{2+} , a Ca^{2+} -dependent transglutaminase-induced polymer formation (Anderson *et al.*, 1977; Siefring *et al.*, 1978) may have reduced the accessibility of the 62000- M_r protein to endogenous proteinases in the presence of Ca^{2+} .

The release of sialoglycopeptides and of proteolytically damaged vesicles was completely inhibited by nutrient deprivation of filtered red cells in buffer containing EDTA and lacking phosphate. These cells nevertheless released spectrin-free vesicles showing no signs of proteolysis. Thus we confirm the earlier finding (Lutz, 1978; Müller *et al.*, 1981) that neither exogenous Ca^{2+} nor endogenous proteolysis was required as a trigger for the release of spectrin-free vesicles containing intact band 3 protein. In addition, we have demonstrated that the enhanced vesiculation observed with Ca^{2+} (Pessina & Skiftas, 1983) cannot be considered an increased release of spectrin-free vesicles, because it involved sialoglycopeptide release and the generation of vesicles that in fact contained band 3 breakdown products.

Density separation of unfiltered red cells by a modified Murphy (1973) procedure resulted in a selective proteolysis of sialoglycoproteins in ghosts from less-dense red-cell subpopulations. This dramatic proteolysis was almost eliminated by using filtered instead of unfiltered red cells. Proteolysis was eliminated altogether when filtered red cells were separated on self-forming Percoll gradients that also contained proteinase inhibitors. The damaging effect of white-cell-derived proteinases occurred during haemolysis rather than with intact red cells, as demonstrated in Fig. 5. This is of particular interest, because it implies that the proteolytic damage occurred after washing of the density-separated red cells. Therefore it is unlikely that the damage was caused by proteinases released from granulocytes during density separation. Whereas these proteinases were washed off (unless they were adsorbed on red cells), the remaining granulocytes co-sedimented with the washed red cells. This means that the co-sedimenting granulocytes released, during hypo-osmotic lysis, some of their enzymes, which then had access to the cytoplasmic domain of integral membrane proteins known to have at least a 100-fold higher sensitivity to proteolytic attack than exoplasmic domains (Steck *et al.*, 1971; Triplett & Carraway, 1972). These results shed some doubts on experiments carried out with unfiltered red cells density-separated by the Murphy (1973) procedure. White-cell contaminations were primarily found in less-dense red-cell fractions and could have affected the significance of the findings thus obtained. It is worth mentioning that decreased sialic acid contents (Danon *et al.*, 1972; Seaman *et al.*, 1977; Gattegno *et al.*, 1976, 1981) and increased accessibility of galactose residues (Aminoff *et al.*, 1981) were reported for dense red cells obtained from unfiltered preparations. Virtually unchanged sialic acid contents per glycophorin molecule and equal numbers of accessible galactose residues were found in light and dense fractions from filtered red cells (Lutz & Fehr, 1979; Schweizer *et al.*, 1982) or red cells washed in buffer containing EDTA and proteinase inhibitors (Miyahara & Spiro, 1984).

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