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CHANGES IN CARBOHYDRATE CONTENT OF SURFACE MEMBRANES OF HUMAN ERYTHROCYTES DURING AGEING

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ABSTRACT

The carbohydrate content (neutral hexoses, fucose, sialic acid, and hexosamine) of human erythrocytes is significantly higher in young than in old erythrocytes. The differences observed in the carbohydrate content of surface glycopeptides, obtained after incubation with either Pronase or trypsin, is still more striking, suggesting important modifications of the carbohydrate moieties of the erythrocyte membrane during *in vivo* ageing.

INTRODUCTION

In rabbits, rats, and man, neuraminidase-treated, sialic acid-free erythrocytes are removed from the circulation significantly faster than intact erythrocytes[1–4].

Young and old erythrocytes are separated by centrifugation according to differences in density[5,6]. The sialic acid content is significantly higher in young erythrocytes[7–9], but the half-life of old erythrocytes is significantly shorter than that of young, neuraminidase-treated erythrocytes having a similar sialic acid content[8]. This suggests that the physiological mechanism of removal of sialic acid is only one of the events leading to the normal elimination of ageing erythrocytes[9]. Since these results suggest that the carbohydrate moiety of the erythrocyte membrane of young and old erythrocytes might be different, we investigated whether sialic acid is the only carbohydrate component of the membrane that is decreased during erythrocyte ageing, and whether the carbohydrate content of surface glycopeptides of young and old erythrocytes is different. We report here a comparative study of the carbohydrate content of young and old human erythrocyte membranes, and of the surface glycopeptides that were obtained by proteolytic digestion of the intact cells with either Pronase[10] or trypsin[11,12]. Differences in age of the erythrocytes separated by centrifugation were controlled by comparing some of the enzymatic activities[13,14].

EXPERIMENTAL

Separation of erythrocytes of different ages. — As the density of erythrocytes is known to increase with ageing[6], human erythrocytes of different age were separated on the basis of density differences by centrifugation for 2 h at 2000*g*. Each blood sample, freshly drawn from normal donors, was collected into heparinized tubes and treated separately. The buffy coat and the plasma were removed. The top and the bottom layers (each representing 16% of the total volume) of the packed red-cells were removed by gentle aspiration with a syringe (1 ml) and an 18-gauge needle (38-mm). The erythrocytes of the middle layer were used as control. The difference of age between the erythrocytes obtained from the top and those obtained from the bottom layer was assessed by comparing the pyruvate kinase[13] (EC 2.7.1.40) and the cholinesterase activities[14] (EC 3.1.1.7).

Survival time of ^{51}Cr-labelled young and old rabbit erythrocytes. — Young and old erythrocytes were incubated for 30 min at 37° with ^{51}Cr (0.05 mCi/ml) in a 0.15M NaCl solution (Commissariat à l'Énergie Atomique, 91 Gif-sur-Yvette, France). The ^{51}Cr-labelled erythrocytes were separated from the unreacted ^{51}Cr by three washes with 0.15M NaCl. The resultant erythrocyte preparation had an activity of 12 μCi per ml of packed erythrocytes. Injections were made in the ear vein of male rabbits weighing from 1 to 1.5 kg. Samples of blood were taken at various time-intervals from the other ear, transferred into heparinized tubes, and the radioactivity was determined with an NaI scintillation counter (Ames, New York, U. S. A.). The accuracy of the separation of the erythrocytes according to age between top and bottom of the tubes was assessed by the difference between their survival time when reinjected into the original animal: T_{50} of ^{51}Cr-labelled top erythrocytes: 11.2$\pm$ 0.25 days (number of experiments 7); T_{50} of ^{51}Cr-labelled bottom erythrocytes: 2.80$\pm$1.53 days (number of experiments 7) ($p<0.001$).

Carbohydrate content of young and old erythrocyte ghosts. — Young and old erythrocytes, washed three times with isotonic phosphate buffer solution (pH 7.4) containing 0.3% of D-glucose, were hemolyzed with hypotonic 25mM $CaCl_2$ and 0.5% heparin. Within 10 min, a precipitate of $Ca_3(PO_4)_2$ appeared which adsorbed the ghosts. Pink ghosts, obtained after centrifugation for 15 min at 3000*g*, became white after several washes with mM phosphate buffer solution[15]. In each ghost preparation, the hexose content was determined by the orcinol–H_2SO_4 reaction[16], the total hexosamine content by the Elson–Morgan reaction[17] after hydrolysis with 3M HCl for 6 h at 100°, the sialic acid content as described by Warren[18] after hydrolysis with 0.05M H_2SO_4 for 1 h at 80°, the fucose content as described by Dische and Shettles[19], and the protein content as described by Lowry *et al.*[20]. The carbohydrate content is expressed in mg of carbohydrate per g of membrane protein.

Carbohydrate content of surface glycopeptides of young and old erythrocytes. — Top and bottom erythrocytes of human origin were washed five times with isotonic NaCl and incubated for 1 h at 37° with either Pronase (EC 3.4.4., Calbiochem, La Jolla, CA 92037,) or trypsin (EC 3.2.1.4., Sigma Chemical Company, St.

Louis, MO 63178,) at an enzyme concentration of 2 mg per ml of packed erythrocytes, in 2 vol. of isotonic Na_3PO_4 buffer (pH 7). In other experiments, the erythrocytes were incubated first with *Vibrio cholerae* neuraminidase (EC 3.2.1.18., Behringwerke, Marburg, West Germany) at an enzyme concentration of 30 units per ml of packed erythrocytes for 1.5 h at 37°, and then with Pronase.

In each case, control experiments were performed with erythrocytes incubated either in the buffer without proteolytic enzymes, or with enzymes that had been heat-inactivated by treatment for 1 h at 80°. The supernatants from each experiment were deproteinized with 12% (v/v) aqueous trichloroacetic acid. The supernatants containing the soluble, heterogenous sialoglycoconjugates were freed of trichloroacetic acid by extraction with ether[21], and then analyzed for their content of hexose, hexosamine, fucose, and bound sialic acid after hydrolysis with 0.05M H_2SO_4 for 1 h at 80°, as just described. In the supernatants of the neuraminidase-treated erythrocytes, free sialic acid was determined without prior acid hydrolysis, as described by Warren[18]. The carbohydrate content is expressed in μg of carbohydrate per 10^{10} red blood-cells (RBC).

Fractionation of the glycopeptides by filtration. — A portion of Pronase-digested glycopeptides or of trypsin-digested glycopeptides was fractionated on a 0.9 cm × 30 cm column of Sephadex G-25 or G-50, eluted with bidistilled water. The flow rate of the column was 30 ml/h. The void volume (Vo) of the column was determined with Dextran Blue. The E_{280} was monitored with the ISCO Absorbance Monitor; 1-ml fractions were collected, each of them being further analyzed for hexose content by the orcinol–H_2SO_4 reaction[16], and for total amino acid content by the ninhydrin reaction. The bound sialic acid content of each peak containing glycopeptides was determined as described by Warren[18], after hydrolysis with 0.05M H_2SO_4 for 1 h at 80°.

RESULTS

Separation of erythrocytes of different ages. — The results (see Table I) obtained with erythrocytes from the top and bottom layers demonstrate that pyruvate kinase and acetylcholinesterase activities were significantly lower in the bottom layers than in the top layers. This suggests that, on average, old cells are obtained in the bottom layer and young ones in the top layer.

Carbohydrate content of young and old erythrocyte ghosts. — The content of neutral hexoses, fucose, hexosamine, and sialic acid was significantly higher in young erythrocyte ghosts than in old ones. Old erythrocyte ghosts contain 84, 87, 77, and 89% of the neutral hexoses, fucose, hexosamine, and sialic acid contents, respectively, of the young ones (see Table II).

Carbohydrate content of surface glycopeptides of young and old erythrocytes. — Surface glycopeptides released from intact, old erythrocytes by proteolytic digestion with Pronase contained 76, 66, 71, and 69% of the neutral hexoses, fucose, hexosamine, and sialic acid content, respectively, of the glycopeptides released from

TABLE I

SEPARATION OF HUMAN ERYTHROCYTES OF DIFFERENT AGES BY CENTRIFUGATION ACCORDING TO DENSITY

Enzymic activity[a]	*Number of paired experiments (n)*	*Top layer*[a]	*Bottom layer*[a]	*P of the differences observed between top and bottom layers*
Cholinesterase	7	13.8 ± 1.8	10.6 ± 2.10	<0.001
Pyruvate kinase	8	3.7 ± 0.5	1.6 ± 0.7	<0.001

[a]Results are expressed, in unit per 10^{10} cells, as mean ± S.E.

TABLE II

CARBOHYDRATE CONTENT OF YOUNG AND OLD HUMAN ERYTHROCYTE GHOSTS

	Neutral hexoses	*Fucose*	*Hexosamine*	*Sialic acid*
Number of paired experiments	10	10	10	11
Top layers[a]	59.7 ± 8.8	22.3 ± 1.8	27 ± 8.0	18.7 ± 1.4
Bottom layers[a]	49.9 ± 5.9	19.4 ± 1.0	20.9 ± 5.0	16.7 ± 1.3
P of the difference observed between top and bottom layers	<0.05	<0.01	< 0.01	0.01
Carbohydrate content of the bottom layer/carbohydrate content of top layer × 100	84	87	77	89

[a]Results are expressed in mg of carbohydrate per g of protein.

TABLE III

CARBOHYDRATE CONTENT OF SURFACE GLYCOPEPTIDES RELEASED FROM YOUNG AND OLD HUMAN ERYTHROCYTES AFTER INCUBATION WITH PRONASE

	Neutral hexoses	*Fucose*	*Hexosamine*	*Sialic acid*
Number of paired experiments	10	6	10	11
Top layers[a]	176 ± 19	24.8 ± 3.3	101 ± 12	150 ± 8
Bottom layers[a]	133 ± 15	16.4 ± 1.5	72 ± 9	104 ± 8
P of the difference observed between top and bottom layers	<0.001	< 0.01	< 0.001	0.001
Carbohydrate content of the bottom layer/carbohydrate content of top layer × 100	76	66	71	69

[a]Results are expressed in μg of carbohydrate per 10^{10} cells.

intact, young erythrocytes under the same experimental conditions (see Table III). Incubation of old and young erythrocytes with neuraminidase prior to Pronase digestion gave similar results (after correction for interference of sialic acid in the orcinol reaction); the neutral hexoses content of surface glycopeptides released from young erythrocytes by sequential neuraminidase and Pronase incubation was 164 ± 20 μg per 10^{10} cells; the neutral hexoses content of surface glycopeptides released from old erythrocytes under the same experimental conditions was: 124 ± 20 μg per 10^{10} cells (p of the difference observed between top and bottom < 0.05, $n = 5$).

The amount of sialic acid released from young erythrocytes by neuraminidase was similar to that released as soluble glycopeptides by Pronase. On the other hand, the amount of sialic acid released as soluble glycopeptides from old erythrocytes by Pronase incubation was significantly lower than that released as the free sugar by neuraminidase (see Table IV).

TABLE IV

PRONASE AND NEURAMINIDASE ACTION ON YOUNG AND OLD ERYTHROCYTES

	Sialic acid released after neuraminidase incubation[a]	*Sialic acid released after Pronase incubation*[a]	*P of the differences observed*
Number of experiments	12	11	
Top layer	160 ± 12	150 ± 8	N.S.
Bottom layer	124 ± 8.5	104 ± 8	< 0.01
P between top and bottom layers	0.001	0.001	
Percentage (%) of the difference between top and bottom layers	22	31	

[a]Results are expressed in μg of sialic acid released per 10^{10} cells.

TABLE V

CARBOHYDRATE CONTENT OF SURFACE GLYCOPEPTIDES RELEASED FROM YOUNG AND OLD ERYTHROCYTES AFTER INCUBATION WITH TRYPSINE

	Neutral hexoses	*Fucose*	*Hexosamine*	*Sialic acid*
Number of paired experiments	8	8	8	8
Top layers[a]	82.5 ± 8	23.4 ± 4.5	82.5 ± 8.7	65.5 ± 17.0
Bottom layers[a]	59 ± 2.5	17.5 ± 3.7	66.0 ± 10.0	38.5 ± 16.0
P of the difference observed between top and bottom layers	< 0.001	0.001	0.02	0.001
Carbohydrate content of the bottom layer/carbohydrate content of top layer × 100	72	75	80	59

[a]Results are expressed in μg of carbohydrate per 10^{10} cells.

Surface glycopeptides released from intact, old erythrocytes by proteolytic digestion with trypsin contained 72, 75, 80, and 59% of the neutral hexoses, fucose, hexosamine, and sialic acid content, respectively, of the glycopeptides released from young intact erythrocytes under the same experimental conditions (see Table V). Trypsin released less than one half of the hexoses and sialic acid content from old and young cells, as compared with Pronase digestion, but almost the same proportions of fucose and hexosamine.

Fractionation of surface glycopeptides from old and young, intact erythrocytes by gel filtration. — Elution with bidistilled water, from a Sephadex G-25 column, of the Pronase-digested, soluble glycopeptides from both old and young erythrocytes gave six peaks containing glycopeptides. No significant difference could be observed between the elution patterns of these two samples (Figs. 1 and 2). However, the material from the young cells that was eluted with the void volume (Peak I) contained a greater proportion of sialic acid than did the comparable fraction obtained from the old cells. The ratio of sialic acid to neutral hexoses (Sia/Neuhex) (w/w) of Peak I was 0.85:1 for the material obtained from the young cells and 0.75:1 for the samples from the old cells. The elution profiles of Figs. 1 and 2 represent one experiment; similar profiles were obtained in five separate experiments.

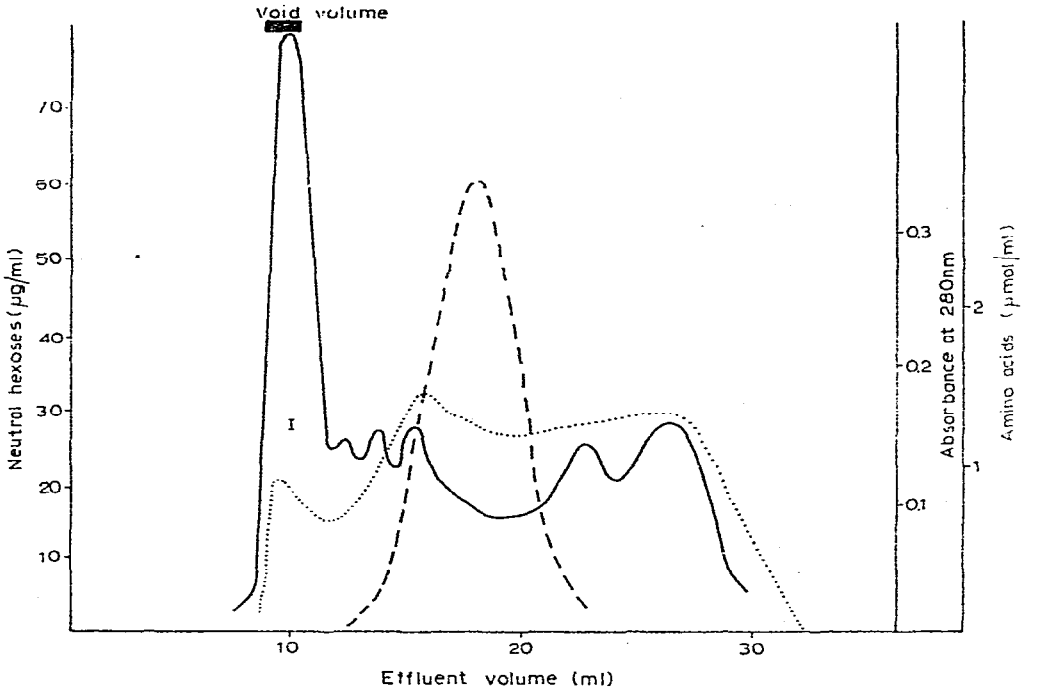

Fig. 1. Elution with bidistilled water, from a 0.9 × 30 cm column of Sephadex G-25, at 4°, of material removed from young erythrocytes by Pronase: ———; neutral hexoses, — — —, amino acids; and ·····, absorbance at 280 nm.

Elution with bidistilled water, from a Sephadex G-25 column, of the trypsin-digested, soluble glycopeptides gave two peaks containing glycopeptides in the samples from old, control, and young erythroctes. The most striking difference between the elution profile of the material from young cells and that from old cells (Figs. 3 and 4) was the decrease in content of neutral hexoses of Peak II in the effluent from old cells. By contrast, the material from young and control cells was eluted as a major glycopeptide peak with a similar elution-volume. The material from young cells that was eluted with the void volume (Peak I, Fig. 3) contained a far greater proportion of sialic acid than did the comparable fraction obtained from old cells (Peak I, Fig. 4). The ratio Sia/Neuhex of Peak I was 1.43:1 for the material obtained from the young cells and 0.81:1 for the old-cell samples; 90% of the sialic acid content of the tryptic glycopeptides was eluted, from each cell line, in the void volume, 10% in Peak II. The elution profiles in Figs. 3 and 4 represent one experiment; similar profiles were obtained in three separate experiments.

Elution with bidistilled water, from a Sephadex G-50 column, of the tryptic-digested, soluble glycopeptides gave three neutral hexose-containing peaks (I, II, III, Fig. 5) for the sample from young erythrocytes. By contrast, the material from old cells gave only two neutral hexose-containing peaks (I, II, Fig. 6). The material from young cells that was eluted with the void volume (Peak I, Fig. 5) contained a

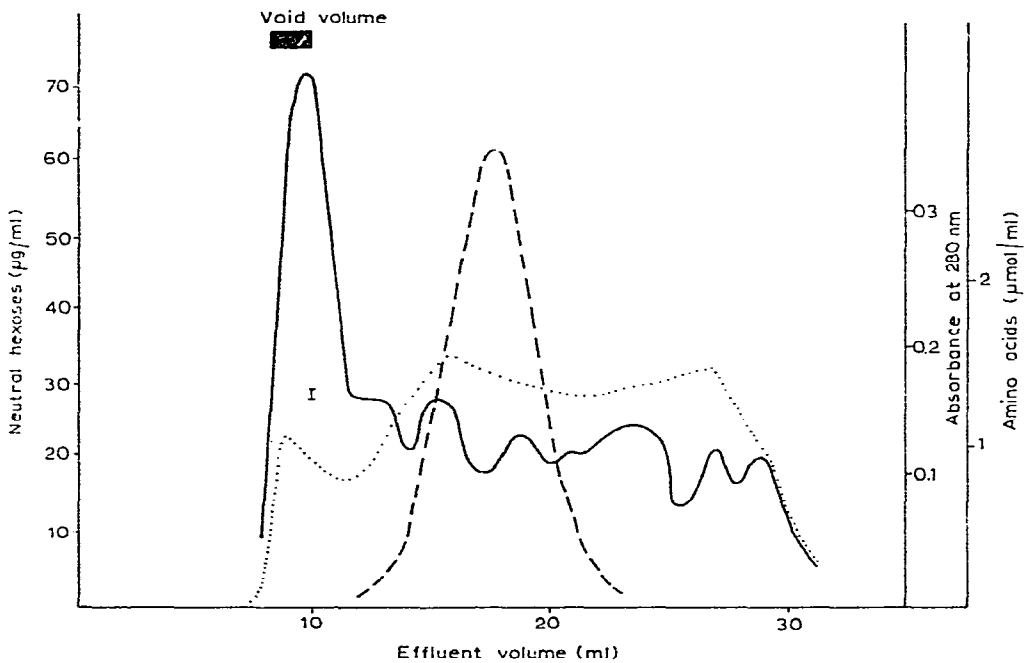

Fig. 2. Elution with bidistilled water, from a 0.9 × 30 cm column of Sephadex G-25, at 4°, of material removed from old erythrocytes by Pronase: ———, neutral hexoses; — — —, amino acids; and ·····, absorbance at 280 nm.

far greater proportion of sialic acid than did the comparable fraction obtained from the old cells (Peak I, Fig. 6). The ratio Sia/Neuhex for this material (Peak I) was 1:1 for the young cells (Fig. 5) and 0.55:1 for the old-cell sample (Fig. 6). The elution profiles in Figs. 5 and 6 represent one experiment. Similar profiles were obtained in two separate experiments.

DISCUSSION

The results reported here show that sialic acid is not the only membrane carbohydrate component that is decreased during *in vivo* ageing of human erythrocytes. Neutral hexoses, fucose, and hexosamine contents are also significantly decreased. The differences between the contents of the carbohydrate constituents (sialic acid, neutral hexoses, fucose, and hexosamines) of surface glycopeptides obtained by proteolytic digestion of young, intact human erythrocytes and those obtained from old, intact erythrocytes are more striking than the same differences between the membrane components. The greatest difference observed in the latter case is a 41% decrease in the sialic acid content of tryptic glycopeptides released from old, intact erythrocytes, as compared to that released from young ones. Furthermore, when the

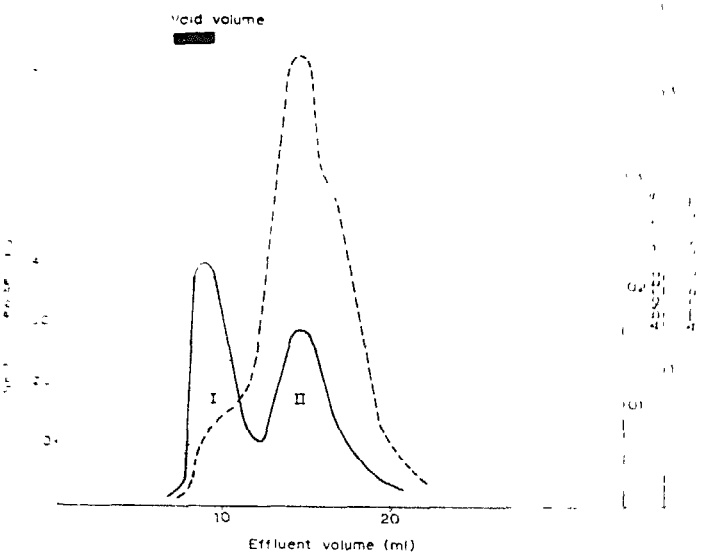

Fig. 3. Elution with bidistilled water, from 0.9 × 30 cm column of Sephadex G-25, at 4°, of material removed from young erythrocytes by trypsin ———, neutral hexoses, – – –, amino acids, and absorbance at 280 nm.

surface-membrane glycopeptides were examined by chromatography on Sephadex G-25 after trypsin digestion, Peak II (Fig. 3) eluted from the young- and control-cells material showed a large amount of neutral hexoses. By contrast, the corresponding Peak II eluted from the old-cells material (Fig. 4) was observed as a minor, neutral hexose-containing fraction. In addition, the glycopeptides that were obtained from the surface of young cells by tryptic digestion and that were eluted with the void-volume after filtration on Sephadex G-25 or Sephadex G-50 contained a far greater proportion of sialic acid than did the comparable fraction obtained from the old cells: the ratio Sia/Neuhex showed a 44–45% reduction for the material eluted from the old cells, as compared to the material eluted from the corresponding young cells.

Each column fraction probably consisted of a mixture of glycopeptides, and further fractionation of Peak I (Figs. 3 and 4) and Peak I (Figs. 5 and 6) of each type of cells was attempted. Sephadex G-100 fractionation gave several peaks that are currently under investigation, but high-voltage electrophoresis did not give consistent results because of insufficient quantities of material. Each experiment was performed with 400 ml of blood from a single donor, which gave only 10 ml of young and old cells, respectively. Trypsin treatment released 50% less of hexoses and sialic acid,

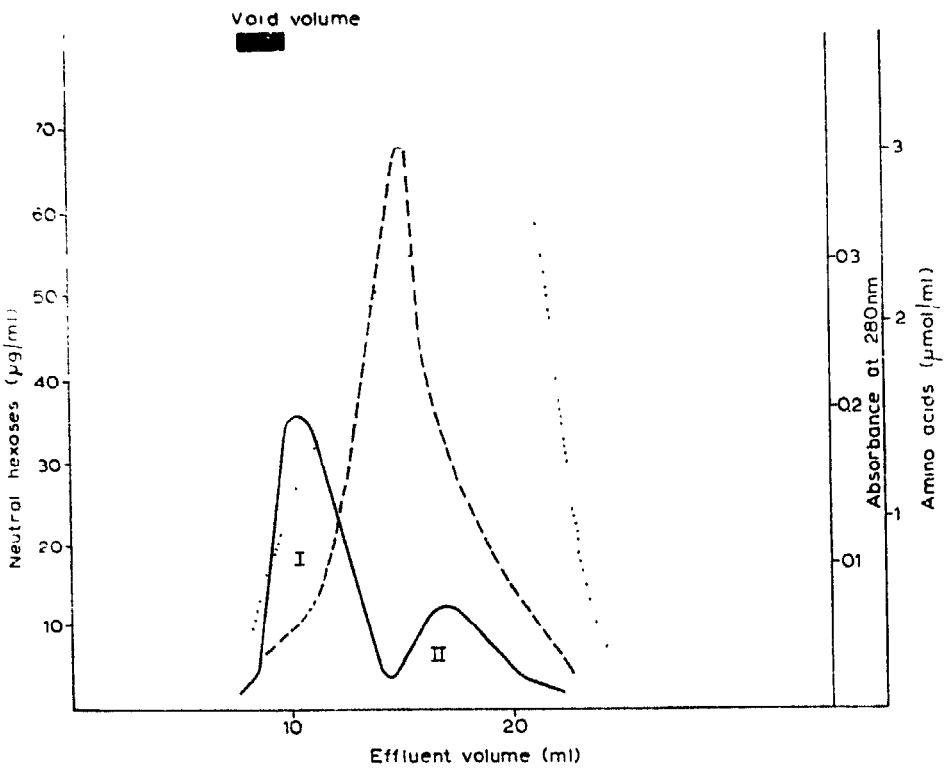

Fig. 4. Elution with bidistilled water, from a 0·9 × 30 cm column of Sephadex G-25, at 4°, of material removed from old erythrocytes by trypsin: ———, neutral hexoses; – – –, amino acids; and ·····, absorbance at 280 nm.

from old and young cells, than Pronase digestion but almost the same proportion of fucose and hexosamines. These results do not agree with the known properties of glycoproteins of the red-cell membrane. Steck and Dawson have identified a multiplicity of glycoproteins heretofore unrecognized in the erythrocyte membrane[22]. These glycoproteins, detected by treatment with D-galactose oxydase, were specifically labelled in the D-galactose and *N*-acetyl-D-galactosamine residues by reduction with alkaline borotritide[22]. The sialoglycoproteins[23] contribute 61% of the total membrane carbohydrates, a minor glycoprotein[22,23] ~10% and the glycosphingolipids[24,25] another 7%. This leaves approximatively 22% of the membrane carbohydrates associated with other glycoproteins. The almost identical proportions of fucose and hexosamine of trypsin-digested and Pronase-digested glycopeptides could be explained by the composition of these heretofore unrecognized glycoproteins.

The results reported here can be compared with those obtained on membranes of young and old rabbit erythrocytes. During *in vivo* ageing of rabbit erythrocytes, a significant reduction of the membrane sialic acid and neutral hexose content was observed, but the fucose and hexosamine content of these membranes remained unchanged[26]. Our results agree with those of Balduini *et al.*[27] which show, with ageing *in vivo*, a significant decrease of the sialic acid and galactosamine content of

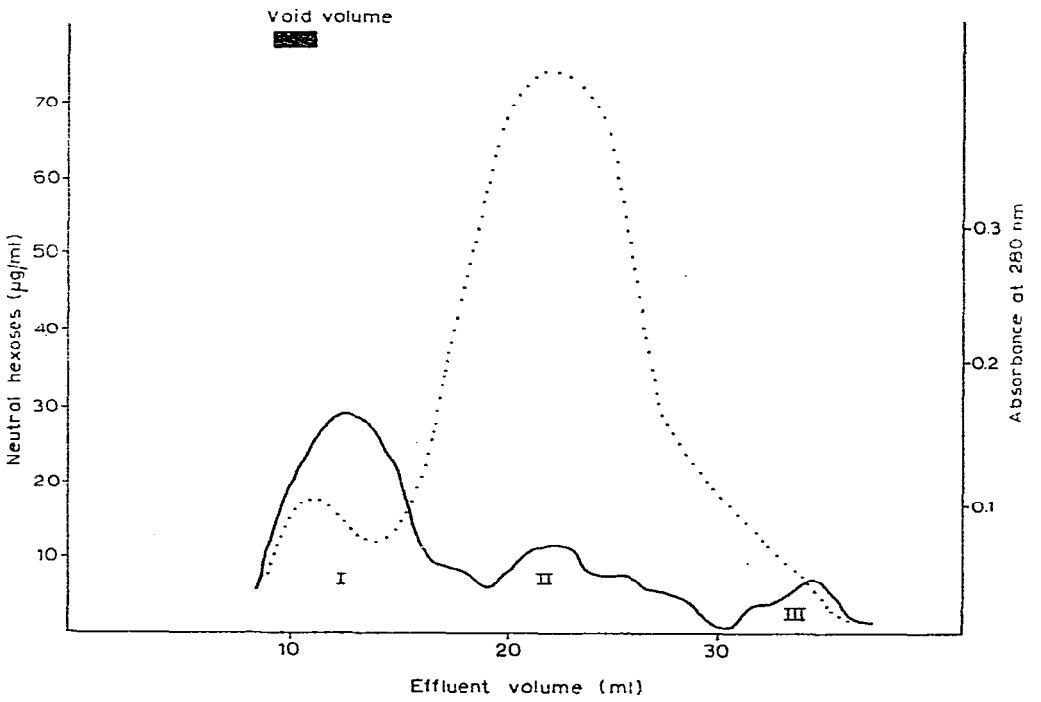

Fig. 5. Elution with bidistilled water, from a 0.9 × 30 cm column of Sephadex G-50, at 4°, of material removed from young erythrocytes by trypsin: ———, neutral hexoses; ·····, absorbance at 280 nm.

the surface glycopeptides obtained by papain digestion of old and young human erythrocyte ghosts.

Our results suggest that there is not only a decrease of the carbohydrate content of erythrocyte membrane with ageing *in vivo*, but also a reduction of its accessibility. Thus, the sialic acid residues of young erythrocytes are equally accessible to Pronase and to neuraminidase degradation, whereas those of old erythrocytes are less accessible to Pronase than to neuraminidase. These results explain previous results[8], obtained with rabbits, that demonstrated a shorter half-life for old, intact erythrocytes than for neuraminidase-treated, young erythrocytes having a similar sialic acid content. During *in vivo* ageing of human erythrocytes, the loss of sialic acid and of other sugars is associated with their eventual rearrangement which could, for instance, unmask either newly-expressed antigenic determinants or a molecular pattern of erythrocyte recognition by the catabolic cells. Indeed, Danon[28] has shown that old-rabbit erythrocytes and neuraminidase-treated erythrocytes have a greater density of antigenic sites than young ones. Furthermore, it has been demonstrated that old rat erythrocytes are more likely to form rosettes with autologous lymphocytes than young ones[8], whereas old human erythrocytes are less likely to form rosettes with autologous lymphocytes[29]. As human, autologous rosetting has been related to carbohydrate structures[29], this latter finding could be explained by the decrease of

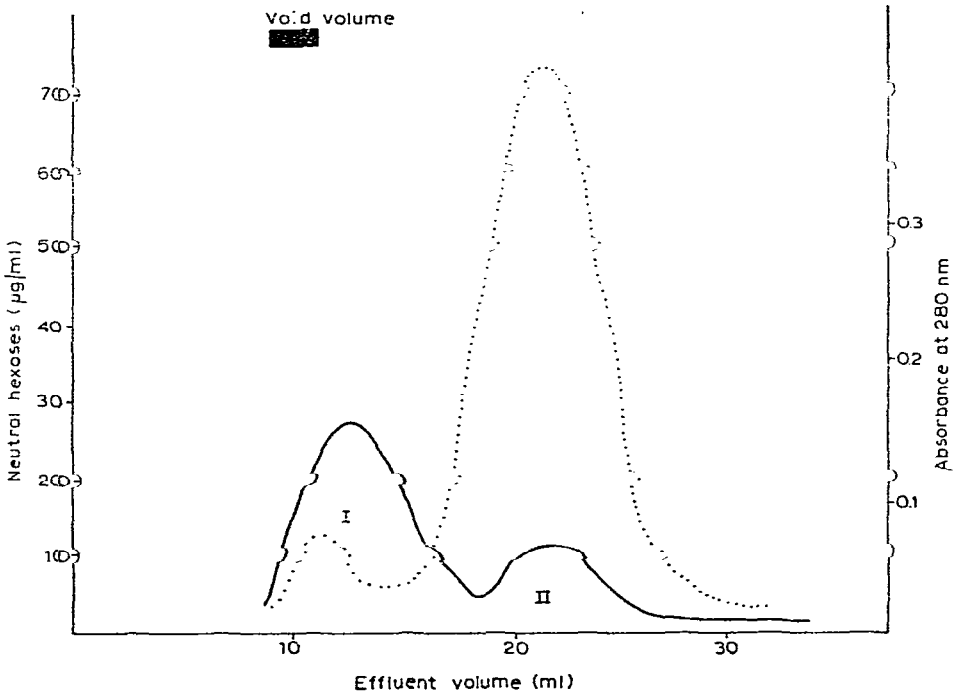

Fig. 6. Elution with bidistilled water, from a 0.9 × 30 cm column of Sephadex G-50, at 4°, of material removed from old erythrocytes by trypsin: ———, neutral hexoses; ·····, absorbance at 280 nm.

surface-membrane carbohydrates in old cells. It is possible that, on ageing, segments of the membrane are pinched off as the red cells are squeezed through small, vascular apertures[30–32], the oldest cells having the smallest surface area[33]. In this case, the total content of surface-membrane carbohydrates per cell must be decreased to a greater degree than the number per unit of surface area. The chemical loss of carbohydrate moieties during the *in vivo* ageing of human erythrocytes might be associated with the conformational rearrangement of these moieties.

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