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ALTERATIONS OF RED CELL MEMBRANES FROM PHENYLHYDRAZINE-TREATED RABBITS *

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Summary

Reticulocytosis was induced in rabbits by two methods: phlebotomy and injection of phenylhydrazine. Normal erythrocytes, reticulocytes from bled rabbits, reticulocytes from phenylhydrazine-treated rabbits, and erythrocytes treated in vitro with phenylhydrazine were compared with respect to their plasma membrane labeling by galactose oxidase and NaB^3H_4, and lactoperoxidase-catalyzed incorporation of ^{125}I. Normal erythrocyte membranes and membranes from reticulocytes of bled rabbits showed almost identical labeling patterns, the presence of 2—3 glycoproteins with moderate to low mobilities on dodecyl sulfate acrylamide gel electrophoresis. Labeling in the absence of enzyme was negligible. In contrast, the reticulocytes from phenylhydrazine-treated rabbits exhibited a large incorporation of tritium without prior treatment with galactose oxidase. Even after prereduction with unlabeled $NaBH_4$ to remove this nonspecific labeling, the labeled glycoprotein components found in normal erythrocytes were not detectable. Normal erythrocytes treated in vitro with phenylhydrazine, washed, and labeled with galactose oxidase had labeling patterns, including high nonspecific incorporation of ^3H, similar to those observed with in vivo phenylhydrazine treatment.

Solubilization of membranes with lithium diiososalicylate followed by partitioning with phenol showed that the same glycoproteins were present in normal or phenylhydrazine membranes, although only the former extract was labeled by galactose oxidase. Individual carbohydrates from the membranes

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were analyzed by gas-liquid chromatography and, in the case of hexosamines, on the amino acid analyzer. The results of these analyses indicated a slight decline in galactose content with phenylhydrazine treatment. Reticulocyte membranes from bled rabbits also showed a decrease in galactose content, although it was less pronounced.

Most of the label incorporated by nonspecific borohydride labeling of membranes from phenylhydrazine-treated animals was found associated with protein. The modified amino acids from labeled proteins are similar to those formed in reactions of oxidized lipids and proteins in model systems.

Introduction

Reticulocytes induced by injection of phenylhydrazine are known to differ from cells produced under more normal conditions, such as bleeding, both in ultrastructure [1] and in the ability of the plasma membrane to interact with transferrin [2]. The latter reaction is probably important as part of the mechanism of iron uptake by the reticulocyte [3]. We have therefore investigated differences between the cell surfaces of reticulocytes from phenylhydrazine-treated animals and those of erythrocytes or reticulocytes from bled animals using cell surface labeling procedures [4].

Experimental procedures

Materials. Trypsin (crystalline, diphenylcarbamyl chloride-treated) was obtained from Miles. Galactose oxidase of *Polyporus circinatus* was obtained from Sigma and had an activity of 75 units per mg of protein. Lactoperoxidase was also purchased from Sigma. $Na^{125}I$ and tritiated potassium borohydride (645 Ci/mol) were purchased from New England Nuclear. Phenylhydrazine hydrochloride was a product of Eastman. Standard carbohydrates were from Supelco and silylation reagents from Applied Science.White albino rabbits weighing approximately 3 kg were obtained from a local supplier.

Preparation of reticulocyte-rich blood. Reticulocytes were obtained from rabbits by four daily subcutaneous injections of a freshly prepared 2.5% solution of phenylhydrazine-HCl in 310 mosM phosphate, pH 7.0, at a dose of 12 mg per kg body weight [5]. Blood was collected 2 days after the final injection. The number of reticulocytes present in the blood samples was determined by the visual counting of one thousand cells on blood smears made after staining with New Methylene Blue. A reticulocyte count of 90% is usually obtained. In some experiments 5—10 ml of blood was removed from an ear vein during the course of the treatment to study the properties of the cells as the reticulocytosis was developing.

Reticulocyte-rich blood was also obtained from rabbits bled via an ear vein. The content of reticulocytes usually was 20% after 4 consecutive bleedings of 15 ml of blood per kg body weight on every other day. Cells thus obtained were washed three times with cold 5 mM phosphate/0.15 M saline, pH 7.4, by centrifugation for 10 min at 2200 × *g*. The plasma and buffy coat were removed by aspiration.

External labeling of glycoproteins of membrane. Tritium labeling of cell surface glycoproteins with galactose oxidase was performed according to the procedure described by Gahmberg and Hakomori [6] with slight modifications. 1 ml packed cells, 1 ml of Hank's solution, pH 7.0, and 20 μg galactose oxidase (1 mg/ml in H_2O) were mixed and incubated at room temperature for 2 h with occasional shaking. After the incubation cells were washed twice in phosphate-buffered saline, pH 8, and suspended in the same medium (50% suspension). To the cell suspension was added 0.01 ml of a freshly prepared tritiated potassium borohydride solution (1 mCi in 0.2 ml of 0.1 N NaOH). After 30 min at room temperature with occasional shaking, the cells were diluted with 30 ml of phosphate-buffered saline (pH 7.4) containing 0.1 mg of unlabeled sodium borohydride, mixed well and centrifuged. Washing by centrifugation in phosphate-buffered saline, pH 7.4, was repeated three times.

For labeling with ^{125}I catalyzed by lactoperoxidase, 1 ml of washed, packed cells was suspended in 2 ml of labeling solution containing 150 mM NaCl, 50 mM Tris, 15 μM KI, 50 μCi/ml $Na^{125}I$ and 0.7 μM lactoperoxidase, pH 7.4, at room temperature. Twenty-five aliquots of 20 μl each of 15 mM H_2O_2 in phosphate-buffered saline (pH 7.4) were added at 15 s intervals to the suspension. At the end of the incubation, 400 μl of 100 mM KI and 10 ml of cold phosphate-buffered saline (pH 7.4) were added and the suspension was allowed to incubate at room temperature for an additional 5 min, then centrifuged at 4°C. The labeled cells were washed twice with 30 ml of cold phosphate-buffered saline before hemolyzing to obtain labeled membrane. Trypsinization was performed on intact cells at a trypsin concentration of 0.1 mg/ml at 37°C for 30 min.

Preparation and purification of erythrocyte and reticulocyte membranes. Cells were lysed by suspending in 30 volumes of hypotonic phosphate solution as described previously [7] and incubated in the cold for 30 min. The cell membranes were separated by centrifugation at 20 000 × *g* for 10 min. To obtain a clean preparation of cell membranes, the ghost pellet was washed 5 times in hemolysis buffer subsequent to the hemolysis. Erythrocyte and reticulocyte membranes were separated by discontinuous sucrose gradient centrifugation [8]. Gradients were centrifuged at 4°C in an SW-27 rotor for 60 min at 25 000 rev./min in a Beckman L5-65 ultracentrifuge. Erythrocyte and reticulocyte membranes were collected at the 32% and 36% sucrose gradient interfaces, respectively, and washed twice with phosphate buffer.

Preparation of phenylhydrazine-treated erythrocytes. Washed erythrocytes from untreated rabbits were treated with phenylhydrazine hydrochloride (0.5 mg per ml of packed erythrocytes) in vitro at 37°C for 60 min in phosphate-buffered saline, pH 7.4. Prior to galactose oxidase labeling treated cells were washed three times in phosphate-buffered saline, pH 7.0.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. The cell ghosts were dissolved in 2% dodecyl sulfate and 5% β-mercaptoethanol and incubated at room temperature overnight. Red cell membranes from normal or bled rabbits were white and dissolved easily in dodecyl sulfate. Membranes from cells exposed in vitro or in vivo to phenylhydrazine were brown and sticky and dissolved only with vigorous mixing in dodecyl sulfate. Electrophoresis was performed with bromophenol blue as tracking dye in 5% or 7.5% acrylamide

gels, run and stained according to previously described methods [9]. The gels containing ^{3}H were sliced into 2 mm slices and the slices were counted in toluene scintillation fluid after NCS solubilization at 50°C overnight [10]. Gels containing ^{125}I were sliced similarly but were counted directly in a well-type gamma counter.

Labeling of membranes without prior galactose oxidase treatment for chromatographic separations. Membranes from erythrocytes or reticulocytes were suspended to 1.0 mg protein/ml in phosphate-buffered saline (pH 8.0) and treated with 200 μC of $NaB^{3}H_4$ (7.8 ml/mmol) in 0.25 ml 0.1 N KOH for 2 h at 4°C. Unlabeled $NaBH_4$ (0.25 ml of 1.0 M) was added and the mixture incubated for 1 h at room temperature and 2 h at 4°C. Samples were lyophilized and dissolved in 0.5 ml phenol/acetic acid/water (1 : 1 : 1, v/v) for chromatography.

Chromatography and amino acid separations. Labeled membranes were solubilized and chromatographed in phenol/acetic acid/water as described previously [11]. Dialyzed, lyophilized fractions were hydrolyzed in 6 N HCl or 4 N methanesulfonic acid [12]. Analysis for amino acids was performed on a single column microanalyzer system [13] equipped with an Autolab integration module. Eluate was collected at 60 s intervals and time monitored precisely by using the run-time function indicator on the integration module.

Analytical methods. Protein assays were performed according to Lowry et al. [14] using bovine serum albumin as a standard. Cholesterol was assayed using the procedure of Zlatkis et al. [15]. Sialic acid was measured using the thiobarbituric acid method [16]. Carbohydrate analysis by gas-liquid chromatography was performed essentially according to Reinhold [17]. The trimethylsilyl derivatives of the methyl glycosides were separated and analyzed on a 6 foot, 1/4 inch (outer diameter) column packed with 3% OV-1 on Chrome-Q (100—120 mesh, Applied Science). The analysis was performed using a Hewlett-Packard research model 5750B chromatograph with a temperature program from 120°C to 250°C at 1°C per min. A computing integrator (Autolab System I) was used to measure peak areas. Recovery was monitored using mannitol as internal standard. Replicate analyses of a standard carbohydate mixture gave standard errors of 3—4% for each sugar. Analysis of purified glycoproteins (ovalbumin, human erythrocyte membrane sialoglycoprotein) gave carbohydrate compositions very similar to those reported in the literature.

Results

Protein and glycoprotein patterns of rabbit erythrocyte and reticulocyte membranes

Hamaguchi and Cleave [18] have reported that rabbit red cell membranes have glycoproteins that are only faintly visible after periodate-Schiff staining. Since a membrane glycoprotein could quite feasibly play a role in the transferrin-membrane interaction, the question of the presence of glycoproteins in reticulocytes and erythrocytes is quite important. Occasionally faint periodate-Schiff-positive bands can be seen in the middle to higher molecular weight region of gels, especially when overloaded. These bands are detected after scanning at 550 nm (Fig. 1). Figure 1a shows a scan of a normal

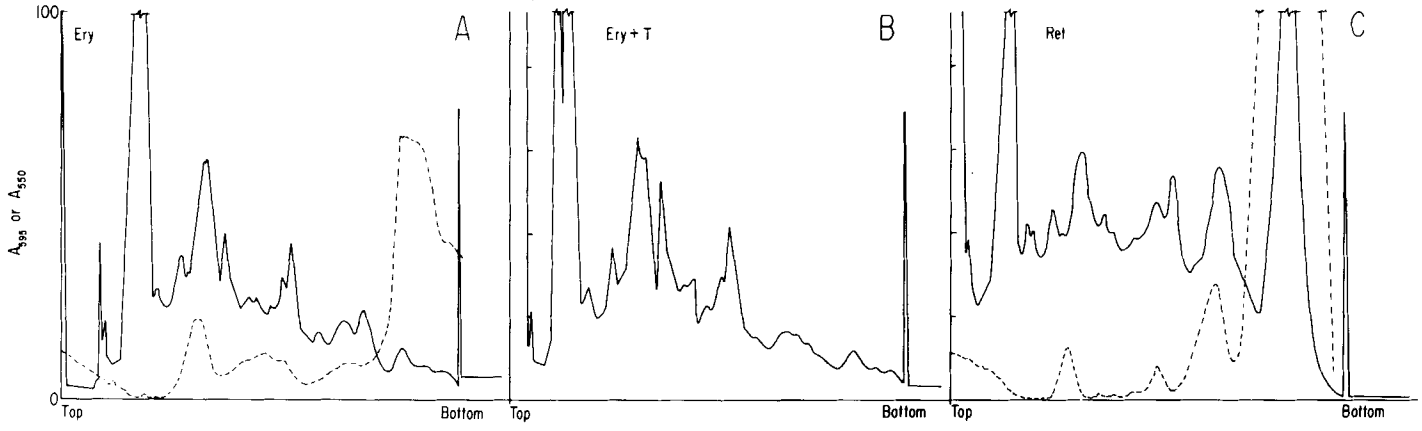

Fig. 1. Polypeptides of rabbit erythrocyte (A), trypsinized rabbit erythrocyte (B) and reticulocyte (C) membranes detected by Coomassie blue (———) and periodate-Schiff (- - - - -) staining. Reticulocytes were obtained from phenylhydrazine-treated rabbits. Acrylamide gels of dodecyl sulfate-solubilized membranes were stained by the periodate-Schiff method and scanned at 550 nm or by Coomassie blue and scanned at 595 nm. The three prominent peaks at the bottom of gel C are due to an opaque white precipitate rather than staining of the periodate-Schiff gels. The Coomassie blue gels indicate that these correspond to hemoglobin and its multimers, which show enhanced association with the reticulocyte membranes. Full scale corresponds to 2.5 and 0.3 absorbance units for the Coomassie blue and periodate-Schiff gels, respectively.

erythrocyte membrane gel stained with periodate-Schiff. It has one major and 3 smaller, overlapping bands in the protein region of the gel. The larger band running approximately with the dye marker is lipid plus glycolipid. The same major protein region band can be seen on gels from reticulocyte membranes prepared from phenylhydrazine-treated animals (Fig. 1c). However, the "absorbance" of the high intensity band at the bottom of the gel is primarily attributable to the large amount of hemoglobin in these membranes. This can easily be seen in gels are stained with Coomassie blue (Fig. 1c), where the two large, higher mobility bands are hemoglobin and (probably) a dimer of hemoglobin. Otherwise, the protein patterns of normal erythrocyte membranes and phenylhydrazine reticulocyte membranes are essentially the same. Trypsinization of the erythrocytes did not change the polypeptide patterns.

Galactose oxidase-borohydride labeling of erythrocytes and reticulocytes

Table I shows the incorporation of label into the cells which have been examined. A clearly specific labeling of erythrocytes from normal rabbits and erythrocytes or reticulocytes from bled rabbits can be demonstrated. In the case of reticulocytes from phenylhydrazine-treated rabbits and of in vitro phenylhydrazine-treated erythrocytes there is a high level of nonspecific incorporation, presumably resulting from the borohydride reduction of oxidized lipids and other species in the membrane. This incorporation can be lowered by prereducing these erythrocytes or reticulocytes with unlabeled borohydride prior to the galactose oxidase modification. In the case of the phenylhydrazine-treated erythrocytes the nonspecific labeling can thus be eliminated. Some nonspecific labeling still remains in membranes from the reticulocytes of phenylhydrazine-treated rabbits. In the phenylhydrazine-treated erythrocyte less than 10% of the incorporated counts are due to galactose oxidase, suggesting that the enzyme may be inhibited by products of this treatment. This effect is not seen in reticulocytes from phenylhydrazine-

TABLE I

INCORPORATION OF RADIOACTIVITY INTO ERYTHROCYTE AND RETICULOCYTE MEMBRANES

Labeling was obtained by reduction with KB^3H_4 after treatment with or without galactose oxidase.

Animal	Cell type	Pre-reduced	Galactose oxidase	Incorporation (cpm/μg protein)
Normal	Erythrocyte	—	+	596
		—	—	4
	Phenylhydrazine-treated erythrocyte	—	+	623
		—	—	563
		+	+	659
		+	—	4
Bled	Erythrocyte	—	+	693
		—	—	11
	Reticulocyte	—	+	634
		—	—	10
Phenylhydrazine-treated	Reticulocyte	—	+	655
		—	—	360
		+	+	407
		+	—	113

treated rabbits. The difference probably resides in the greater severity of the direct treatment with phenylhydrazine.

Electrophoresis profiles of tritium labeled components of erythrocytes and reticulocytes

Since the amount of label incorporated into the membranes is relatively the same for the different cell preparations, we examined the labeling profiles obtained by dodecyl sulfate electrophoresis of membranes from the treated cells. Erythrocytes from bled rabbits show the labeling profile in Fig. 2, which is essentially the same as that obtained from labeled erythrocytes from normal, untreated rabbits. A complex pattern of at least five bands is observed. These have been denoted as I—V. The reticulocytes from bled rabbits show a similar pattern of five bands (Fig. 3). The glycoprotein nature of bands I-III is indicated by their susceptibility to trypsin treatment of intact cells, which hardly alters the Coomassie blue pattern of electrophoresed membranes (Fig. 1b).

The labeling of reticulocytes from phenylhydrazine-treated animals shows significant differences. If the cells are not prereduced with unlabeled borohydride, the labeled membranes exhibit a high nonspecific incorporation into

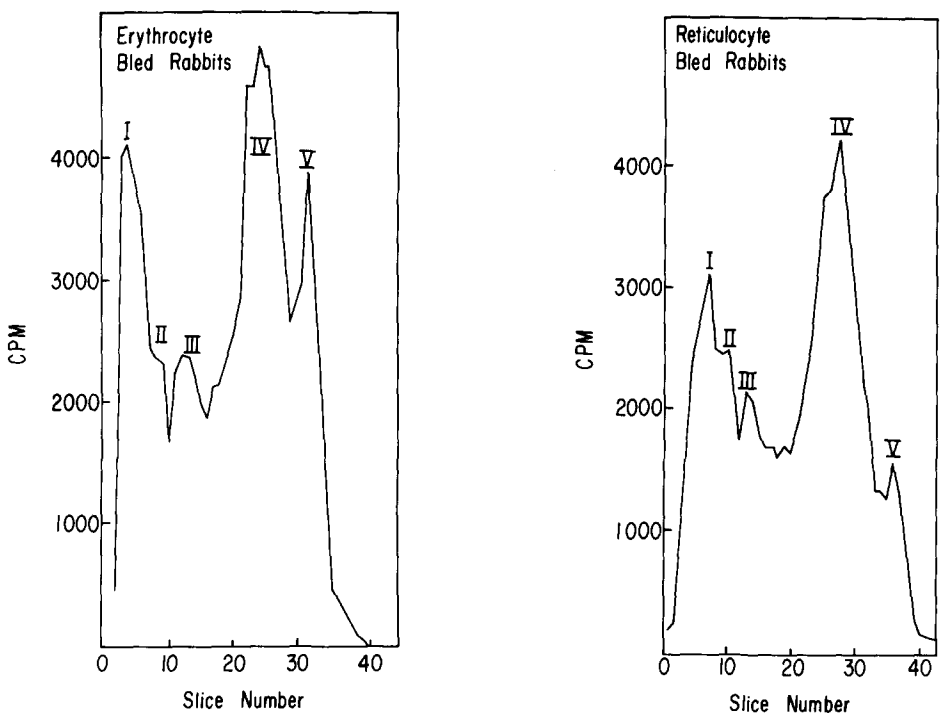

Fig. 2. Galactose oxidase labeling of erythrocytes from bled rabbits. Cells were labeled as described in Experimental Procedures. Membranes were isolated by density gradient centrifugation and subjected to electrophoresis in dodecyl sulfate. Gels were sliced and counted.

Fig. 3. Galactose oxidase labeling of reticulocytes from bled rabbits. Reticulocyte membranes were separated from erythrocyte membranes of labeled cell population by density gradient centrifugation.

three rapidly moving bands both with and without galactose oxidase treatment (Fig. 4). Prior reduction before galactose oxidase treatment reduces these bands to an acceptable level, but the major labeled peaks are still in the lower region of the gel near the tracking dye. There is virtually no labeling in the region of the glycoprotein bands shown previously (Fig. 3) for normal erythrocytes or reticulocytes. However, these glycoproteins are still present because after extraction of both erythrocyte and phenylhydrazine reticulocyte membranes with lithium diiodosalicylate (LIS) according to Marchesi and Andrews [19], the aqueous phases containing the glycoproteins show a single periodate-Schiff band of similar intensity and migration for each. However, only the erythrocyte membrane extract is labeled (Fig. 5).

These results suggest that phenylhydrazine oxidation of the reticulocytes may cause a surface change which decreases the reactivity of glycoproteins toward the galactose oxidase. To check this possibility rabbit erythrocytes were incubated directly in vitro with phenylhydrazine. As shown in Fig. 6, this procedure also reduces the labeling of glycoprotein components I-III, although the labeling was not completely eliminated. Labeling of these glycoproteins can be eliminated by incubation with higher concentrations of phenylhydrazine, but the nonspecific incorporation is unacceptably high even after prereduction with unlabeled borohydride under the conditions we have investigated to date. The decreased glycoprotein labeling of reticulocytes from phenylhydrazine-treated rabbits was not due to a much smaller amount of membrane protein

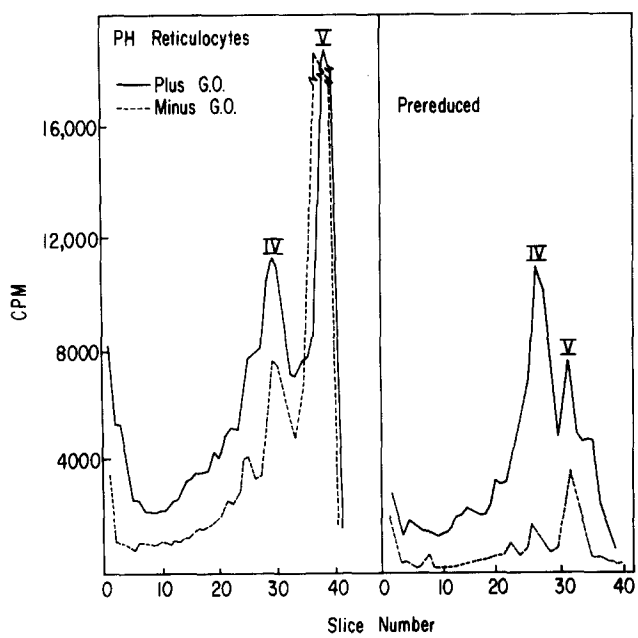

Fig. 4. Radioactivity profiles of reticulocyte membranes from phenylhydrazine-treated rabbits whose erythrocytes were labeled with boro[^{3}H]hydride with (———) or without (- - - - -) prior galactose oxidase treatment. Figure on left shows reticulocytes not pre-reduced with unlabeled borohydride, while that on right shows cells that were prereduced. Prereduction was performed by treatment of 1.0 ml of packed cells with 0.2 mg of unlabeled $NaBH_4$ in 1.0 ml phosphate-buffered saline (pH 8.0).

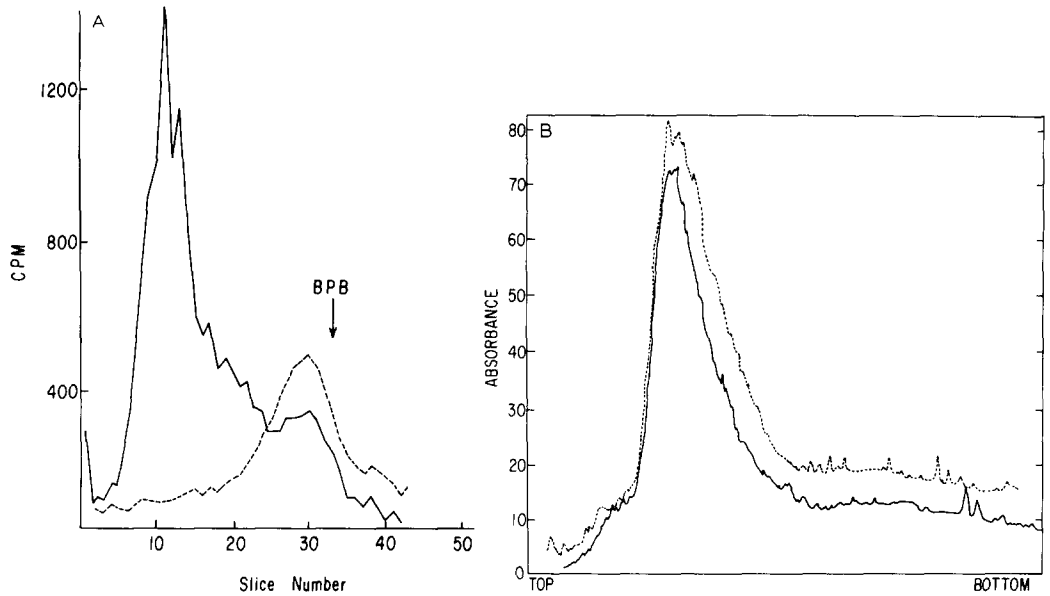

Fig. 5. Radioactivity (A) and periodate-Schiff (B) profiles of aqueous phase from lithium diiodosalicylate extract of galactose oxidase labeled membranes. (A) Patterns from bled rabbits before (———) and after (- - - - -) treatment with trypsin. No label was detected in the corresponding extract from phenylhydrazine-treated rabbits. (B) Patterns from erythrocyte membranes of bled rabbits (———) and reticulocyte membranes of phenylhydrazine-treated rabbits (. . . . .).

applied to the gels, since control gels stained with Coomassie blue showed approximately the same quantities of the stained proteins (cf. Fig. 1). The decreased labeling also cannot be due solely to competition of the oxidized products for borohydride, since prereduction does not cause any substantial increases in glycoprotein labeling.

Lactoperoxidase-catalyzed labeling of erythrocytes and reticulocytes

Lactoperoxidase catalyzes the iodination of any protein on the cell surface having accessible tyrosyl residues [20]. The proteins (glycoproteins) modified by lactoperoxidase and galactose oxidase need not be the same. Thus we decided to examine the labeling of these same kinds of cells by lactoperoxidase. Erythrocytes or reticulocytes from bled rabbits, incubated under conditions leading to high incorporation of label, show a simpler labeling pattern than the same cells treated with galactose oxidase [4] but no significant differences between erythrocytes and reticulocytes. If the incubation conditions are altered to give lower total incorporation, multiple labeled bands are seen on electrophoresis of normal red cell membranes, but there is a decreased labeling of the slower moving components of reticulocyte membranes from phenylhydrazine-treated rabbits. These studies support the galactose oxidase experiments in that they also point to altered properties of the membranes of phenylhydrazine-treated animals.

Carbohydrate analysis of membranes

It is possible that the decreased specific labeling of glycoproteins could be

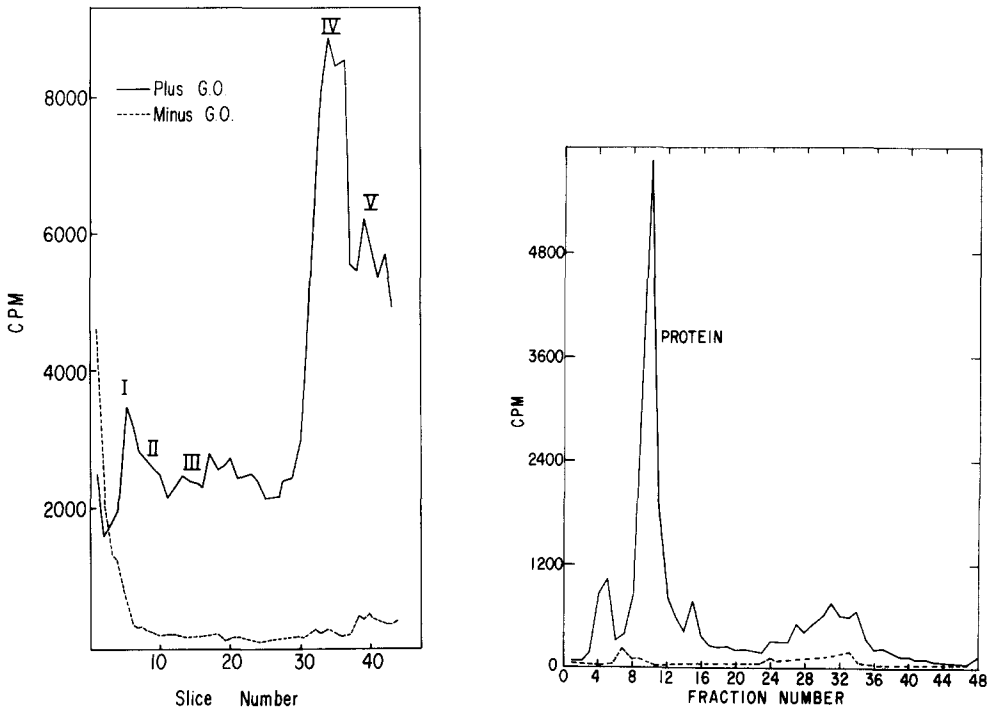

Fig. 6. Radioactivity profiles of rabbit erythrocyte membranes from erythrocytes treated in vitro with phenylhydrazine and reduced with NaB^3H_4. (- - - - - -) control (no galactose oxidase); (———) plus galactose oxidase.

Fig. 7. Separation of protein and lipid components from borohydride-labeled membranes of cells from bled (- - - - - -) and phenylhydrazine-treated (———) rabbits. Cells were treated with radioactive borohydride without prior galactose oxidase treatment. Membranes were isolated, dissolved in phenol/acetic acid/water and chromatographed as previously described [14].

attributed to a lesser amount of galactose (or *N*-acetylgalactosamine) in red cell membranes from phenylhydrazine-treated animals. Carbohydrate analyses of the membranes are shown in Table II. There is a decrease in galactose content which could be detected as the degree of reticulocytosis induced by phenylhydrazine increased, but it is uncertain if this is sufficient to affect the galactose oxidase labeling patterns. The content of *N*-acetylgalactosamine residues in the membrane at any time was much less than the galactose and is unlikely to influence the results.

The sialic acid content of the membranes was very small (less than 30 nmol per mg cholesterol), and thus it is very unlikely that the galactose residues were protected from galactose oxidase by sialic acid. In fact, prior neuraminidase treatment did not affect the galactose oxidase labeling pattern of normal rabbit erythrocytes. Also, most of the thiobarbituric acid-positive material (after periodate oxidation and assay according to Warren [16]) detected in membranes from phenylhydrazine-treated rabbits was probably a chromogen arising from oxidized lipids [21,22], as its visible absorption spectrum was different from that arising from sialic acid. Despite the demonstration of the probable presence of oxidized lipids, most of the radioactivity in phenylhydrazine mem-

TABLE II

CARBOHYDRATE AND PROTEIN ANALYSIS OF RED CELL MEMBRANES FROM NORMAL, PHENYLHYDRAZINE-TREATED AND BLED RABBITS

Sugar analyses were done on 3—5 samples from two separate preparations by gas-liquid chromatography as described in Methods. Amino sugar values were also obtained by analysis on an amino acid analyzer. Values are expressed as nmol sugar/mg cholesterol. Values reported are the mean ± standard error of the mean. N.D., not detected.

Membrane sample	Fucose	Mannose	Galactose	Glucose	*N*-Ac-glucosamine	*N*-Ac-galactosamine	Sialic acid
Normal erythrocyte	55 ± 10	284 ± 4	1339 ± 174	378 ± 70	851 ± 36	213 ± 62	84 ± 36
Phenylhydrazine reticulocyte	162 ± 45	363 ± 25	1076 ± 123	N.D.	719 ± 99	202 ± 57	N.D.
Bled reticulocyte	25 ± 7	232 ± 54	1269 ± 244	334 ± 79	1645 ± 418	142 ± 32	101 ± 32

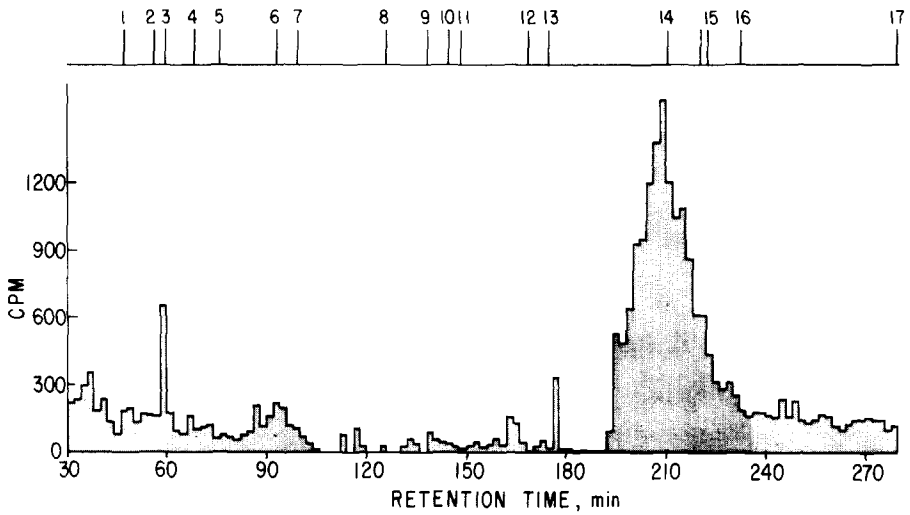

Fig. 8. Elution of radioactive products from hydrolyzed protein fraction (Fig. 7) of borohydride-labeled membranes from cells of phenylhydrazine-treated rabbits. Amino acids of the protein hydrolysate were separated on a single column, small bore microanalyzer. Effluent fractions were collected at 60 s intervals and counted. Lines and numbers at the top of the graph show the elution positions for the common amino acids: 14-histidine; 15-lysine; 16-ammonia. The unlabeled line before lysine is the position of the new ninhydrin positive peak.

branes reduced with NaB^3H_4 is not extractable with a mixture of chloroform and methanol.

Borohydride-labeled products from phenylhydrazine-treated animals

To determine the types of products obtained during the oxidation reactions, membranes were isolated from rabbits treated for various intervals with phenylhydrazine. Protein and lipid components were separated by chromatography in phenol/acetic acid/water (Fig. 7). Most of the radioactivity is present in the protein. Amino acids, from the hydrolyzed protein were fractionated by ion exchange chromatography. The radioactivity profile of the eluate shows a broad peak in the region of histidine elution, indicative of multiple labeled products (Fig. 8). However, only one prominent new product is noted on the ninhydrin profiles, and it elutes just prior to lysine (unmumbered marker). The differences in the ninhydrin and radioactivity profiles are as yet unexplained.

Discussion

Using galactose oxidase-borohydride or lactoperoxidase labeling no significant differences in surface components could be shown between erythrocytes and reticulocytes from bled rabbits. Differences were demonstrated, however, between reticulocytes from phenylhydrazine-treated rabbits and erythrocytes from normal or bled rabbits. Since these reticulocytes had clearly been subjected to oxidative stress, as evidenced by the presence of Heinz bodies, it appears that the surface differences might have resulted from oxidation changes in the cell membrane.

Products deriving from the oxidative modification of the membranes can be

detected by borohydride labeling, and are found associated predominantly with the membrane protein fraction. The low molecular weight of the labeled products suggests that they may be hemoglobin subunits and their multimers rather than intrinsic membrane proteins. Goldberg and Stern [23] have suggested that lipid peroxidation does not occur in cells which have been treated to produce superoxide. However, the analysis of malonaldehyde may not be able to detect lipid peroxidation if the oxidized lipid intermediates rapidly react with proteins. The analysis of stable oxidation products may be more useful in analyzing perturbations caused by oxidant stress of membranes.

Acknowledgements

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References

1 Bessis, M. (1973) Living Blood Cells and Their Ultrastructure, p. 215, Springer-Verlag, New York
2 Workman, Jr., E.F., Graham, G. and Bates, G.W. (1975) Biochim. Biophys. Acta 399, 254—264
3 Walsh, R.J., Thomas, E.D., Chow, S.K., Fluharty, R.G. and Finch, C.A. (1949) Science 110, 396—398
4 Carraway, K.L. (1975) Biochim. Biophys. Acta 415, 379—410
5 Borsook, H., Deasy, C.L., Haagen-Smit, A.J., Keighley, G. and Lowry, P.H. (1952) J. Biol. Chem. 196, 669—694
6 Gahmberg, C.G. and Hakomori, S.-I. (1973) J. Biol. Chem. 248, 4311—4317
7 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119—130
8 Percy, A.K., Schmell, E., Earles, B.J. and Lennarz, W.J. (1973) Biochemistry 12, 2456—2461
9 Carraway, K.L., Colton, D.G., Shin, B.C. and Triplett, R.B. (1975) Biochim. Biophys. Acta 382, 181—192
10 Carraway, K.L. and Shin, B.C. (1972) J. Biol. Chem. 247, 2102—2108
11 Carraway, K.L., Triplett, R.B., Summers, J. and Ellis, D.E. (1972) Biochim. Biophys. Acta 266, 484—493
12 Liu, T.Y., Simpson, R.J. and Neuberger, M.R. (1976) J. Biol. Chem. 251, 1936—1940
13 Liao, T.-H., Robinson, G.W. and Salnikow, J. (1973) Anal. Chem. 45, 2286—2288
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Zlatkis, A., Zak, B. and Boyle, A.J. (1953) J. Lab. Clin. Med. 41, 486—493
16 Warren, L. (1959) J. Biol. Chem. 234, 1971—1975
17 Reinhold, V.N. (1972) Methods Enzymol. 25, 244—249
18 Hamaguchi, H. and Cleve, H. (1972) Biochem. Biophys. Res. Comm. 477, 459—464
19 Marchesi, V.T. and Andrews, E.P. (1971) Science 174, 1247—1248
20 Morrison, M., Bayse, G. and Danner, D.J. (1970) in Biochemistry of the Phagocytic Process (Soultz, J., ed.), p. 51, North-Holland, Amsterdam
21 Grinna, L.S. and Barber, A.A. (1973) Biochem. Biophys. Res. Comm. 55, 773—779
22 Barber, A.A. (1966) Lipids 1, 146—151
23 Goldberg, B. and Stern, A. (1976) J. Biol. Chem. 251, 6468—6470