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Red Cell Hydrolases: Glycosidase Activities in Human Erythrocyte Plasma Membranes

H. Bruce Bosmann*

Department of Pharmacology and Toxicology, University of Rochester, School of Medicine and Dentistry, Rochester, New York 14620

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Summary. Human erythrocyte plasma membranes were found to contain the following glycosidases: α- and β-glucosidase, α- and β-galactosidase, α- and β-fucosidase, β-N-acetylglucosaminidase, β-N-acetylgalactosaminidase, β-xylosidase and α-mannosidase. All the enzymes except β-fucosidase had activity interpreted to be on the external surface of the plasma membrane. The enzymes had optimum pH values of 4.2 to 5.0 and temperatures of 37 to 40 °C. The enzymes were not greatly activated by divalent cations but Hg^{++} and Pb^{++} were inhibitory. The enzyme extract of the human erythrocyte plasma membranes liberated carbohydrate from intact red cells, which lead to the speculation that the glycosidases might function to modify the erythrocyte plasma membrane.

The prevention of hemolysis *in vitro* of the red cell membrane is of importance in the storage of blood-banked whole human blood. Attention has recently been focused on the presence of various degradative enzymes in the erythrocyte plasma membrane, enzymes which perhaps are in part capable of degrading portions of the erythrocyte membrane. Initially, Morrison and Neurath (1953) reported that proteolytic enzymes were present in the erythrocyte, and recently Moore, Kocholaty, Cooper, Gray and Robinson (1970) have reported on the isolation of a proteinase from human erythrocyte membranes. Finally, a membrane acid phosphatase has been identified in rabbit erythrocyte membranes (Berry & Hochstein, 1969). This communication describes yet another group of degradative enzymes – the glycosidases – associated with the plasma membrane of the human erythrocyte.

The glycosidases are thought to be responsible for the hydrolysis of bonds between sugars (hexoses, pentoses and hexosamines, primarily) and

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amino acids (primarily asparagine, but also serine, threonine and hydroxylysine), between sugars and lipid moieties, and between adjacent sugars primarily in glycoproteins, glycolipids, oligo- and polysaccharides and glycosaminoglycans (Bosmann & Merritt, 1969).

The glycosidases studied in this report were N-acetyl-β-glucosaminidase (E.C. 3.2.1.30), α-D-glucosidase (E.C. 3.2.1.20), β-D-glucosidase (E.C. 3.2.1.21), α-D-galactosidase (E.C. 3.2.1.22), β-D-galactosidase (E.C. 3.2.1.23), α-L-fucosidase (E.C. 3.2.1.–), β-L-fucosidase (E.C. 3.2.1.–), β-D-xylosidase (E.C. 3.2.1.37), α-D-mannosidase (E.C. 3.2.1.24), and N-acetyl-β-D-galactosaminidase (E.C. 3.2.1.–). In addition, the erythrocyte plasma membrane locations of acid phosphatase (E.C. 3.1.3.2), β-glucuronidase (E.C. 3.2.1.31) cathepsin (E.C. 3.4.4.9), trypsin (E.C. 3.4.4.4), general proteolytic activity and collagenase (E.C. 3.4.4.19) were determined.

Materials and Methods

Blood

Units of acid-citrate-dextrose blood from young healthy male donors were used. Blood was used immediately after drawing.

Preparation of Erythrocyte Membranes

Erythrocyte membranes were prepared either exactly by the dialysis method of Schrier (1963) or exactly by the osmotic lysis method of Weed, Reed and Berg (1963) and Van Stevenurck, Weed and Rothstein (1965). Both methods give similar results, and the work reported herein is based on membranes prepared by the latter method.

Fractions Tested for Enzyme Activity

Whole blood cells washed six times with isotonic saline, erythrocyte membranes extracted for 30 strokes with a Ten Broeck Homogenizer in 0.1% Triton X-100, a 20,000 × *g* supernatant of the 0.1% Triton X-100 extract, and a Sephadex G-100 column eluate were tested for enzyme activity.

Sephadex G-100 Chromatography

Routinely, erythrocyte membranes were homogenized in five volumes of 0.1% Triton X-100 for 30 strokes with a Ten Broeck homogenizer at 4 °C and then centrifuged at 20,000 × *g* for 20 min; the supernatant was used as the starting material for purification of glycosidases by Sephadex G-100 column chromatography. Sephadex G-100 was prepared in 0.1% Triton X-100 as recommended by the manufacturers. The 20,000 × *g* supernatant of the 0.1% Triton X-100 extract was placed on the gel column. The column (83.0 × 3.4 cm) was eluated at 4 °C with 0.1% Triton X-100 with a flow rate of 10 ml/hr; fractions of 6 ml were collected. These fractions were kept at 4 °C during collection and assayed immediately.

Protein

Total protein in any of the various enzyme preparations was determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Crystalline bovine serum albumin was used as a standard. In each instance of protein determination, the sample for analysis was precipitated with 30% trichloracetic acid (TCA), and washed twice with 10% TCA and once with ethanol: diethyl ether (2:1 v/v); the resultant insoluble material was dissolved in NaOH for analysis. Protein in column eluents was determined by measuring the optical density at 280 nm against a 0.1% Triton X-100 blank.

Enzyme Assay

The amount of a given glycosidase, acid phosphatase, or β-glucuronidase activity at pH 4.3 in any of the various enzyme preparations was determined using the p-nitrophenyl derivative in the following manner for the optimal conditions (Bosmann, 1969; Bosmann & Hemsworth, 1970*a*). A 50-μliter portion (in some instances of high activity, 10 μliters) of the 0.1% Triton X-100 enzyme extract of the fraction to be tested or the whole blood (50 μliters of 0.1% Triton X-100 was added to these assays; the blood cells were not homogenized or extracted) was incubated with 6.0 μmoles of the para-nitrophenyl derivative (the final volume was made up to 0.050 ml with 0.05 M citrate buffer, adjusted to pH 4.3) for 1 hr at 37 °C. The amount of 0.1% Triton X-100 was constant in all assays at 50 μliters. The reaction was terminated by the addition of 2 ml of 0.4 M glycine: NaOH buffer, pH 10.5. The reaction mixtures were then centrifuged at 5,000 × *g* for 10 min, and the optical density of the released para-nitrophenol in the supernatant was measured at 420 nm. From these data and a standard p-nitrophenol curve run simultaneously, the nmoles hydrolyzed per hour were calculated. In each instance, reactions were terminated at 10-min intervals to insure the linearity of the reactions up to 1 to 6 hr. The substrates used were p-nitrophenyl phosphate, p-nitrophenyl-β-D-glucuronide, p-nitrophenyl-N-acetyl-β-D-glucosamide, p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-N-acetyl-β-D-galactosaminide, p-nitrophenyl-α-D-mannopyranoside, p-nitrophenyl-β-D-xyloside, p-nitrophenyl-α-L-fucopyranoside, p-nitrophenyl-β-L-fucopyranoside, p-nitrophenyl-β-D-galactopyranoside, and p-nitrophenyl-α-D-galactopyranoside. P-nitrophenol was used as a standard. Each of the above compounds was purchased from Pierce Biochemicals (Rockford, Ill.). In each experiment, assays and controls were run in duplicate or triplicate. Controls consisted of assays in which glass-distilled water was substituted for either the p-nitrophenyl derivative substrate or the enzyme in the reaction mixture. These control values from triplicate assays were averaged, added together, and subtracted from the appropriate assays. Enzyme blank values were always less than 1% of the assay, and substrate blank values were never greater than 10% of the total assay value; these blank values were always subtracted from the assay values run simultaneously. Thus, light-scattering contributions from the enzyme preparations or optical density contributions from nonspecific hydrolysis of the p-nitrophenyl derivative were subtracted from the results presented herein. Each experiment was performed at least four times. All solutions, suspensions, and buffers were made in distilled water that was deionized by an ion-exchange column and then distilled in a glass still.

General Proteolytic Activity

Azocoll (25 mg) was incubated at 37 °C with 25 μg of each of the extracts in 2 ml of 0.05 M citrate buffer, pH 7.5, for 60 min. Pronase controls were run simultaneously, and the results are expressed as the fraction of pronase activity found with each extract.

Cathepsin

Cathepsin activity was measured by the method of Anson (1938).

Trypsin

Trypsin activity was measured by the method of Anson (1938).

Collagenase

Collagenase was measured by the method of Mandl, Mac Lennan and Howes (1953).

Carbohydrate

Total carbohydrate released from whole blood cells by the 0.1% Triton X-100 extract of human erythrocyte plasma membranes was measured by the anthrone procedure (Dische, 1962).

Results

Enzyme Activities

The data presented in Table 1 indicate that α- and β-glucosidase, α- and β-galactosidase, α-mannosidase, β-xylosidase, α-fucosidase, β-N-acetyl-glucosaminidase, β-N-acetyl galactosaminidase, acid phosphatase and general proteolytic activities were present in whole blood when tested with intact erythrocytes; β-fucosidase, β-glucuronidase, cathepsin, trypsin and collagenase activities were absent. The highest activities of the glycosidases were found for α-fucosidase and β-N-acetyl-galactosaminidase. These results demonstrate not only that these enzymes are present in the red cell but also that their activity is expressible from intact cells presumably because the enzymes are located on the external surface of the erythrocyte plasma membrane. An alternative explanation, equally plausible, is that the substrates enter the red blood cell where they are hydrolyzed by enzymes on the inner surface of the plasma membrane; however, the released *p*-nitrophenol would then have to diffuse out for measurement. When erythrocyte plasma membranes were prepared and extracted with 0.1% Triton X-100, a large increase in specific activity of the enzymes was observed. α-Galactosidase was purified 500-fold by this procedure, and β-fucosidase and β-glucuronidase (neither of which exhibited any activity when assayed for with the intact cells) showed activities of 61 and 48 nmoles/hr per mg protein, respectively. These data indicate that β-fucosidase and β-glucuronidase are probably located internally in the plasma membrane of the human erythrocyte. An alternative explanation is that the non-ionic detergent causes solubilization of β-fucosidase and β-glucuronidase located within the membrane or that

Table 1. *Glycosidase, acid phosphatase, β-glucuronidase and proteolytic activity of human erythrocyte plasma membranes. Units for proteolytic activity are ng pronase equivalents per mg protein. Units for other enzyme activities are nmoles/hour per mg protein. Preparation of the various fractions is as given in Materials and Methods*

Enzyme	Whole blood	0.1% Triton X-100 extracts of erythrocyte plasma membranes			20,000 × *g* supernatant	Sephadex G-100 column chromatography fractions
		Complete	0 °C[a]	Boiled extract[b]		
α-glucosidase	0.6	43	2	11	46	62
β-glucosidase	0.4	82	2	0	65	74
α-galactosidase	0.4	200	3	4	208	229
β-galactosidase	0.9	10	0[c]	6	12	12
α-fucosidase	1.3	30	2	1	42	48
β-fucosidase	0[c]	61	4	1	69	84
β-N-acetyl-galactosaminidase	1.3	36	2	2	17	26
β-N-acetyl-glucosaminidase	0.1	55	3	4	66	86
α-mannosidase	0.3	39	0[c]	1	59	69
β-xylosidase	0.1	14	0[c]	2	23	40
Acid phosphatase	0.1	96	12	11	59	76
β-glucuronidase	0[c]	48	0[c]	2	42	49
Cathepsin	0[c]	0[c]	0[c]	0[c]	—[d]	—[d]
Trypsin	0[c]	0[c]	0[c]	0[c]	—[d]	—[d]
General proteolytic	22.0	406	21	12	0[c]	—[d]
Collagenase	0[c]	0[c]	0[c]	0[c]	—[d]	—[d]

[a] Incubation performed at 0 °C.
[b] Extracts boiled 10 min before assay.
[c] No measurable activity was found.
[d] Experiment was not performed.

it exposes enzyme previously inaccessible on the outer surface of the membrane. The great increase in specific activity of the enzymes in the erythrocyte plasma membranes attests to their tight binding to this structure. Centrifugation of the 0.1% Triton X-100 at 20,000 × *g* increased the specific activities of some of the glycosidases slightly in the supernatant but decreased the activity of acid phosphatase, β-glucuronidase, and general proteolytic activity, indicating that these enzymes were preferentially bound to material sedimenting at this force (Table 1). Sephadex G-100 column chromatography increased slightly the specific activities of the human erythrocyte plasma membrane enzymes (Fig. 1). In general, the enzymes

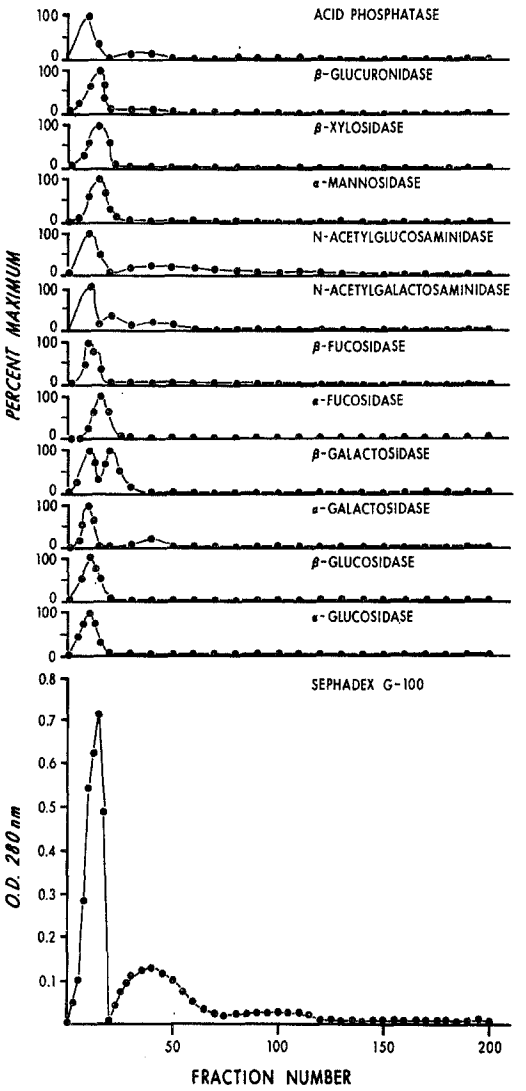

Fig. 1. Sephadex G-100 column chromatogram of human erythrocyte plasma membrane glycosidases. The bottom curves represents protein; the upper curves represent the indicated enzyme. The column was eluted with 0.1% Triton X-100 at 4 °C. The flow rate was 10 ml/hr, and 6-ml fractions were collected. Activities are expressed as percentage of the rate observed in the fraction with the maximal activity

were eluted near the void volume of the column (Fig. 1). Several of the enzymes eluted in multiple peaks on the Sephadex G-100, a phenomenon reported for these same enzymes from other sources (Bosmann & Merritt, 1969).

Ion Effects on Human Erythrocyte Plasma Membrane Glycosidases

The figures presented in Table 2 demonstrate that ethylenediaminetetraacetate (EDTA) increased the activity of the glycosidases slightly, perhaps by chelation of inhibitory ions. Of the other ions tested, in general, Mg^{++}, Cd^{++} and Ca^{++} were without effect, Co^{++} was slightly acceleratory, Ba^{++} and Cu^{++} were mildly inhibitory, and Hg^{++} and Pb^{++} were very inhibitory.

Table 2. *Effect of ions on human erythrocyte plasma membrane glycosidases. The incubation mixture contained 6.0 μmoles of substrate in 0.2* M *citric acid: sodium citrate buffer (pH 4.3), 25 μliters of the 0.1% Triton X-100 human erythrocyte plasma membrane extract plus the EDTA and cation tested to a final volume of 1.675 ml. The incubation mixture was incubated at 37 °C for 1 hr and the activity determined*

Enzyme	Activity[a] (mean ± 1 SE)								
	Substance added[b]								
	EDTA	Mg^{++}	Ca^{++}	Ba^{++}	Cu^{++}	Cd^{++}	Hg^{++}	Co^{++}	Pb^{++}
α-D-galactosidase	107±2	114±7	101±2	88±8	80±4	100±2	20±1	100±2	42±7
β-D-galactosidase	118±3	111±2	100±3	72±6	82±3	103±6	19±2	82±1	39±8
α-D-glucosidase	119±4	102±6	98±2	90±1	76±1	102±1	27±4	104±2	48±3
β-D-glucosidase	129±7	101±7	96±3	101±2	100±1	101±1	41±6	106±2	49±2
α-L-fucosidase	140±8	90±2	101±4	103±2	76±2	103±2	37±2	111±4	61±2
β-L-fucosidase	126±7	86±3	102±3	86±3	82±4	104±2	40±1	100±1	40±1
α-D-mannosidase	182±16	71±4	96±2	91±1	76±2	96±2	20±2	136±2	46±3
β-D-xylosidase	104±2	102±1	101±4	88±6	78±1	101±6	14±3	140±3	49±2
N-acetyl-β-D-glucosaminidase	96±4	103±2	102±6	80±2	83±2	102±6	16±2	127±2	27±2
N-acetyl-β-D-galactosaminidase	110±8	100±1	98±1	81±3	88±6	101±2	31±7	106±2	29±3

[a] Activities for the EDTA assays are expressed as percentages of controls assayed with water substituted for the EDTA, and for the cations as percentages of the EDTA assays with deionized water substituted for the cations. Both controls and tests were assayed in duplicate in each experiment. The experiment was repeated four times.

[b] EDTA had a final concentration of 16 mM. All divalent cations were added as the chloride and were present at a concentration of 1 mM.

Optimum pH, Temperature and Michaelis Constants for Glycosidases of Human Erythrocyte Plasma Membranes

The data in Table 3 show that the optimum pH for the glycosidases ranged from 4.2 to 5.0, with many of the enzymes having a pH 4.3 optimum. None of the enzymes had activity below pH 3.0 or above pH 8.0. The optimum temperatures for the enzymes ranged from 37 to 40 °C. Calcula-

Table 3. *Optimum pH, temperature, K_m and V_{max} of the glycosidases of human erythrocyte plasma membranes*[a]

Enzyme	Optimum pH	Optimum temp. (°C)	K_m (mM)	V_{max} (nmoles/hr)
α-D-glucosidase	4.3	40	21	29
β-D-glucosidase	4.8	37	6	40
α-D-galactosidase	4.6	40	3	20
β-D-galactosidase	5.0	38	50	28
α-L-fucosidase	4.2	39	12	20
β-L-fucosidase	4.2	37	10	13
α-D-mannosidase	4.3	38	41	28
β-D-xylosidase	4.3	40	49	40
N-acetyl-β-D-galactosaminidase	4.3	40	40	24
N-acetyl-β-D-glucosaminidase	4.3	37	21	28

[a] In the instances of pH and temperature, if the condition was optimum for a range of values the mean value of this range is given. The enzyme source for these determinations was the 0.1% Triton X-100 extract of the human erythrocyte.

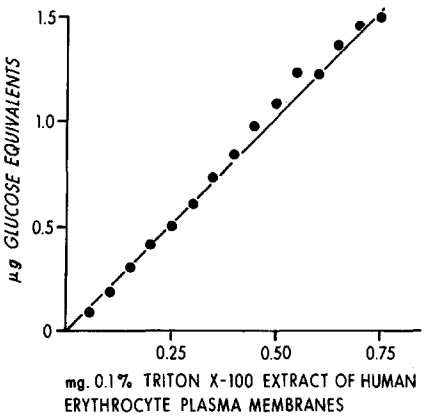

Fig. 2. Release of carbohydrate from intact red blood cells by 0.1% Triton X-100 extracts of human erythrocyte plasma membranes. The complete system contained 50 μliters of the 0.1% Triton X-100 extract of erythrocyte membranes containing the indicated amount of extract, and 15 mg of whole intact blood cell protein (final volume 1.050 ml, 0.05 M in citrate buffer, pH 4.3) and was incubated at 37° for 1 hr. After incubation, 2 ml of 20% TCA was added, and the mixture was centrifuged at $2000 \times g$ for 10 min. The decanted supernatant was analyzed for carbohydrate by the anthrone procedure. Controls consisting of the enzyme source (0.1% Triton X-100 extract) and substrate (intact red blood cells), incubated and precipitated as above, were subtracted from the incubated reactions. In all instances, these controls were less than 4% the test values. Experiments were performed in triplicate. If the 0.1% Triton X-100 extract of human erythrocyte plasma membrane was boiled for 10 min before incubation, no release of carbohydrate occurred

tion of K_m and V_{max} values from linear double-reciprocal plots resulted in the figures given in Table 3 for the glycosidases of human erythrocyte plasma membranes.

Release of Carbohydrate from Human Red Blood Cells by a 0.1% Triton X-100 Extract of Human Erythrocytic Plasma Membranes

It was deemed necessary to determine if the glycosidases present in the 0.1% Triton X-100 extract of human erythrocyte membranes were capable of releasing carbohydrate from intact human erythrocytes. The data in Fig. 2 show that carbohydrate was released by the extract, and that the release was linear with added amounts of the 0.1% Triton X-100 extract of human erythrocyte plasma membranes.

Discussion

The results presented herein demonstrate the following: (1) glycosidases are present in the human erythrocyte; (2) the enzymes are associated with the plasma membrane of the erythrocyte and, in most instances, the activity may be expressible on the outer surface of this membrane; and (3) these enzymes can release carbohydrate from intact erythrocytes.

All of the proteins isolated from plasma with the exception of albumin have been demonstrated to have covalently linked carbohydrate (Spiro, 1969). Since the erythrocyte contains presumably surface-active glycosidases, these might be functional in degrading these plasma glycoproteins if the correct conditions of pH and temperature were met. This would seem to be of prime importance for the plasma immunoglobulins. Furthermore, the plasma lipoproteins have been described as glycolipoproteins (Marshall & Kummerow, 1962), and these erythrocyte glycosidases could conceivably alter these lipoproteins. It has been demonstrated herein that release of carbohydrate would occur by the action of the erythrocyte plasma membrane glycosidases. The blood group ABH and Lewis antigens are glycosphingolipid components of the red cell membrane (Hakomori & Strycharz, 1968), and glycoproteins containing MM, NN and MN blood group substances have been isolated from red cell stroma (Kathin, Winzler & Johnson, 1961). Thus the human erythrocyte glycosidases might be capable of modifying the very surface membrane to which they are attached by altering the blood group substances located thereon.

The glycosidases are thought to be exclusively lysosomal enzymes (Aronson & de Duve, 1968), so it is surprising to find these enzymes located on the surface membrane of human erythrocytes. A similar situation is true for acid phosphatase (Berry & Hochstein, 1969) and to some extent for proteases. This leads one to conclude that in cells devoid of lysosomal particles, enzymes necessary for catabolism are located either in soluble form or attached to membranous structures.

It is of interest that the glycosidases of the human erythrocyte plasma membrane are quite similar to those of lysosomal origin from other tissues (Bosmann & Merritt, 1969; Bosmann & Hemsworth, 1970*b*; Bosmann 1969) with regard to optimum pH, temperature, solubilization and effect of divalent cations. Finally, it should be noted that the glycosidases of the human erythrocyte plasma membrane are present at lower specific activity than those of liver lysosomes (Aronson & de Duve, 1968).

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