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Isolation and Preliminary Characterization of Two Glycophorins from Rabbit Erythrocyte Membranes

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Two different glycophorins were isolated from rabbit erythrocyte membranes. Crude glycophorin fraction prepared by extraction with lithium diiodosalicylate and partition in aqueous phenol contained water-soluble glycolipid in addition to glycophorins. After removal of the glycolipid from glycophorins by ion-exchange chromatography, glycophorins were effectively fractionated by two different techniques: gel chromatography on Bio-Gel A 1.5m and ion-exchange chromatography in the presence of a nonionic detergent, Ammonyx LO. Two glycophorins were isolated in a ratio of about 1:2 (10 mg and 18 mg/3 g lyophilized erythrocyte membranes). The minor component, designated glycophorin RA, was a sialoglycoprotein (42% protein and 58% carbohydrate) and the major one, designated glycophorin RB, was of smaller size and lower carbohydrate content (57% protein and 43% carbohydrate). Glycophorins RA and RB were also different in chemical composition and amino-terminal sequence. Further evidence that the structures of the two glycophorins are unrelated to each other was obtained by Ouchterlony immunodiffusion with lectins; glycophorin RB precipitated with concanavalin A and *Ricinus communis* agglutinin, whereas glycophorin RA did not.

Keywords——rabbit erythrocyte; membrane glycoprotein; glycophorin; precipitation reaction; concanavalin A; *Ricinus communis* agglutinin

Introduction

In spite of the importance of rabbit erythrocytes as a model to study immunological processes, the surface structure of rabbit erythrocytes remains uncertain. Recent studies indicated that the surface glycoconjugates of rabbit erythrocytes are markedly different from those of erythrocytes from other species. Firstly, the sialic acid content of rabbit erythrocyte membranes is about one-eighth of that of human erythrocyte membranes.[1] This low content of sialic acid is related to the finding that only rabbit erythrocytes among various animal erythrocytes are susceptible to human alternative complement pathway.[2] Secondly, rabbit erythrocyte membranes do not show an unequivocal band positive to periodic acid-Shiff reagent (PAS) staining as detected by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS).[3] This result suggested that glycophorin (the major sialoglycoprotein) found in other animal erythrocytes might not be present in rabbit erythrocytes. Finally, rabbit erythrocyte membranes contain large amounts of macroglycolipids.[4] Therefore, it is of interest to characterize glycophorins from rabbit erythrocyte membranes.

In this paper, we describe the isolation and characterization of two glycophorins from rabbit erythrocyte membranes.

Experimental

Materials——Erythrocyte membranes were prepared from fresh rabbit blood by the method of Dodge *et al.*[5] Ammonyx LO (*N*,*N*-dimethyllaurylamine *N*-oxide) was obtained from Onyx Chemical Company. Diethylaminoethyl (DEAE)-cellulose DE 52 was purchased from Whatman Inc, Bio-Gel A 1.5m from Bio-Rad Laboratories and polyamide sheets from Cheng Chin Trading Co. Concanavalin A was prepared from jack bean meal by the method of Agrawal and Goldstein.[6] *Ricinus communis* agglutinin was prepared by the method of Tomita *et al.*[7] All other chemicals used were of reagent grade.

Preparation of Rabbit Glycophorin Fraction——Crude glycophorin fraction was prepared from rabbit erythrocyte membranes by extraction with lithium diiodosalicylate and partition in aqueous phenol as described by Marchesi and Andrews.[8] Separation of glycophorin and water-soluble glycolipid was achieved by DEAE-cellulose column chromatography.[4] The crude glycophorin fraction (38 mg) was dissolved in 5 ml of 0.1% Ammonyx LO and applied to a DEAE-cellulose column (HCO_3^- form, 1.0×15 cm) equilibrated with 0.1% Ammonyx LO. The column was eluted with 0.1% Ammonyx LO (230 ml), followed by 0.3 M NH_4HCO_3 containing 0.1% Ammonyx LO (200 ml). Fractions (4.5 ml) were collected and assayed by measurement of absorbance at 280 nm and by the phenol–H_2SO_4 method for carbohydrate.[9] Fractions were combined into two major pools; unadsorbed fraction (DE 1) containing water-soluble glycolipid and adsorbed fraction (DE 2) containing glycophorin. Pool DE 2 was dialyzed against water for 2 d and lyophilized.

Separation of Glycophorins by Gel Chromatography on Bio-Gel A 1.5m——The glycophorin fraction (DE 2, 34 mg) obtained by the DEAE-cellulose chromatography was dissolved in 10 ml of 5 mM phosphate buffer, pH 8.0, containing 25 mM NaCl, 0.015% NaN_3 and 0.1% Ammonyx LO. The solution was applied to a Bio-Gel A 1.5m column (5.0×130 cm) equilibrated with the same buffer. After elution with 720 ml of the buffer, fractions (10 ml) were collected and analyzed by measurement of absorbance at 226 nm and 280 nm. The elution profile is shown in Fig. 1. Fractions were combined into three pools, labeled Vo, A and B. The three pools were dialyzed against water, concentrated by evaporation under reduced pressure and lyophilized.

Separation of Glycophorins by DEAE-Cellulose Chromatography——The glycophorin fraction (DE 2, 25 mg) was dissolved in 3 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 0.1% Ammonyx LO, and the solution was loaded on a column (1.0×15 cm) of DEAE-cellulose (DE 52) equilibrated with the same buffer. The elution of glycophorin was done stepwise with 135 ml of the same buffer, 90 ml of 60 mM sodium phosphate buffer (pH 6.5) and 70 ml of 300 mM sodium phosphate buffer (pH 7.0). All of these buffers contained 0.1% Ammonyx LO. Fractions (4.5 ml) were collected and analyzed by measurement of absorbance at 226 nm and 280 nm. The elution profile is shown in Fig. 2. Fractions were combined into two major pools, labeled I and II. These pools were dialyzed against water, concentrated by evaporation under reduced pressure and lyophilized.

Amino Acid Analysis——Dry samples (50—100 μg) were hydrolyzed with 200 μl of 6 N HCl at 110°C for 22 h and 72 h. The amino acid analyses were performed on a Hitachi 835 amino acid analyzer. The values of residues mol/100 mol amino acids were obtained after hydrolysis for 72 h with the exception of threonine and serine, the values of which were obtained after hydrolysis for 22 h.

Carbohydrate Analysis——Carbohydrate composition was determined by gas–liquid chromatography (GLC) (Shimadzu GC-4CM) of trimethylsilyl derivatives of methyl glycosides released by methanolysis in 0.5 N methanolic HCl for 16 h at 65°C on a column of 3% OV-17, as reported by Reinhold.[10] Samples containing 20—50 μg of carbohydrate were used with 5 μg of inositol as an internal standard. Amino sugars were also determined on the Hitachi 835 amino acid analyzer after hydrolysis with 4 N HCl at 100°C for 4 h.

Edman Degradation and Dansyl-Edman Degradation——Direct manual Edman degradation was carried out by the method of Peterson *et al.*[11] with a minor modification described by Tomita *et al.*[12] Phenylthiohydantoin (PTH)-amino acids were identified by GLC on a 10% DC-560 column,[13] thin layer chromatography (TLC) on polyamide plates[14] and back-hydrolysis with 6 N HCl containing 0.1% $SnCl_2$ at 150°C for 4 h followed by amino acid analysis.[15] Dansyl-Edman degradation was done by the method of Gray.[16]

Polyacrylamide Gel Electrophoresis——Slab polyacrylamide gel electrophoresis of glycophorin in the presence of SDS was carried out by the method of Laemmli using 10% gel.[17] The gel was stained with Coomassie blue for proteins and with periodic acid-Shiff for carbohydrate.

Ouchterlony Immunodiffusion with Lectins——Double immunodiffusion was carried out on microscope slides covered with 1% agarose in 50 mM phosphate-buffered saline (pH 7.5). Each glycophorin dissolved in water was loaded into peripheral wells. A center well was filled with lectin solution (concanavalin A or *Ricinus communis* agglutinin). The plates were left at room temperature for 2—3 d.

Results

Separation of Glycophorins by Gel Chromatography on Bio-Gel A 1.5m

Crude glycophorin fraction was obtained in a yield of 40—50 mg from 3 g of lyophilized rabbit erythrocyte membranes by extraction with lithuim diiodosalicylate and partition in aqueous phenol. The glycolipid, accounting for one-third of the crude glycophorin fraction, was separated from glycophorin by DEAE-cellulose chromatography in the presence of Ammonyx LO as described elsewhere.[4] The glycophorin fraction, a mixture of two glycophorins, was eluted from the DEAE-cellulose column with 0.3 M NH_4HCO_3.

Separation of the two glycophorins was achieved by gel chromatography on Bio-Gel A 1.5 m in the presence of Ammonyx LO. Three pools; labeled Vo, A and B, were collected

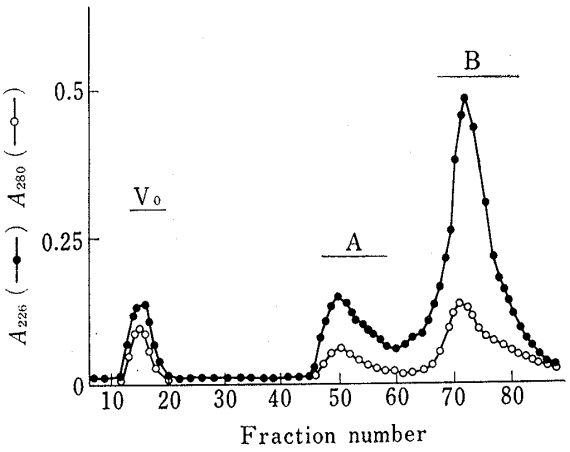

Fig. 1. Separation of Rabbit Glycophorins by Gel Chromatography on Bio-Gel A 1.5 m

The glycophorin fraction (34 mg) was dissolved in 10 ml of 5 mM sodium phosphate buffer (pH 8.0) containing 25 mM NaCl, 0.015% NaN_3 and 0.1% Ammonyx LO. The solution was applied to a Bio-Gel A 1.5 m column (5.0×130 cm) equilibrated with the same buffer. After elution with 720 ml of the buffer, fractions (10 ml) were collected and analyzed by measurement of absorbance at 226 nm and 280 nm.

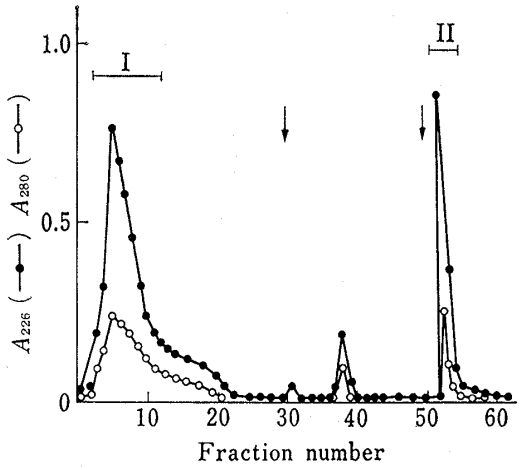

Fig. 2. Separation of Rabbit Glycophorins by DEAE-Cellulose Chromatography

The glycophorin fraction (25 mg) was dissolved in 3 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 0.1% Ammonyx LO, and the solution was loaded on a column (1.0×15 cm) of DEAE-cellulose (DE 52) equilibrated with the same buffer. The elution of glycophorin was done stepwise with 135 ml of the same buffer, 90 ml of 60 mM sodium phosphate buffer (pH 6.5) and 70 ml of 300 mM sodium phosphate buffer (pH 7.0). All of these buffers contained 0.1% Ammonyx LO. Arrows in the figure indicate the positions at which the elution buffer was changed. Fractions (4.5 ml) were collected and analyzed by measurement of absorbance at 226 nm and 280 nm.

as shown in Fig. 1. The pool Vo contained only trace amounts of amino acids and carbohydrate. Therefore, further characterization of pool Vo was not attempted. Pools A and B were obtained in dry weights of 10 mg and 18 mg, respectively, from 3 g of the lyophilized membranes.

Separation of Glycophorins by Ion-Exchange Chromatography on DEAE-cellulose

Alternative separation of the two glycophorins was performed by EDAE-cellulose column chromatography. The column was equilibrated with 50 mM phosphate buffer (pH 8.0) containing 0.1% Ammonyx LO. The glycophorin fraction free from glycolipid was applied to the column, and eluted with stepwise changes of pH and salt concentration as shown in Fig. 2. Two pools, I and II, were obtained in the ratio of 2:1 by dry weight. The same procedure has been used successfully for the separation of horse glycophorins.[18] The pools I and II were virtually identical with the pools B and A, respectively. in amino acid and carbohydrate compositions as well as mobility on SDS-polyacrylamide gel electrophoresis (data not shown). Indeed, when each of the pools I and II was subjected to gel chromatography on Bio-Gel A 1.5 m, each was eluted as a single peak in the position corresponding to those of pools B and

Table I. Amino-Terminal Sequences of Two Glycophorins from Rabbit Erythrocyte Membranes[a)]

	1	2	3	4	5	6	7
Glycophorin RA	H–$\frac{\text{Gly}}{\text{Ala}}$–	Phe–	(X)–	Ala–	(X)–	(X)–	Leu–
Glycophorin RB	H–	Leu–	Asn–	Glu–	Thr–	Gul–	(X)–Gly–
				CHO			

a) (X) indicates that no PTH-amino acid was identified.
CHO; oligosaccharide unit.

A, respectively, (data not shown). Hence, it is evident that pool A corresponds to pool II while pool B corresponds to pool I. The pools A and B were designated glycophorins RA and RB, respectively.

Purity of Glycophorins RA and RB

Definitive data on the purity of each glycophorin preparation were obtained from amino acid sequence analysis; we could unambiguous determine the amino-terminal sequences of glycophorins RA and RB by Edman degradation (Table I). No PTH-amino acid was detected at the third, fifth or sixth position of glycophorin RA, indicating that these positions might be glycosylated. Similarly, no PTH-amino acid was detected at the second or sixth position of glycophorin RB. Dansyl-aspartic acid was detected at the second position of glycophorin RB by dansyl-Edman degradation, indicating that the second residue is probably *N*-glycosylated aspargine. The other residues, at which no PTH-amino acid was detected, appeared to be *O*-glycosylated serine or threonine, but unequivocal data could not be obtained by dansyl-Edman degradation. The findings that the amino-terminal sequences of both glycophorins could be determined indicate that the glycophorin preparations are homogeneous, at least as regards the peptide moiety.

When rabbit glycophorins were subjected to SDS-gel electrophoresis, glycophorins RA and RB gave very diffuse bands, as shown in Fig. 3. It is well-known that human glycophorins give diffuse bands on SDS-gel electrophoresis because of the microheterogeneity in carbohydrate units.[19] However, the bands of rabbit glycophorins are much more diffuse than those of human glycophorins. A possible explanation for these diffused bands may be greater microheterogeneity in carbohydrate units of rabbit glycophorins, whereas the oligosaccharide structures of rabbit glycophorins have not been elucidated. Figure 3 also shows that glycophorin RA has minor bands besides a major band. The minor bands are probably aggregated forms of glycophorin RA, because human glycophorins also show two bands corresponding to a monomeric form and a dimeric form. Efforts are being directed to verify these tentative conclusions unambiguously.

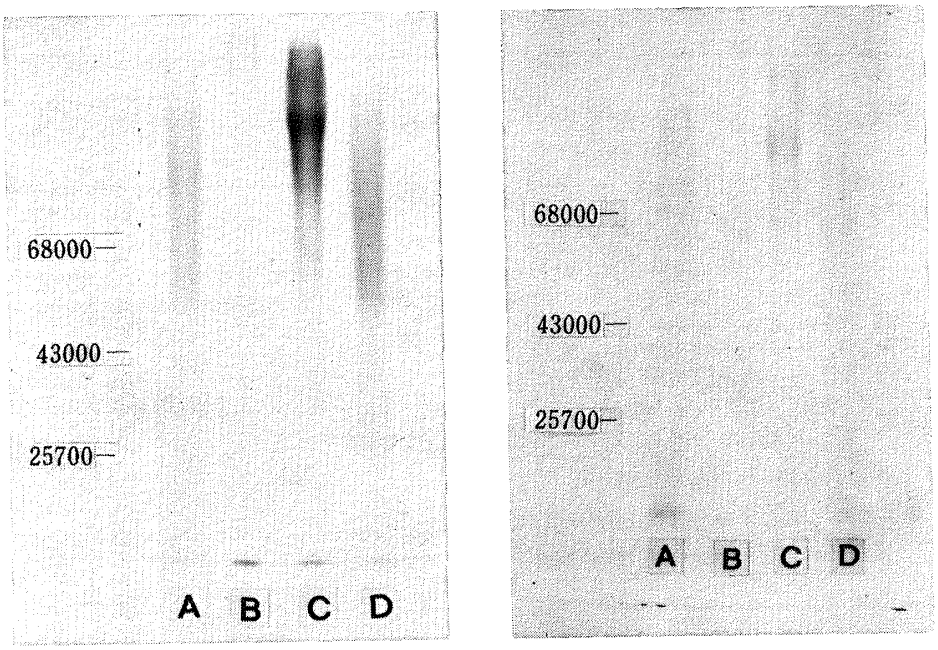

Fig. 3. Slab Gel Electrophoresis of the fractions prepared by Bio-Gel A 1.5 m Chromatography (Fig. 1)

Sample A represents the rabbit glycophorin fraction. Samples B, C and D represent pools Vo, A (glycophorin RA) and B (glycophorin RB), respectively. The gel on the left was stained with Coomassie brilliant blue and the gel on the right was stained with periodic acid-Shiff.

Chemical Properties of Glycophorins RA and RB

Table II shows the amino acid compositions of two glycophorins. The two glycophorins are similar in amino acid composition, though some differences are observed in the contents of serine and threonine. Striking differences between the two glycophorins were observed in carbohydrate composition (Table III). Glycophorin RA contained fucose, mannose, galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine and sialic acid. On the other hand, glycophorin RB did not contain significant amounts of *N*-acetylgalactosamine and sialic acid. Since *O*-glycosidic oligosaccharide units of glycophorins are attached to serine or threonine residues of the peptide backbone through *N*-acetylgalactosamine,[19] glycophorin RB has no *O*-glycosidic oligosaccharide unit. Glycophorin RA is similar in carbohydrate composition to glycophorins from other animal species.[19,20]

Table II. Amino Acid Compositions of Rabbit Glycophorins isolated by Gel Chromatography on Bio-Gel A 1.5 m

(mol %)	Pool A Glycophorin RA	Pool B Glycophorin RB
Aspartic acid	9.7	10.7
Threonine	10.4	6.3
Serine	11.8	7.0
Glutamic acid	10.0	12.1
Proline	5.6	6.0
Glycine	8.3	10.5
Alanine	6.6	7.3
Valine	7.9	8.8
Methionine	0.8	1.2
Isoleucine	6.2	7.4
Leucine	8.5	8.6
Tyrosine	1.2	1.6
Phenylalanine	1.8	2.1
Lysine	6.5	4.6
Histidine	1.7	1.8
Arginine	2.9	4.0

Table III. Carbohydrate Compositions of Rabbit Glycophorins Isolated by Gel Chromatography on Bio-Gel A 1.5 m

(mol %)	Pool A Glycophorin RA	Pool B Glycophorin RB
Fucose	2.7	2.9
Mannose	8.6	18.6
Galactose	42.9	43.3
N-Acetylgalactosamine	15.1	0
N-Acetylglucosamine	12.1	32.1
Sialic acid	18.6	3.0
Weight %		
Protein	42	57
Carbohydrate	58	43
Yield (mg)[a]	10	18

a) Yield from 3 g of lyophilized membranes.

When the apparent molecular weights of the two glycophorins were determined by SDS-gel electrophoresis, those of glycophorins RA and RB were roughly estimated to be 120000 and 70000, respectively (Fig. 3), but precise values could not be determined because the bands

were diffuse bands on gle electrophoresis. These results clearly indicate that glycophorin RB has a structure distinct from that of glycophorin RA, and is not a partially degraded form of glycophorin RA.

Precipitation Reactions of Glycophorins RA and RB with Lectins

Since concanavalin A strongly agglutinates only rabbit erythrocytes among various of animal erythrocytes, it seemed interesting to investigate the binding activity of rabbit glycophorins to concanavalin A. Figure 4 shows the results of Ouchterlony analysis with two lectins (Concanavalin A and *Ricinus communis* agglutinin) and several animal glycophorins. Indeed, only the crude glycophorin fraction of rabbit erythrocyte membranes gave precipitin lines with both lectins. Figure 5 shows the binding activities of glycophorins RA and RB to the two lectins. Only glycophorin RB gave precipitin lines with both lectins. These results indicate that glycophorin RB has a structure different from that of the other glycophorin.

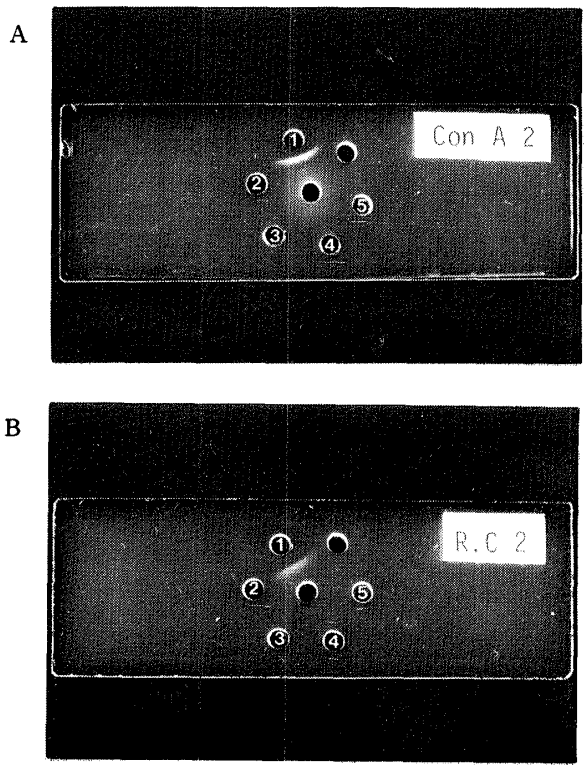

Fig. 4. Precipitation Reaction of Mammalian Glycophorins with Concanavalin A (A) and *Ricinus Communis* Agglutinin (B) by the Ouchterlony Method

A: center well, concanavalin A; peripheral wells, (1) rabbit glycophorin, (2) bovine glycophorin, (3) horse glycophorin, (4) human glycophorin, (5) porcine glycophorin.
B: center well, *Ricinus communis* agglutinin; Peripheral wells were filled with glycophorin solutions in the same order as in A.

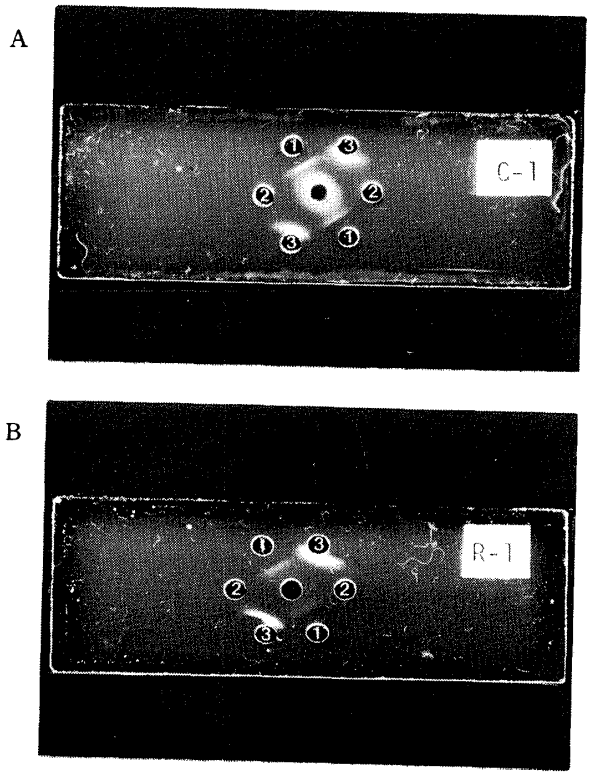

Fig. 5. Precipitation Reaction of Two Rabbit Glycophorins with Concanavalin A (A) and *Ricinus communis* Agglutinin (B) by the Ouchterlony Method

A: center well, concanavalin A; peripheral wells, (1) crude glycophorin fraction, (2) glycophorin RA, (3) glycophorin RB.
B: center well, *Ricinus communis* agglutinin; Peripheral wells were filled with glycophorin solutions in the same order as in A.

Discussion

The crude glycophorin fraction from rabbit erythrocyte membranes contains water-soluble glycolipid (macroglycolipid) in addition to glycophorins. Isolation and preliminary characterization of the macroglycolipid have been reported by our group.[4] The fraction free from the macroglycolipid contained two distinct species of glycophorins. Difficulties in isolating membrane glycoproteins such as glycophorins are well-known. The difficulties

are mainly due to the marked tendency of the membrane glycoproteins to occur in associated form.[19] We were able to isolate the two glycophorins by the use of a nonionic detergent Ammonyx LO, which prevented the glycophorins from associating throughout the purification procedure.

Glycophorin RA, containing a significant amount of sialic acid, showed typical properties of sialoglycoproteins. It is now evident, despite earlier studies describing the absence of sialoglycoprotein,[3] that rabbit erythrocyte membranes do have a sialoglycoprotein. Probably earlier workers were led to the false conclusion not only by evidence that rabbit erythrocyte membranes have the least amount of sialic acid among animal erythrocytes so far examined, but also by the result that glycophorin RA shows a very diffuse band on SDS-gel electrophoresis.

Glycophorin RB, the major glycophorin of rabbit erythrocyte membranes, does not contain a significant amount of sialic acid and shows some properties distinct from those of other glycophorins. For instance, glycophorin RB binds to concanavalin A. This binding activity of glycophorin RB is consistent with evidence that only rabbit erythrocytes are strongly agglutinated with concanavalin A. Podolsky and Weiser have isolated galactosyltransferase from rabbit erythrocyte membranes as a major receptor for concanavalin A.[21] The properties of the enzyme are somewhat different from those of glycophorin RB. The total yield of glycophorins RA and RB was about 10 mg from 1 g of the lyophilized membranes. This yield is the least among those of glycophorins from animal erythrocyte membranes. Rabbit erythrocyte membranes contain a large amount of macroglycolipid, probably to compensate for the small amount of glycophorins. Since, rabbit erythrocytes show unique immunological properties, it is of interest to investigate how rabbit glycophorins participate in those properties. As a prerequisite to elucidation of the immunological roles of the glycophorins in detail, further structural characterizations of the two glycophorins are in progress.

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