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ISOLATION OF GLYCOPHORIN WITH DEOXYCHOLATE

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Summary

In a previous communication we reported that human erythrocyte glycophorin prepared by the lithium diiodosalicylate phenol procedure contains approximately 10 mol of lithium diiodosalicylate per mol of glycophorin, and further we showed that this bound lithium diiodosalicylate is difficult to remove by detergents or organic solvents (Romans, A.Y. and Segrest, J.P. (1978) Biochim. Biophys. Acta 511, 297—301). In the present communication we report an alternative purification procedure for glycophorin in which sodium deoxycholate is substituted for lithium diiodosalicylate; the sodium deoxycholate is subsequently removed by gel filtration. Utilizing this procedure, 25—30 mg glycophorin are obtained per gram of lyophilized erythrocyte ghosts. The glycophorin prepared by the sodium deoxycholate procedure, after a single gel filtration step, contains less than 1 mol of sodium deoxycholate per mol glycophorin and is colorless compared with glycophorin prepared by the lithium diiodosalicylate procedure, which has a distinct reddish-brown cast.

Human erythrocyte glycophorin has proven to be a popular integral membrane protein for physical chemical studies [2—5] and especially for membrane reconstitution studies [6—10]; the glycophorin used in most of these studies has been isolated by the lithium diiodosalicylate (LIS)-phenol procedure [11]. Because glycophorin isolated by the LIS-phenol procedure contains close to 10 mol of LIS per mol glycophorin [1], a decision was made to develop an alternate method for purification of glycophorin. We report such a method here.

Abbreviations: LIS, lithium diiodosalicylate; SDS, sodium dodecyl sulfate.

Sodium deoxycholate was substituted for LIS at concentrations of 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 M in a sodium deoxycholate-phenol extraction of six 0.75-g samples of fresh human erythrocyte ghosts identical in every other way to a parallel extraction by the LIS-phenol procedure [11]. The 0.25 M sodium deoxycholate solution gave generally better results than the other sodium deoxycholate concentrations and resulted in a final glycophorin preparation which, on the basis of sodium dodecylsulfate-polyacrylamide gel electrophoresis [12] is identical to glycophorin isolated by the LIS-phenol procedure.

In order to measure the degree of retention of sodium deoxycholate by the final glycophorin product, 20 μCi sodium deoxy[^{14}C]cholate (California Bionuclear Corporation, Sun Valley, CA) was added to 5.20 g sodium deoxycholate (Sigma Chemical Co., Elk Grove Village, IL) and the total dissolved in 50 ml of 25 mM Tris-HCl buffer, pH 7.4, to make a 0.25 M sodium deoxycholate solution. To this solution was added 1.5 g-ghosts and the suspension stirred 5 min at room temperature. The suspension was then placed in ice and 100 ml cold distilled water was added in 25-ml increments while stirring. The final suspension was filtered through cheese cloth and centrifuged for 80 min at 48 000 × *g* and 4°C.

The supernatant was removed and an equal volume of cold 50% phenol was added to the supernatant and the suspension stirred on ice for 15 min. The suspension was then centrifuged for 1 h at 6500 × *g* and 0°C.

The upper water layer was removed by pasteur pipette to within 1 cm of the lower phenol layer and dialyzed against two changes (20 l each) of deionized water at 4°C and lyophilized.

The lyophilized material was suspended in 60 ml cold 90% ethanol, stirred for 1 h at 4°C, and centrifuged for 30 min at 16 500 × *g* and 4°C. The precipitate was then suspended in 60 ml 100% ethanol and stirred for 1 h at 4°C and centrifuged as before. The washing in 100% ethanol was repeated three times.

The final precipitate was dissolved in 100 ml distilled water and dialyzed against two changes (2 l each) of distilled water. The dialysate was centrifuged 30 min at 48 000 × *g* and 0°C to remove any precipitate and the supernatant lyophilized.

The lyophilized preparation (47.7 mg) was dissolved in 5 ml glass distilled water and a 100 μl fraction counted in Aquasol solution on a Searle Isocap/300. This count indicated that there was significant binding of sodium deoxycholate to the glycophorin preparation following the ethanol washing procedure (Table I). Because of this sodium deoxycholate retention, a gel filtration step was added to the isolation protocol.

TABLE I

SODIUM DEOXYCHOLATE-PHENOL PROCEDURE FOR GLYCOPHORIN PURIFICATION

Preparation	Yield of glycophorin (mg/g lyophilized ghosts)	mol DOC*/ mol glycophorin	mg DOC*/mg glycophorin
Following ethanol wash	28	12.2	0.14
Following G-50 column chromatography	25	0.96	0.013

*DOC, deoxycholate.

The 5 ml sample of glycophorin dissolved in glass distilled water was subjected to gel filtration on a 2.5 X 120 cm column of Sephadex G-50 (Pharmacia) equilibrated with distilled water and 5-ml fractions collected. The fractions were analyzed for protein by measuring absorbance at 280 nm and for sodium deoxycholate by counting 100 μl samples in Aquasol. As can be seen from Fig. 1, glycophorin is well separated from free sodium deoxycholate; only a small portion of the sodium deoxycholate still remains with the glycophorin fractions.

Fractions 28—35 were pooled and lyophilized. The results of the analysis of this lyophilized material are shown in Table I. Glycophorin was obtained from this fraction at a yield of 25 mg per gm lyophilized erythrocyte ghosts and contained less than 1 mol sodium deoxycholate per mol glycophorin. In comparison, a LIS extracted preparation of glycophorin was obtained at a yield of 35 mg per gm ghosts. Fig. 2 shows the results of sodium dodecylsulfate polyacrylamide gel electrophoresis of the two glycophorin preparations.

A concentration of 0.25 M sodium deoxycholate is optimal for the sodium deoxycholate preparation of glycophorin described here. Lower concentrations of sodium deoxycholate result in lower yields of glycophorin and contamination by several periodic acid-Schiff negative peptides. Concentrations higher than 0.25 M sodium deoxycholate inhibit the formation of two phases from the phenol/water mixture and are therefore unsatisfactory.

The exact concentration of sodium deoxycholate required for optimal results varies between 0.25 and 0.3 M depending upon the purity of the sodium deoxycholate used. For consistent results recrystallization of commercial sodium deoxycholate may prove desirable.

It has been our experience that a second gel filtration of the glycophorin preparation will reduce the residual sodium deoxycholate well below 1 mol sodium deoxycholate per mol glycophorin. However, such an additional step has the disadvantage of decreasing the yield of glycophorin.

The lithium diiodosaliclylate-phenol method [11] for glycophorin preparation appears to be the most universally used of the methods described in the literature [6—10]. The advantage of the LIS method is its high yield (35

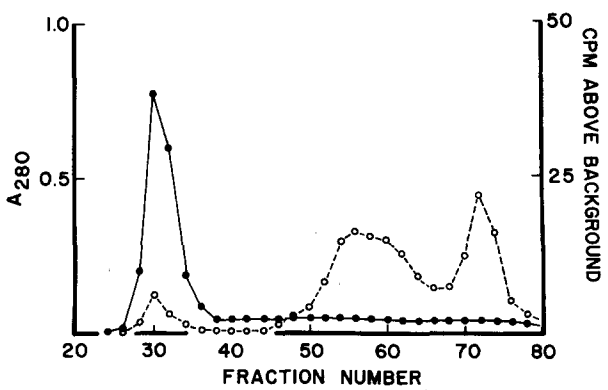

Fig. 1. Gel filtration of sodium deoxycholate-phenol glycophorin preparation on 2.5 X 120 cm column of Sephadex G-50. The column was equilibrated with distilled water and the sample dissolved in 5 ml of distilled water. Fractions (5 ml) were analyzed for protein by A_{280} (●—●) and for sodium deoxy[^{14}C]-cholate by liquid scintillation counting (○—○).

mg/g ghosts); the major disadvantage is retention of up to 10 mol LIS per mol glycophorin [1]. Further, the LIS method does a poor job of removing pigments, such as heme, from the preparation.

Table II gives the estimated yields of glycophorin in mg per g of lyophilized ghosts for a number of published preparation methods for glycophorin [11,13–17]. The LIS-phenol procedure gives almost twice the yield of the best of the other published methods. The hot phenol procedure used by the

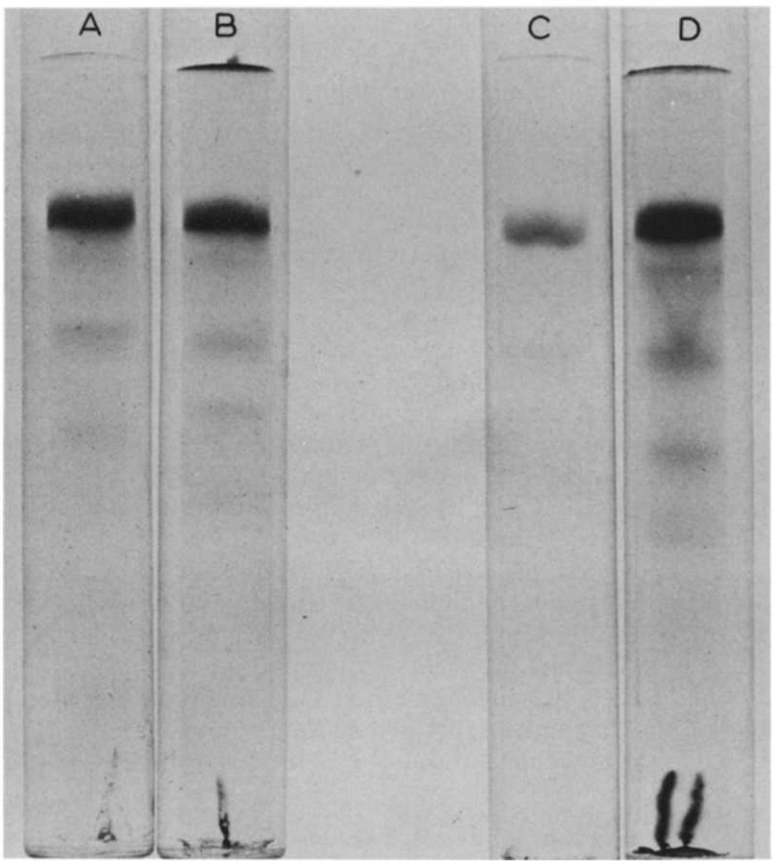

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 5% gels of sodium deoxycholate-phenol extracted (A and B) and LIS-phenol extracted (C and D) glycophorin. (A) Periodic acid-Shiff stain. (B) Coomassie blue stain. (C) Periodic acid-Schiff stain. (D) Coomassie blue stain.

TABLE II

YIELDS OF GLYCOPHORIN FROM PUBLISHED PURIFICATION PROCEDURES

Gp, glycophorin; DOC, deoxycholate; WGA, wheat germ agglutinin.

Procedure	Yields (mg GP/g ghosts)
LIS-phenol [11]	35
DOC-phenol-gel filtration	25
Hot phenol [13]	20
Chloroform: methanol-SDS gel filtration [14]	<20
Aqueous pyridine [15]	14
Triton-SDS-phenol [16]	8
Triton-WGA affinity chromatography [17]	3

earlier workers, Morawiecki [18] and Winzler and coworkers [19] gives the best yields of the latter methods (20 mg/g ghosts) but suffers from the high temperature (65—70°C) required; Winzler was never able to demonstrate an NH_2-terminal residue for his glycophorin preparations, perhaps due to chemical modification [19].

The yield of the chloroform: methanol method of Hamaguchi and Cleve [14] is uncertain due to a failure to report the yield of the product following the final SDS gel filtration step. The remaining methods for glycophorin preparation reported in the literature have yields considerably less than 20 mg glycophorin per g ghosts [15—17].

The advantage of the sodium deoxycholate-phenol-gel filtration method for glycophorin preparation is the purity of the product (it contains negligible amounts of lipids, pigment and detergent) and the relatively high yield (25 mg glycophorin per g ghosts). While the yield is not as high as for the LIS procedure, the slightly lower yields are considered justified by the improved purity of the product.

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