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COMBINED LECTIN-AFFINITY AND METAL-INTERACTION CHROMATOGRAPHY FOR THE SEPARATION OF GLYCOPHORINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Human erythrocyte sialoglycoproteins, or glycophorins, were chromatographed by lectin-affinity and metal-interaction chromatography on high-performance liquid chromatographic columns. Glycophorins A, B and C were separated from other proteins and from glycophorin E by using a column containing wheat germ agglutinin, immobilized on a microparticulate silica support. The glycophorins were adsorbed on the lectin column from a mobile phase containing 0.25 *M* sodium chloride and recovered by stepwise desorption with 0.2 *M* N-acetylglucosamine solution. Glycophorins A, B and C were separated into the individual components on a silica-bound iminodiacetic acid stationary phase in the copper(II) chelate form. The separation of the glycophorins by metal-interaction chromatography was accomplished by decreasing salt gradient elution. Retention times and resolution of the individual glycophorins were sensitive to the initial sodium chloride concentration and the pH of the eluent. Addition of methanol to the eluent increased the resolution. The effects of linear, decreasing gradients of pH and methanol in 25 m*M* phosphate buffer on the resolution of glycophorins were also investigated. In both types of chromatography the mobile phases contained 0.05% (w/v) sodium dodecyl sulfate. With octylglycoside or CHAPS in the eluent glycophorins A and C could not be eluted. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to analyze all the chromatographic results.

INTRODUCTION

High-performance liquid chromatography (HPLC) is increasingly employed in the isolation and separation of membrane proteins[1]. These high-molecular-weight

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amphiphilic molecules often contain a highly hydrophilic glycan moiety in the extracellular domain and a strongly hydrophobic amino acid sequence in their membrane-spanning domain. The peculiar molecular architecture of membrane proteins engenders particular problems in their chromatography and necessitates the use of aqueous eluents that contain surfactants to keep them in molecular dispersion and to improve their recovery[2]. Size-exclusion[2–6], ion-exchange[5,6] and hydrophobic-interaction chromatography[7,8] have traditionally been employed for the purification. In the reversed-phase chromatography[9,10] of membrane proteins hydro-organic eluents are usually much stronger, and therefore more denaturing, than those otherwise employed in the HPLC of proteins. Affinity chromatography on lectin stationary phases has also been used for the purification of membrane proteins[1,11,12]. Recently affinity chromatography and metal-interaction chromatography have been employed in the HPLC of such proteins[13–15].

In this study, glycophorins, the erythrocyte membrane sialoglycoproteins, were separated by HPLC employing the two chromatographic techniques mentioned above. These integral membrane proteins contain almost 60% (w/w) glycan, which is highly sialylated, and a hydrophobic domain of about 23 amino acids that forms the intramembraneous segment[16,17]. According to Anstee[18], human erythrocyte membranes contain at least four sialoglycoproteins, the glycophorins A, B, C and E. They have different amino acid contents and electrophoretic mobilities in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). In another nomenclature the letters α, δ, β and γ, are used instead of A, B, C and E, respectively[18,19].

In addition to their attraction as readily available members of an important class of membrane proteins, glycophorins are of interest because they carry a number of antigenic determinants, which are involved in blood types[18]. Further, they provide an anchoring site for the stabilizing membrane skeleton[20,21] and hence they contribute to the reversible deformability of erythrocytes. The structure of glycophorins A, B and C has been investigated and their amino acid sequences and glycosylation sites have been determined[22,23].

The interest in glycophorin structure and function prompted us to examine the possibility of a relatively rapid and straightforward HPLC procedure for the separation of the individual glycophorins. Traditional approaches by size-exclusion[24] and lectin-affinity chromatography[25] and also by preparative SDS-PAGE[19] are not only cumbersome but also rather ineffective for purifying the less abundant B, C and E forms. Recently, Blanchard *et al.*[23] have developed a method for the preparative isolation of glycophorin B and a mixture of glycophorins C and E by a combination of high-performance size-exclusion and ion-exchange chromatography with Triton X-100 in the eluent.

This paper presents the results of a study of the chromatographic conditions for the separation of glycophorins by a dual approach with high-performance metal-interaction chromatography and lectin-affinity chromatography, in which columns with microparticulate, macroporous siliceous stationary phases were used. Both techniques have already been successfully used for the HPLC of glycoproteins[26–28] and immobilized wheat germ agglutinin has been a suitable stationary phase for the isolation of glycophorins[25]. As the various glycophorins differ in their histidine contents, a Cu^{II}–iminodiacetic acid (IDA) column, which separates proteins according to their histidine content[29,30], was selected for metal-interaction chromatography.

The combination of the two HPLC techniques resulted in a relatively fast analytical and micropreparative procedure for the assay and/or purification of glycophorins.

EXPERIMENTAL

Materials

Wheat germ agglutinin (WGA), sodium chloride, *n*-octyl-β-D-glucopyranoside (octyl glucoside), N-acetyl-D-glucosamine (GlcNAc), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] (CHAPS), 2-(N-morpholino)ethanesulfonic acid (MES), 3,5-diiodosalicylic acid (DIS) and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) were obtained from Sigma (St. Louis, MO, U.S.A.). Phosphoric acid, acetic acid, sodium and lithium hydroxide, nickel sulfate, copper(II) nitrate, the disodium salt of ethylenediaminetetraacetic acid (EDTA) and HPLC-grade methanol were purchased from Fisher (Pittsburgh, PA, U.S.A.). SDS, iron(III) chloride and IDA were obtained from Aldrich (Milwaukee, WI, U.S.A.). Two spherical silica gels having nominal particle and pore diameters of 7 μm and 300 Å, respectively, were used. They were supplied under the respective trade names of Nucleosil and Zorbax by Macherey, Nagel & Co. (Düren, F.R.G.) and DuPont (Wilmongton, DE, U.S.A.). Lithium diiodosalicylic acid (LIS) was prepared by twice recrystallizing DIS from methanol, then allowing the recrystallized DIS to react with an aqueous solution of lithium hydroxide and recrystallizing the resulting salt.

Instruments

The liquid chromatograph was assembled from a Perkin-Elmer (Norwalk, CT, U.S.A.) Series 4 solvent delivery pump, controlled by an LC terminal, Model LC-85B spectrophotometric detector, Model LCI-100 laboratory computing integrator with thermal printer and a Model 7500 laboratory computer with Chromatographics 3 software for data acquisition and reprocessing. Samples were injected by a Model 7010 sampling valve with a 100-μl sample loop from Rheodyne (Berkeley, CA, U.S.A.). A Model 110B solvent delivery pump from Beckman (San Ramon, CA, U.S.A.) was used to load the metal-interaction column with the metal of interest.

Columns

The IDA stationary phase was prepared from Nucleosil silica and the WGA was immobilized on the Zorbax silica support according to methods outlined elsewhere[26–31]. All columns were made of 100 or 60 mm × 4.6 mm I.D. No. 316 stainless-steel tubing (Handy and Harman, Morristown, PA, U.S.A.) and packed at 8000 p.s.i. Metal-interaction and lectin columns were packed from a methanol slurry and a slurry in 25 m*M* phosphate buffer (pH 6.0) containing 0.5 *M* sodium chloride and 50% (w/v) sucrose, respectively.

Protein isolation

Outdated units of erythrocytes were obtained from the blood bank of the Yale-New Haven Hospital. Erythrocytes were washed, and hemoglobin-free membranes were prepared by the method of Dodge *et al.*[32]. The LIS–phenol method[33] was employed to obtain a lyophilized extract of the glycophorins from the erythrocyte membranes.

Procedure

The IDA–silica column was washed successively with 50 ml each of water, 50 m*M* EDTA solution, methanol and water again. Thereafter, it was perfused with 100 ml of 15 m*M* copper(II) chloride solution and then washed with water to remove the unbound metal. From time to time the Cu^{II}–IDA column was regenerated by washing with 50 m*M* disodium EDTA and subsequently reloading it with the metal.

Gel electrophoresis

In each chromatographic run 10–25 fractions ranging from 0.5 to 2.2 ml were collected. They were dialyzed against water to remove excess of salt, concentrated to dryness under vacuum and subjected to discontinuous SDS-PAGE[34] with a 3.5% acrylamide stacking gel and a 10% acrylamide running gel. All reagents were of electrophoresis grade from Bio-Rad Labs. (Richmond, CA, U.S.A.). The proteins bands were rendered visible by silver staining[35].

RESULTS AND DISCUSSION

Lectin-affinity chromatography

The glycophorin extract from human red blood cells was chromatographed on the WGA column that was pre-equilibrated with 25 m*M* phosphate buffer (pH 6.0) containing 0.05% (w/v) SDS and 0.25 *M* sodium chloride. In a typical chromatographic run, 0.7 mg of glycoprotein were applied to the column, which was washed with the above buffer for 15 min in order to elute all non-glycophorins components of the sample and glycophorin E, before the tightly bound glycophorin A, B and C were eluted in a single desorption step with 0.2 *M* N-acetylglucosamine in the equilibrating buffer. The fractions taken throughout the run were analyzed by SDS-PAGE. This confirmed the composition of the peaks and showed that glycophorin E was eluted during the preceding washing step, as illustrated in Fig. 1. The results show that lectin chromatography on a WGA–silica column is an effective means of purifying the glycophorins from other proteins and of isolating pure glycophorin E.

The salt concentration in the eluent is very critical in this kind of chromatographic separation, because electrostatic interactions between the sample components and the lectin play an important role in the retention process and they are strongly affected by the salt concentrations[36]. Therefore, a certain ionic strength of the mobile phase was required in order to elute the glycophorins from the WGA column. On the other hand, when the buffer contained sodium chloride concentrations higher than 0.7 *M*, the glycophorins were very tightly bound to WGA, presumably by hydrophobic interactions, and could not be desorbed with GlcNAc. Buffers containing 0.2–0.5 *M* sodium chloride were the most appropriate in each of the pre-equilibration, washing and desorption steps.

Metal-interaction chromatography

An IDA acid column in the copper(II) chelate form was used for the separation of glycophorins A, B and C with 0.05% (w/v) of SDS in the mobile phase and a linear gradient of decreasing sodium chloride concentration. In order to find the optimal conditions, the influence of the mobile phase pH and composition, *i.e.*, concentration of methanol, nature of the surfactant and sodium chloride concentration in the starting eluent, was examined.

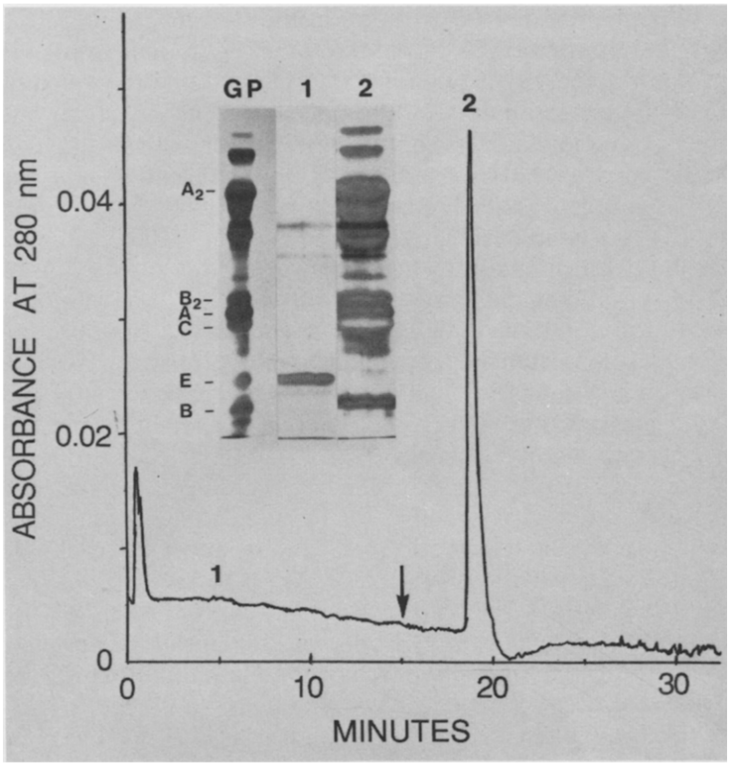


Fig. 1. Isolation of glycophorins by lectin affinity chromatography and SDS-PAGE analysis of glycophorin extract (GP) and fractions of chromatographed glycophorins. Column, 60 × 4.6 mm I.D., packed with silica-bound WGA; temperature, 25°C; flow-rate, 1 ml/min; equilibrating buffer, 25 m*M* phosphate (pH 6.0), containing 0.25 *M* sodium chloride and 0.05% (w/v) SDS. The arrow indicates the desorption step with 0.2 *M* GlcNAc in the equilibrating buffer. Sample, 0.7 mg of glycophorins in 100 μl. Inset: electropherograms of unfractionated glycophorin extract (GP), fractions containing glycophorin E (1) and the glycophorins eluted with the haptenic sugar (2). A_2, glycophorin A dimer; B_2, glycophorin B dimer; A, glycophorin A monomer; B, glycophorin B monomer; C, glycophorin C monomer; E, glycophorin E monomer.

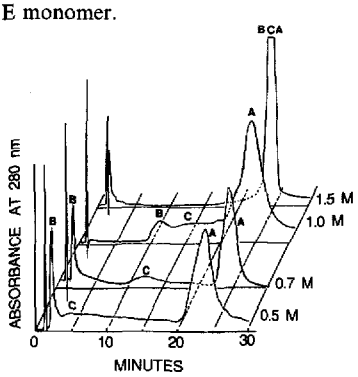


Fig. 2. Separation of glycophorins by metal-interaction chromatography with starting eluents having different sodium chloride concentrations. Column, 100 × 4.6 mm I.D., packed with Cu^{II}–IDA silica; temperature, 25°C; flow-rate, 1 ml/min; 20-min linear gradient with decreasing salt concentration from 0.5, 0.7, 1.0 or 1.5 to 0 *M* sodium chloride in 25 m*M* phosphate buffer (pH 6.0) containing 0.05% (w/v) SDS, followed by a 10-min isocratic elution with the gradient former. Sample, 0.7 mg of glycophorins in 100 μl.

The effect of the sodium chloride concentration in the starting eluent on the retention and resolution of glycophorin is evident from Figs. 2 and 3. As the chromatogram in Fig. 2 shows, the starting sodium chloride concentration significantly affects the retention and resolution of glycophorins. The effect on the chromatographic behavior of the individual glycoproteins is more clearly illustrated in Fig. 3 by plots of the adjusted retention volume *versus* the salt concentration in the starting eluent. Only glycophorin C yielded a linear plot. The adjusted retention volume of glycophorin B first slightly increases with increasing sodium chloride concentration and then increases dramatically when the latter exceeds 0.7 *M*. In contrast, the adjusted retention volume of glycophorin A shows the opposite dependence on salt concentration. Without added salt in the eluent, glycophorins were not retained on the Cu^{II}–IDA column. On the other hand, with 1.5 *M* sodium chloride in the starting eluent they were all strongly retained and were eluted together at the end of the gradient run. Fig. 3 suggests that the optimal difference in retention times is achieved when the salt concentration in the starting eluent is about 0.7 *M*. From the results, we may infer that at high salt concentrations in the eluent, electrostatic repulsion between the sialic acid-rich glycoproteins and the slightly acidic stationary phase is attenuated and, in addition to specific metal interactions, hydrophobic interactions also contribute to the retention. However, it must be pointed out that increasing salt concentrations reduce the critical micellar concentration of the surfactant and may promote association of the hydrophobic domains of the proteins. Either of these factors may affect the retention behavior of glycophorins on the Cu^{II}–IDA column.

The effect of eluent pH on the separation of glycophorins is illustrated in Figs. 4 and 5. The chromatograms in Fig. 4 show that the retention and resolution of glycophorin C is particularly pH dependent. On decreasing the pH of the eluent from 7.0 to 5.3, the retention of all glycophorins decreased, as seen in Fig. 5. At pH 5.0 or below, they all were eluted as a single peak without retention. This is in agreement with the findings[29,30] that protein retention on chelates of "soft" metals, such as copper, decreases with the pH in the range where the protonation of the amino groups in histidine residues (which are believed to be mainly responsible for retention) changes appreciably with pH. Glycophorin A was recovered in relatively pure form when the pH of the eluent ranged from 5.3 to 6.5, the best results being obtained at pH 5.3.

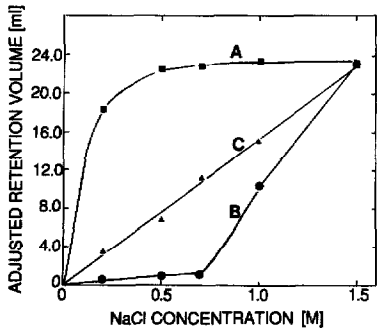
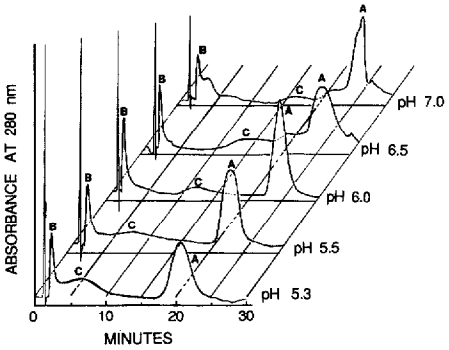


Fig. 3. Plots of adjusted retention volume of glycophorins against the concentrations of sodium chloride in the starting eluent. Conditions as in Fig. 2.



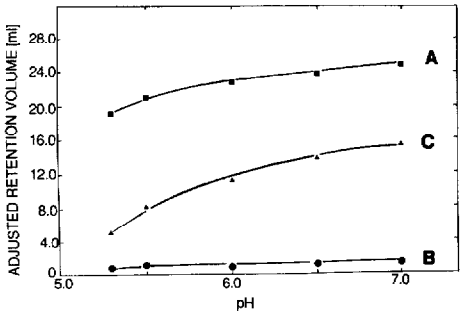


Fig. 4. Separation of glycophorins by metal-interaction chromatography with eluents of different pH. Conditions as in Fig. 2 except the starting eluent contained 0.7 *M* sodium chloride and the pH of the 25 m*M* phosphate buffer was varied.

Fig. 5. Plots of adjusted retention volume of glycophorins against the pH of the eluent. Conditions as in Fig. 4.

However, at this particular pH, glycophorins B and C were not well resolved. On the other hand, glycophorin B was obtained in pure form at pH 7.0, whereas glycophorin A and C were not well resolved. A compromise was reached by using pH 6.0, where all glycophorins were recovered in relatively pure form.

A decreasing pH gradient was also employed in some experiments. Fig. 6 illustrates the separation of glycophorins on the Cu^{II}–IDA column with a gradient of linearly decreasing pH (from 7.0 to 5.0) and salt concentration (from 0.7 to 0 *M* sodium chloride) in the eluent. According to SDS-PAGE, the first peak was pure glycophorin B and the second was relatively pure glycophorin C, whereas the third peak of glycophorin A contained traces of glycophorin C.

The effect of the addition of methanol to the eluent was examined under the conditions described above, except that the buffer was 25 m*M* phosphate, the decrease in pH was from 6.5 to 5.0 and 5 or 10% (v/v) methanol was added to the starting eluent. The results are presented in Figs. 7 and 8. With 5% (v/v) methanol in the starting eluent both the retention and resolution of glycophorins C and A increased. However, even under these conditions, no separation was obtained for glycophorins C and E. With 10% (v/v) methanol, the resolution of glycophorins A and C was poor, owing to the increased retention of glycophorin C. At the higher methanol concentration, glycophorin B present in the sample was not eluted as a sharp peak, and traces of it were found in all fractions collected throughout the chromatogram (see Fig. 8, panel 3). These results suggested the use of 5% methanol in the starting eluent. Generally, as seen in Fig. 7, the retentions of all glycophorins increase with increasing methanol concentration. This in agreement with previous observations[31] that in metal-interaction chromatography, protein retention increases with increasing organic solvent content of the mobile phase. This is probably due to weaker hydration of the metal chelate.

The ionic and non-ionic surfactants SDS, CHAPS and octylglucoside were compared for their effectiveness in the separation of glycophorins on a Cu^{II}–IDA column at concentrations of 0.05%, 0.085% and 0.87% (w/v), respectively. In these experiments, a decreasing linear salt gradient from 1.0 to 0 *M* sodium chloride in 25

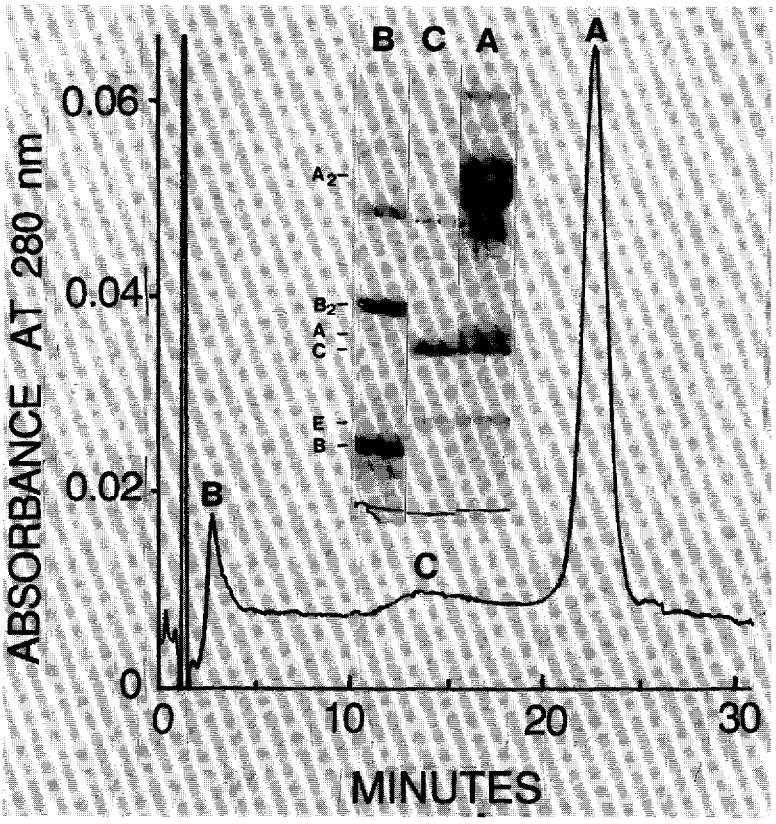


Fig. 6. Separation of glycophorins by metal-interaction chromatography. Conditions as in Fig. 2, except the starting eluent was 25 m*M* HEPES–MES–acetic acid (pH 7.0) containing 0.7 *M* sodium chloride and the gradient former was 25 m*M* HEPES–MES–acetic acid buffer (pH 5.0); both eluents contained 0.05% (w/v) SDS. Inset: electropherogram of representative fractions from peaks B, C and A. The positions of glycophorin bands are as in Fig. 1.

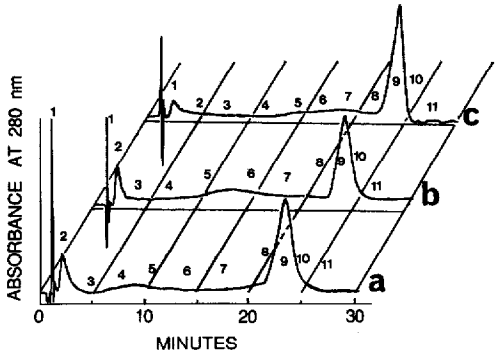


Fig. 7. Separation of glycophorins by metal-interaction chromatography with or without methanol in the starting eluent. Conditions as in Fig. 2, except starting eluent, 25 m*M* phosphate buffer (pH 6.5) containing 0.7 *M* sodium chloride, 0.05% (w/v) SDS and (a) 0%, (b) 5% and (c) 10% (v/v) methanol; gradient former, 25 m*M* phosphate buffer (pH 5.0) containing 0.05% (w/v) SDS. Numbers in the chromatogram indicate the collected fractions. Sample, 0.8 mg of glycophorins in 100 μl.

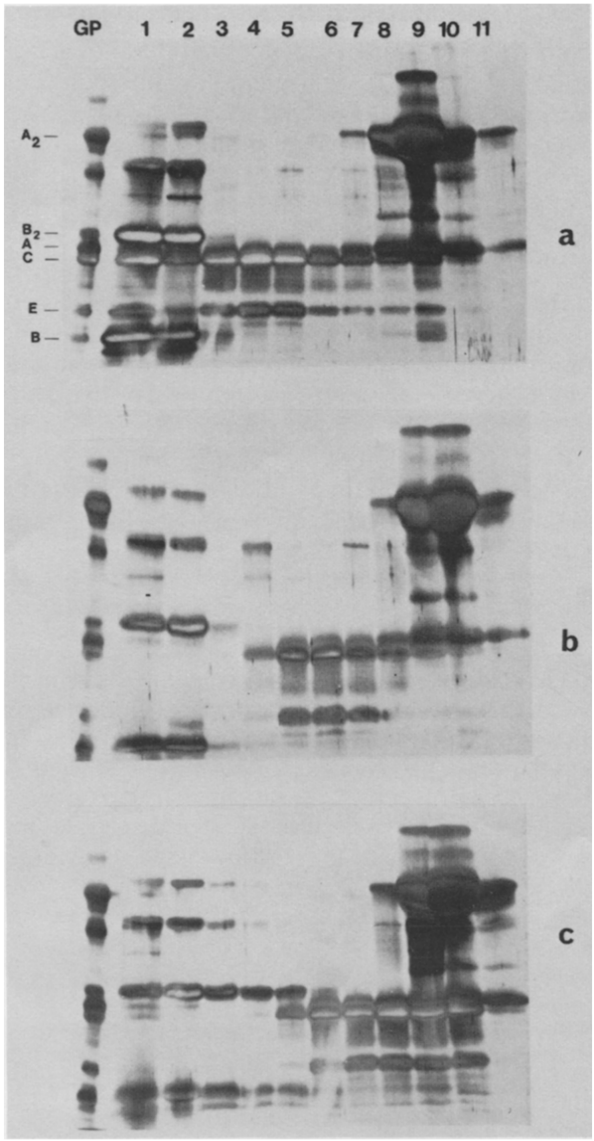


Fig. 8. SDS-PAGE analysis of fractions obtained in the metal-interaction chromatography of glycophorins under the conditions shown in Fig. 7. Panels a, b and c correspond to the chromatogram obtained with 0%, 5% and 10% (v/v) methanol, respectively, in the starting eluent. The codes for the individual glycophorins are given in Fig. 1.

m*M* phosphate (pH 6.0) was used with a 10-min isocratic elution at the end of the chromatographic run. Whereas glycophorin B was eluted close to the mobile phase hold-up time of the column with all of the surfactants tested, only eluents containing SDS were effective at desorbing glycophorins A and C from the column under our experimental conditions.

The effectiveness of different immobilized metals in separating the glycophorins was examined. Metal-interaction columns were prepared by loading the IDA–silica column with Fe^{III} and Ni^{II}. In both instances, the glycophorins were not retained at either low or high salt concentrations in the eluent in the pH range 5.0–7.0.

Our study has shown that metal-interaction chromatography is an effective method for separating integral membrane glycoproteins. The results confirmed the expectation that glycophorins A, C and B having five, three and two histidines, respectively, were retained in order of increasing number of histidine residues on the Cu^{II}–IDA column under all conditions of our experiments. The lack of separation of glycophorins C and E on the Cu^{II}–IDA column under any of the conditions tested supports previous suggestions[18] that these two glycophorins are very closely related. Depending on the desired separation, conditions can be varied to adjust the retention of glycophorin C to favor complete isolation of either glycophorin A or B or to obtain significant portions of all three glycoproteins in the pure form. When used in conjunction with the WGA column, which separates glycophorin E from the other glycophorins, the Cu^{II}–IDA column permits the rapid separation of the erythrocyte glycophorins, thus facilitating the analysis and purification of these important membrane proteins.

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