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Isolation of an Abnormally Phosphorylated Erythrocyte Membrane Band 3 Glycoprotein from Patients with Myotonic Muscular Dystrophy

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Summary. A fraction of erythrocyte Band 3 (M_r, 93,000) glycoprotein that demonstrates decreased autophosphorylation in membranes from myotonic muscular dystrophy patients is demonstrated. Sequential affinity chromatography of Triton X-100 solubilized erythrocyte membrane proteins separated three specifically retained glycoprotein fractions on a Ricin Communis I-Sepharose 4B column. One fraction contains a portion of the major sialoglycoprotein (apparent M_r, 78,000) and is specifically eluted from the column by 10 mM NaCl and 100 mM D-galactose (10/100). The two other glycoprotein fractions are eluted by 100 mM NaCl, 10 mM D-galactose (100/10) and 100 mM NaCl, 100 mM D-galactose (100/100). The composition of both fractions contains greater than 95% Band 3 (apparent M_r, 93,000) glycoprotein.

The quantities of glycoprotein in each fraction obtained from erythrocytes of myotonic dystrophy patients did not differ from the quantities obtained from control erythrocytes. Following endogenous protein kinase incubations of ghosts with [γ-^{32}P]ATP, the specific [^{32}P] phosphorylation of the 10/100 and 100/10 fractions are identical. The 100/100 fraction, which makes up approximately 3% of the total erythrocyte membrane protein, demonstrates a different pattern for myotonic dystrophy patients; specific phosphorylation was reduced by 50% relative to activity in control experiments. These findings are consistent with previous experiments that demonstrated decreased autophosphorylation of the glycoprotein portion of Band 3 (Roses & Appel, 1975, *J. Membrane Biol.* **20:** 51) and are consistent with the autosomal dominant mode of inheritance in this disease.

Myotonic muscular dystrophy (MyD) is the most prevalent of the human muscular dystrophies and is inherited as an autosomal dominant trait (Thomasen, 1948; Caughey & Myrianthopoulos, 1963; Roses, Harper & Bossen, 1978). The cause of the disease, which affects many different organ systems, has recently been proposed to be a widespread abnormality of cellular membranes (Roses *et al.*, 1978). We have used the erythrocyte membrane as an available biopsy source of purified human membrane material. We have demonstrated decreased phosphorylation in the glycoprotein component of Band 3 of erythrocytes from

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MyD patients. Our experiments measured membrane protein [^{32}P]autophosphorylation during endogenous cyclic AMP independent protein kinase assays, using [γ-^{32}P]ATP[1] as a substrate (Roses & Appel, 1975). The experiments reported below describe the fractionation and purification of erythrocyte Band 3 glycoproteins. We have identified a difference in phosphorylation in a single fraction from MyD patients. There is a 50% reduction in phosphorylation of this fraction as compared to the activity of the control fraction. This fraction accounts for approximately 3% of the total erythrocyte membrane protein. The findings are consistent with previous experiments and with the autosomal dominant pattern of inheritance (Roses & Appel, 1975; Appel & Roses, 1978).

We used Ricin I as the lectin for separation of glycoproteins. Ricin I, also called RCA_{120} or Ricin Communis hemagglutinin, is a plant lectin from *Ricinus Communis* castor bean which has *specific binding properties for galactose* and oligosaccharides having galactose as the sugar terminal (Nicolson, Blaustein & Etzler, 1974). Affinity chromatography of the proteins of erythrocyte membranes on a Ricin I column has been reported by Adair and Kornfeld (1974), but their specific methods were not directly applicable to the problem we are investigating. Adair and Kornfeld used erythrocyte membranes that had not been phosphorylated with [γ-^{32}P]ATP. Their methodology does not allow the quantitative analyses needed to compare MyD patients and controls since the solubilization of erythrocyte membranes was only partial. They applied large amounts of protein to the Ricin I column in order to increase the specificity of the column, and some of the protein receptors may have been eluted by the relatively high salt concentrations used to wash the column. In addition, the borate buffer used can form complexes with carbohydrate and inhibit the binding of some of the protein receptors to the Ricin I column. In this series of experiments, we have defined the conditions that allow a more complete solubilization of the erythrocyte membrane proteins with a nondenaturing detergent and have developed an affinity chromatography procedure that uses sequential elution from a Ricin I-Sepharose 4B column. Our method is based on increasing salt concentration in the absence and presence of an appropriate concentration of D(+) galactose in order to separate the nonspecifically bound glycoprotein from the specifically bound. Using this approach, we have isolated three definable glycoprotein fractions, one of which demonstrates decreased phosphorylation in MyD.

Materials and Methods

Materials

Blood was withdrawn by venipuncture in the presence of heparin and used within a period of 24 hr. CNBr[2]-activated Sepharose 4B was obtained as a lyophilized powder from Pharmacia Fine Chemicals; Triton X-100 was purchased from Rohm and Haas; ATP disodium salt, EGTA[3], D(+)galactose and trizma base and ovomucoid were obtained from

[1] ATP—adenosine 5′ triphosphate.

[2] CNBr—cyanogen bromide.

[3] EGTA—ethylene glycol bis (β-aminoethyl ether) N,N′-tetra acetic acid.

Sigma Chemical Company; [γ-^{32}P]ATP was prepared from ATP and [^{32}P]orthophosphoric acid according to a modified procedure of Glynn and Chappell (1964). Asialofetuin was a generous gift from Dr. Peter Jeffrey. Ricin I was purified from castor bean according to a procedure of Nicolson *et al.* (1974) and was stored as a crystalline suspension in 60% $(NH_4)_2SO_4$. Ricin I thus prepared was very stable, and material stored longer than 6 months required no higher concentration to agglutinate human erythrocyte cells than that of freshly isolated Ricin I. Ricin I free of $(NH_4)_2SO_4$ was obtained by gel filtration through a calibrated Sephadex G-25 (Coarse) column. The purified Ricin I was greater than 97–98% pure, as determined by densitometry measurement of Coomassie blue stained SDS[4]-polyacrylamide gel (Fig. 1, gel *A*). In the absence of β-mercaptoethanol, Ricin I migrated to the expected position on SDS-polyacrylamide gel, i.e., one related to an apparent molecular weight of 120,000 (Nicolson *et al.*, 1974). In the presence of β-mercaptoethanol, Ricin I fragmented into polypeptides having apparent molecular weights similar to those reported by Nicolson *et al.* (1974) (Fig. 1, gel *B*). Bovine *serum albumin Fraction IV* (M_r, 68,000), carbonic anhydrase (M_r, 29,000), β-galactosidase grade *IV* (M_r, 115,000), glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle (M_r, 36,000), ovalbumin grade *VI* (M_r, 43,000) and phosphorylase *a* from rabbit muscle (M_r, 100,000) were used as molecular weight markers for SDS-PAGE[5] and were purchased from Sigma Chemical Company. Reagents for separation of SDS-polyacrylamide gels were ultra pure and were obtained from Bio-Rad Laboratories. Coomassie blue was purchased from Schwarz/Mann. Other reagents were reagent grade.

Preparation of Erythrocyte Membranes and Phosphorylation

Erythrocyte membranes were prepared from freshly withdrawn blood according to a procedure of Dodge, Mitchell and Hanahan (1963) with 5 mM sodium phosphate buffer, pH 8.0, as the lysis medium. The phosphorylation of erythrocyte membranes was carried out according to a slightly modified procedure of Roses and Appel (1975). Erythrocyte membranes were washed once with 10–12 volumes of cold 50 mM sodium acetate, 10 mM $MgCl_2$, 0.6 mM EGTA buffer, pH 6.5, and diluted to a protein concentration of 2–2.5 mg/ml. A solution of [γ-^{32}P]ATP (50 nm/ml, 1.6–2.4 $\times$ 10^7 cpm/nm) was added to a final concentration of 1.1–3.5 nm/ml and the resulting mixture gently stirred for 30 min at room temperature. Erythrocyte membranes were centrifuged at 27,000 $\times$ *g* for 15 min, washed twice with 10–12 volumes of 5 mM cold sodium phosphate, 10 mM EDTA buffer, pH 8.0, and three times with 10–12 volumes of 5 mM cold sodium phosphate buffer, pH 8.0. This washing procedure was essential for the complete solubilization needed for affinity chromatography. *[^{32}P]phosphoserine bonds were* demonstrated by high voltage electrophoresis using previously reported methods (Roses & Appel, 1973; Allerton & Perlmann, 1965).

Coupling of Ricin I to Sepharose 4B

The procedure followed was based on a procedure provided by Pharmacia Fine Chemicals. Fifteen grams of lyophilized powder of CNBr-activated Sepharose 4B were suspended in one liter of 1 mM HCl for 15 min and filtered on a sintered glass funnel under vacuum. The wet gel was rapidly washed three times with 100–150 ml of deionized water

[4] SDS—sodium dodecyl sulfate.

[5] SDS-PAGE—sodium dodecyl sulfate polyacrylamide gel electrophoresis.

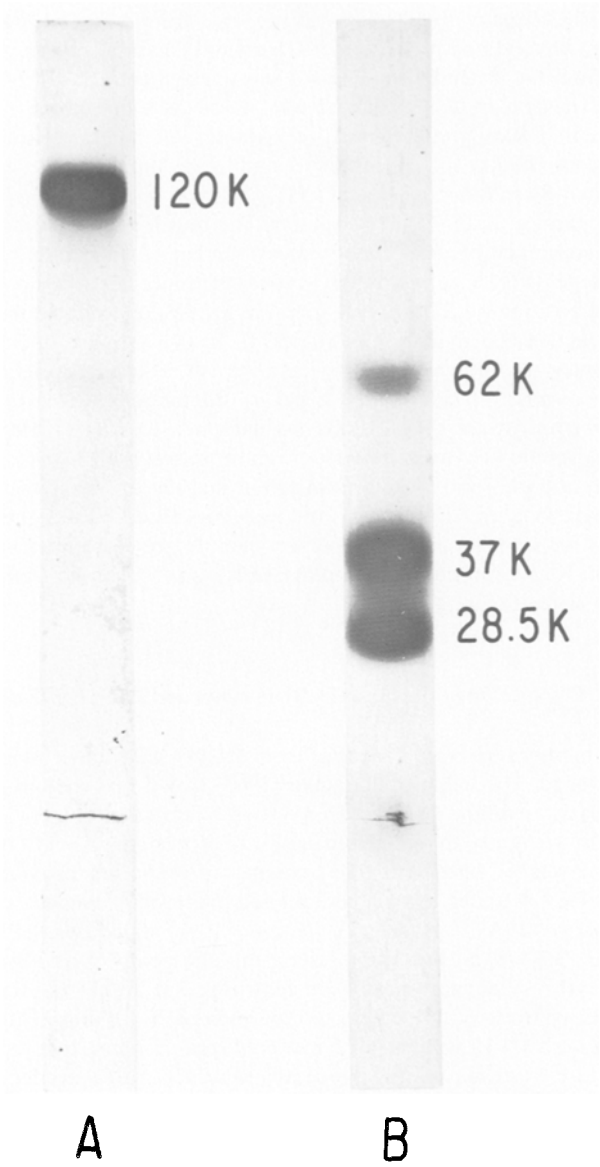


Fig. 1. SDS-PAGE of Ricin I in the absence (*A*) and presence (*B*) of β-mercaptoethanol

and mixed with 75 ml of 500 mM NaCl, 100 mM D(+)galactose, 100 mM $NaCHO_3$ buffer, pH 8.5, containing 150 mg of Ricin I. After a period of 2 hr at room temperature with continuous shaking, the gel was spun at 1000 × *g* for 1 min and suspended in 60 ml of 1 M ethanolamine solution, pH 8.0, for a period of 2 hr at room temperature or overnight at 4°C. The gel was then washed by three cycles of 200 ml in each solution of 1 M NaCl, 100 mM sodium acetate buffer, pH 4.0, deionized water, 1 M NaCl, 100 mM sodium borate buffer, pH 8.0, deionized water, and was equilibrated with 0.625 mM sodium phosphate, 0.1%

Triton X-100 buffer, pH 8.0. Under the above conditions, greater than 99% of Ricin I was coupled to Sepharose 4B.

Spectrin Extraction

Spectrin and Band 5 were extracted before affinity chromatography (Roses *et al.*, 1976). The procedure followed began with mixing the washed erythrocyte membranes with seven volumes of deionized water. After the mixture stood for a period of 20 min at room temperature or overnight at 4°C, the mixture was centrifuged at $1.44 \times 10^5 \times g$ for 20 min. Under these conditions, only minimal extraction of Band 3 and other proteins was observed. The pellet after extraction was suspended in 5 mM sodium phosphate buffer, pH 8.0, at a protein concentration of approximately 2 mg/ml and solubilized with seven volumes of cold 0.114% Triton X-100 solution. The solution was filtered by gravity through glass wool, and the filtrate was applied on the Ricin I-Sepharose 4B column. We initially attempted to extract spectrin with seven volumes of 0.1 mM EDTA solution, pH 8.0, but this method resulted in extraction of some Band 3 material as well as the major glycoprotein.

Affinity Chromatography on Ricin I-Sepharose 4B

The affinity chromatography was carried out at 4°C with a column of Ricin I-Sepharose 4B of 1.5 × 15 cm. The solubilized pellet, from which spectrin and Band 5 had been extracted, was applied on the Ricin I-Sepharose 4B column at a flow rate of 20–25 ml/hr, and the column was washed with 0.625 mM sodium phosphate, 0.1% Triton X-100 buffer, pH 8.0, until the radioactivity was that of the background. The column was then eluted in stages with freshly prepared buffer solutions of 0.625 mM sodium phosphate, 0.1% Triton X-100, pH 8.0, that contained varying concentrations of NaCl as well as the haptenic sugar of Ricin I, D(+)galactose. The fractions collected during elution with salt and sugar solutions were dialyzed against two changes of 4 liters of 0.5 mM sodium phosphate buffer, pH 8.0, at 4°C for 16–20 hr and lyophilized. The residues obtained were dissolved in an appropriate volume of deionized water for storage at −20°C.

To regenerate the Ricin I-Sepharose 4B column, two procedures were followed: in one procedure the column was eluted with 300–400 ml of 0.625 mM sodium phosphate, 0.1% Triton X-100 buffer, pH 8.0. The second procedure was to wash the column 3 times and centrifugate at approximately $3000 \times g$ for 1 min with 5 to 10 volumes of 0.625 mM sodium phosphate, 0.1% Triton X-100 buffer, pH 8.0. Under the above conditions the affinity column was found to be stable at 4°C for a period of at least six months.

SDS-PAGE

SDS-polyacrylamide gels were prepared according to a procedure of Weber and Osborn (1969). The electrophoresis was carried out at room temperature and at a constant voltage of 25 V until the tracking dye was approximately 1 cm from the lower edge of the gel. Samples before electrophoresis were heated at 90°C for 5 min in a freshly prepared solution of 3% SDS, 1% β-mercaptoethanol, 5 mM EDTA, 10 mM sodium phosphate, pH 7.2. To stain the proteins, the gels were transferred into test tubes containing 15 ml of 0.25%

Coomassie blue solution in methanol/water/acetic acid (227:227:46, v/v) and shaken for 3 hr at 37°C; they were then transferred in 25 ml erlenmeyer flasks containing 12 ml of 5% methanol and 7.5% glacial acetic acid solution and 0.5–1.0 g of AG 501-X-8 resin (Bio-Rad Laboratories) and shaken at 37°C until the background was free of Coomassie blue (12–24 hr). Glycoproteins were stained with PAS[6] according to a procedure of Zacharius *et al.* (1969). To measure the radioactivity along the gels, gels were lightly frozen after electrophoresis, cut into slices of 0.2 cm, oxidized to a soluble form with 0.2 ml of 30% hydrogen peroxide at 90°C for 4 hr [modification of the method of Tishler and Epstein (1968)] and counted in an appropriate scintillation fluid.

Protein and Sialic Acid Determination

Protein was determined by the method of Lowry *et al.* (1951). In samples containing Triton X-100, SDS and Triton X-100 were added at a final concentration of 5% and 0.1%, respectively (to the sodium carbonate solution). Sialic acid was determined by the thiobarbituric assay after acid hydrolysis with 0.1 N H_2SO_4 for 1 hr at 80°C (Warren, 1959).

Results

Scheme Used for Purification of Glycoprotein Fractions

Scheme I was the standard procedure used for the purification of glycoproteins that specifically bind to Ricin I. After the erythrocyte membranes were prepared, an endogenous protein kinase incubation was performed. Spectrin (Bands 1 and 2) and Band 5 were extracted and the pellet was solubilized as described under *Materials and Methods* in *Spectrin Extraction.* The solubilized material was applied to the Ricin I-Sepharose 4B column for sequential elution and analyses.

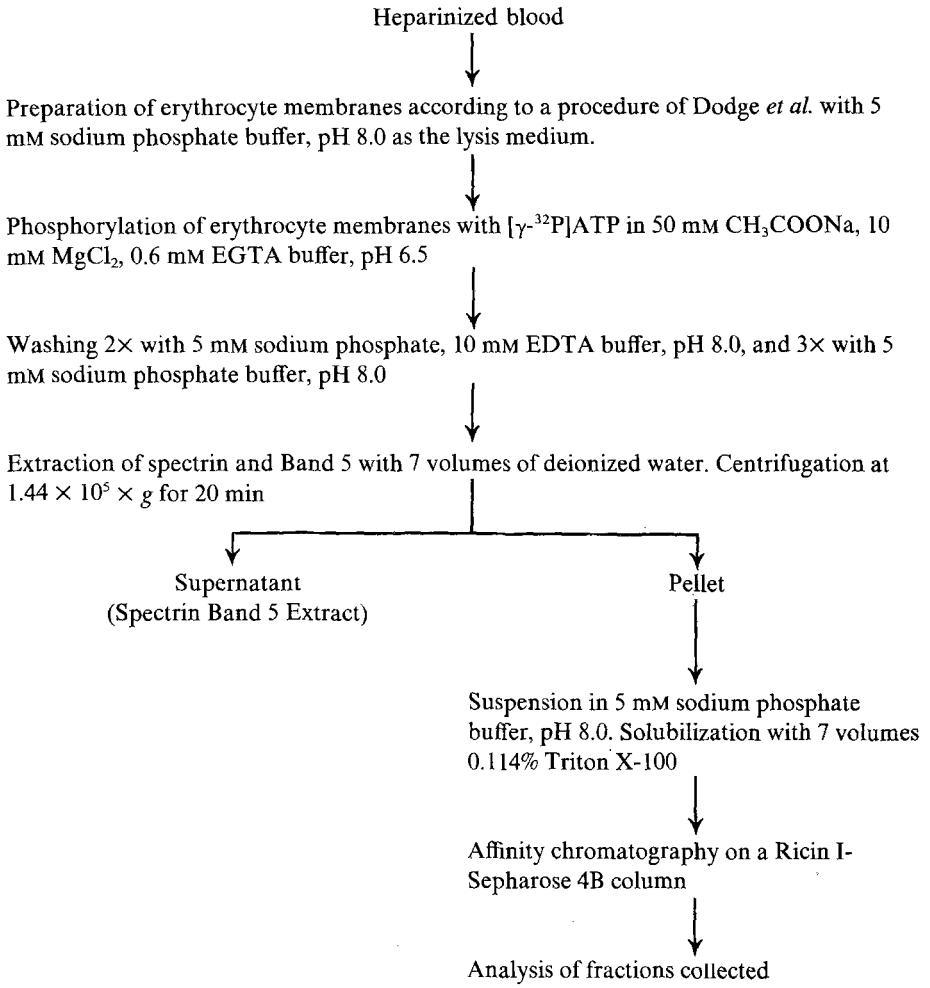
Solubilization Procedures

Several methods for the solubilization of erythrocyte membranes have been published. Complete solubilization in the presence of nonionic detergents that would not interfere with affinity chromatography procedures required initial spectrin and Band 5 extraction. Figure 2 illustrates that spectrin and Band 5 extraction was selective and almost complete (Gels *2* and *3*). The remaining pellet was soluble in 0.1% Triton X-100 (Fig. 2, gels *4* and *5*), but not in 0.001% or 0.01% Triton X-100, as indicated by the protein profiles on SDS-PAGE (Fig. 2, gels *6–9*).

Conditions reported by Yu, Rischman and Steck (1973) to allow selective solubilization of Band 3 were effective but not satisfactory for the purpose of comparing MyD and control erythrocytes since Band 3 was incompletely extracted by these methods. Indeed, under certain conditions of extraction, such as in the pres-

[6] PAS—periodic acid Schiff base.

Scheme I.



ence of 5 or 36 mM sodium phosphate buffer, the solubilization of Band 3 was relatively specific but very limited (Fig. 3, gels *5–8*). Furthermore, there was an irreversible precipitation of the proteins in the extract. Under other conditions using borate buffer, an extensive solubilization occurred but it was not selective, compared to the solubilization of the pellet after spectrin and Band 5 extraction (Fig. 3, gels *3* and *4 vs.* Fig. 2, gels *4* and *5*). Table I summarizes the solubilization of the erythrocyte membrane under the various conditions investigated.

Affinity Chromatography in the Presence of Detergent

Ovomucoid, a glycoprotein used to purify Ricin I (Lotan *et al.*, 1977), and asialofetuin, a glycoprotein having galactose as the terminal sugar, were quan-

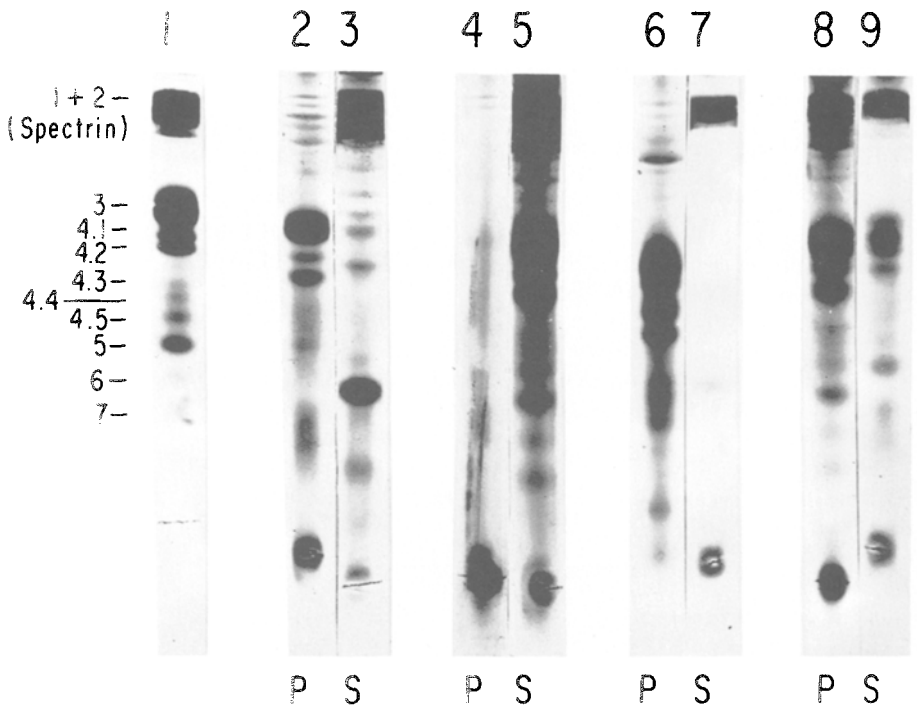


Fig. 2. Extraction of the phosphorylated proteins of the human erythrocyte membranes (gel *1*) with deionized water (gels *2* and *3*), 0.1% Triton X-100 (gels *4* and *5*), 0.001% Triton X-100 (gels *6* and *7*) and 0.01% Triton X-100 (gels *8* and *9*). *P* and *S* refer to proteins of pellet and supernatant, respectively, after extraction. Conditions for extraction are described in Table 1

titatively and specifically retained on the Ricin I-Sepharose 4B column when applied at levels below the capacity of the column in the presence of 0.1% Triton X-100 (capacity: approximately 0.5 and 1.0 mole of ovomucoid and asialofetuin per mole of Ricin I bound covalently to the Sepharose 4B column, respectively). These experiments demonstrated that this concentration of 0.1% Triton X-100 did not affect the binding properties of Ricin I. Lotan *et al.* (1977) have recently reported the effects of detergent concentration on the ability of various lectins to agglutinate erythrocytes as well as the effect of detergent and salt concentrations on the ability of various lectin columns to bind asialofetuin. They also demonstrated no effect of 0.1% Triton X-100 on the specific binding of Ricin I.

Affinity Chromatography on Ricin I-Sepharose 4B

The sequential elution profile of the Triton X-100 solubilized proteins of the erythrocyte membranes is demonstrated in Fig. 4. Radioactivity measurements rather than protein determinations were used routinely because of the absorbance

of Triton X-100 at 280 nm and the interference of D-galactose with the Lowry protein assay. Table 2 presents a summary of recovery of proteins during the sequential affinity chromatography procedure. A recovery of 85–90% of Triton X-100 solubilized protein applied to the Ricin I-Sepharose 4B column was routinely achieved. The remainder was either retained irreversibly on the column or lost during subsequent dialysis for protein determination. Approximately 60% of the retained material eluted specifically with D-galactose. Glucose, which does not bind to Ricin I, was found to have no effect on the elution profile (Fig. 5).

Table 2 also reports the sialic acid recovery during sequential elution affinity chromatography. Most of the sialic acid of the erythrocyte membrane is associated with the major sialoglycoprotein, and not with Band 3 glycoprotein. Approximately 80% of the total sialic acid was routinely recovered, with 26% eluting

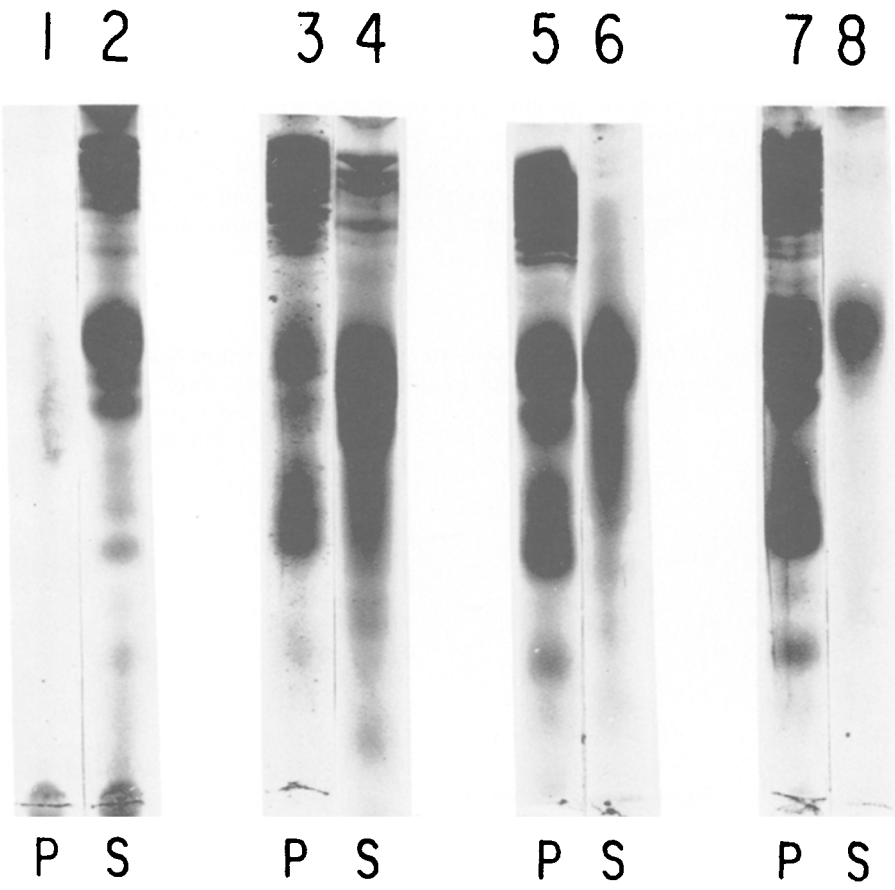


Fig. 3. Extraction of the phosphorylated proteins of the human erythrocyte membranes in the presence of 0.5% Triton X-100 (gels *1* and *2*); 0.5% Triton X-100, 56 mM sodium borate buffer, pH 8.0 (gels *3* and *4*); 0.5% Triton X-100, 5 mM sodium phosphate buffer, pH 8.0 (gels *5* and *6*); 0.5% Triton X-100, 36 mM sodium phosphate buffer, pH 7.5 (gels *7* and *8*). *P* and *S* refer to proteins of pellet and supernatant, respectively, after extraction. Conditions for extraction are described in Table 1

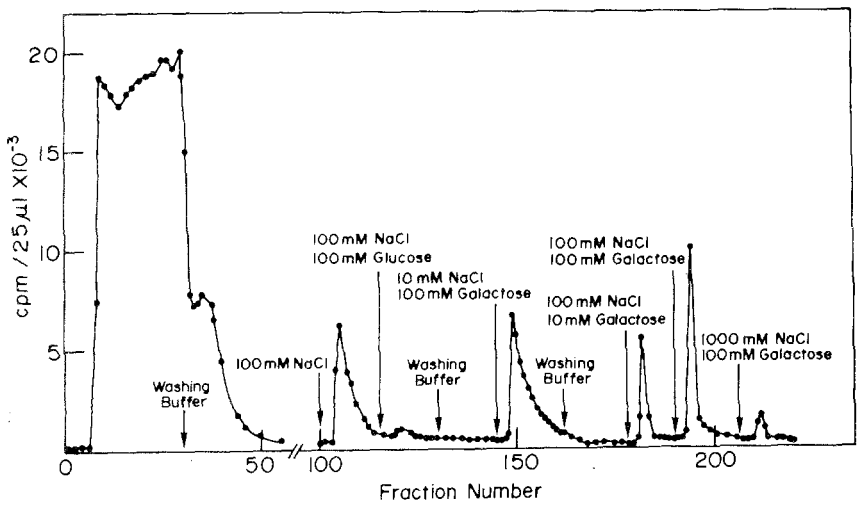


Fig. 4. Radioactivity profile of solubilized proteins of the erythrocyte membranes eluted on a Ricin I-Sepharose 4B column after phosphorylation with [γ-^{32}P]ATP. After spectrin and Band 5 extraction, the pellet (40 mg protein) was suspended in 5 mM sodium phosphate buffer, pH 8.0, solubilized with seven volumes of 0.114% Triton X-100, and applied on a Ricin I-Sepharose 4B column. Column was then eluted as described

Table 1. Effects of Triton X-100 concentration and buffered Triton X-100 solution on the extraction of the proteins of erythrocyte membranes

Extracting solution	μ	Protein distribution (%)	
		Pellet	Supernatant
Deionized H_2O 4 °C	.00175	74	26
Deionized H_2O room temperature	.00175	76	24
Deionized H_2O 37°	.00175	73	27
0.001% Triton X-100	.00175	84	16
0.01% Triton X-100	.00175	64	36
0.1% Triton X-100	.00175	1.3	98.7
0.5% Triton X-100	.00175	3.1	96.9
0.5% Triton X-100 56 mM sodium borate buffer, pH 8.0	.0027	38	62
0.5% Triton X-100 5 mM sodium phosphate buffer, pH 8.0	.0138	58	42
0.5% Triton X-100 36 mM sodium phosphate buffer, pH 7.5	.080	57	43

One volume of erythrocyte membranes in 5 mM sodium phosphate buffer, pH 8.0, was mixed with seven volumes of extracing solution. After 20 min at room temperature, or 37° or overnight at 4°, the mixture was centrifuged at $1.44 \times 10^5 \times g$ for 20 min. Pellets and supernatant were analyzed for protein according to Lowry's assay.

Table 2. Protein and sialic acid recovery during affinity chromatography on a Ricin I-Sepharose 4B column

Fraction	Recovery (%)	
	Protein	Sialic acid
Fraction applied	100	100
Fraction nonretained	69.8 ± 6.4 (11)	43.0 ± 6.9 (5)
Fraction eluted with 100 mM NaCl	7.3 ± 1.8 (11)	11.9 ± 1.3 (5)
Fraction eluted with 10 mM NaCl, 100 mM D(+)galactose	5.4 ± 1.5 (11)	25.5 ± 4.2 (5)
Fraction eluted with 100 mM NaCl, 10 mM D(+)galactose	3.3 ± 1.9 (14)	1.0 ± 0.6 (3)
Fraction eluted with 100 mM NaCl, 100 mM D(+)galactose	2.5 ± 1.6 (14)	0.5 ± 0.5 (3)
Fraction eluted with 1000 mM NaCl, 100 mM D(+)galactose	0.72 ± 0.6 (6)	0.25 ± 0.25 (2)

specifically with the 10 mM NaCl, 100 mM D-galactose (10/100) fraction and 54% with the nonretained fractions. Rechromatography of the proteins specifically eluted with D-galactose was attempted but was unsuccessful using the elution conditions of the first chromatography. The rechromatographed material was irreversibly retained on the column and could not be eluted with a solution of high salt concentration or solutions buffered at pH 4.0–5.0. The basis of this strong binding is not clearly understood. However, the retention of these proteins compared to those in the original procedure that were not retained suggests that dialysis done between the two chromatography procedures (in order to remove D-galactose) causes the formation of aggregates. These aggregates would bind more strongly than the individual glycoprotein molecules because they have more carbohydrate side chains (Helenius & Simons, 1975).

SDS-PAGE of Fractions Collected During Affinity Chromatography

The fractions obtained by the sequential elution affinity chromatography procedure were submitted to SDS-PAGE in order to analyze the polypeptides. The results are summarized in Figs. 5 and 6, in which identical gels are stained with Coomassie blue and the PAS reagent, respectively. A portion of the major sialoglycoprotein which stains with PAS and migrates with an apparent mol wt of 76–80,000 daltons was retained on the Ricin I. The specifically retained major sialoglycoprotein was concentrated in the fraction eluted with 10 mM NaCl, 100 mM D-galactose (10/100) and was absent in later elutions with 100 mM NaCl. Gel *5* in Figs. 5 and 6 demonstrates the 10/100 fraction. Significant amounts of sialic acid were present in nonretained fractions, but the concentration was not as high as in the 10/100 fraction, and PAS staining could not be appreciated in Gel *2* of Fig. 6. This major sialoglycoprotein fraction was specifically retained since glucose had no effect on its elution (Gel *3*, Figs. 5 and 6). Prior elution with 100 mM

NaCl removed additional nonspecifically retained glycoprotein (Gel *4*, Figs. 5 and 6).

Two distinct fractions of Band 3 (M_r 93,000) were also retained on the Ricin I column and could be specifically eluted at moderate salt concentrations (100 mM NaCl) in the presence of 10 mM D-galactose (100/10) and 100 mM D-galactose (100/100), respectively (Table 2). The gel-staining characteristics of both fractions were identical (Gel *6*, Figs. 5 and 6). Glucose had no effect on the elution of either fraction. Differences between the 100/10 and 100/100 fractions will be discussed below under *Comparison of Control and MyD Patients.*

Phosphorylation of Proteins Specifically Eluted with D-galactose

Following SDS-PAGE the gels were cut and radioactivity was measured. Figure 7 illustrates the typical radioactive profile. The major sialoglycoprotein re-

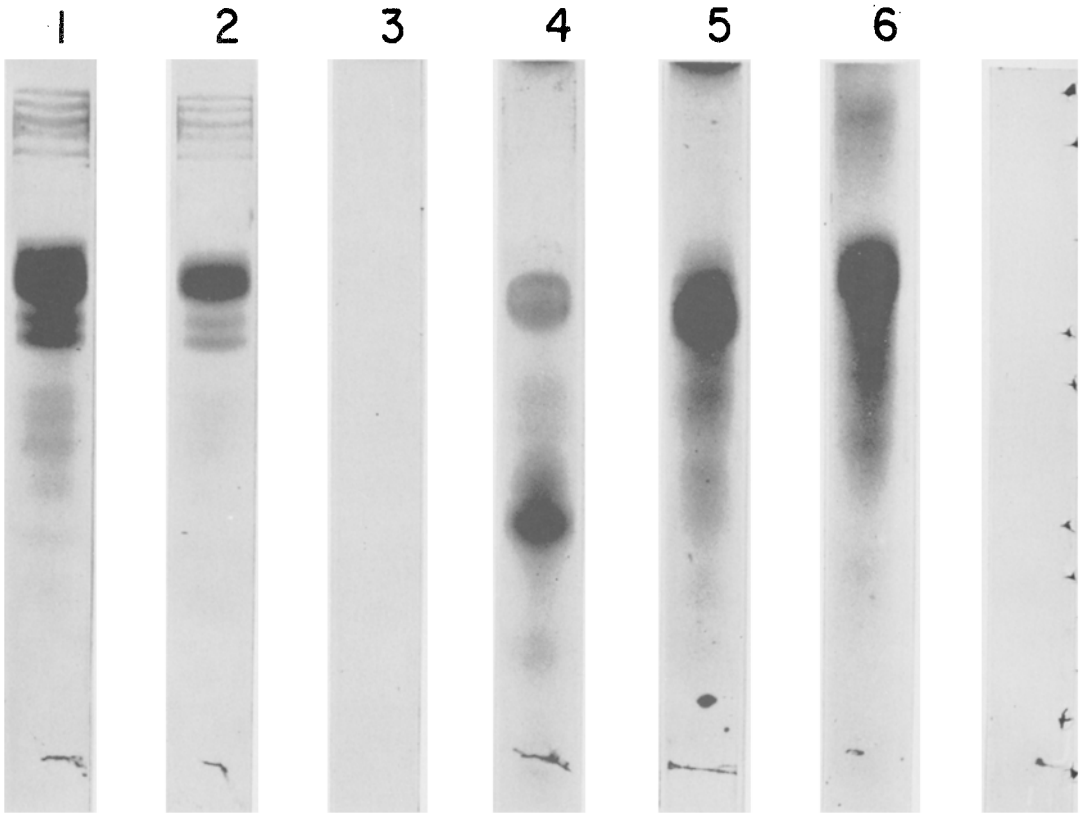


Fig. 5. SDS-PAGE of fractions collected during affinity chromatography on a Ricin I-Sepharose 4B column. Staining with Coomassie blue. Gel *1*, fraction applied; gel *2*, fraction nonretained; gel *3*, elution with 100 mM NaCl, 100 mM glucose; gel *4*, elution with 100 mM NaCl; gel *5*, elution with 10 mM NaCl, 100 mM D(+)galactose; gel *6*, elution with 100 mM NaCl, 100 mM D(+)galactose; gel *7*, elution with 1000 mM NaCl, 100 mM D(+)galactose

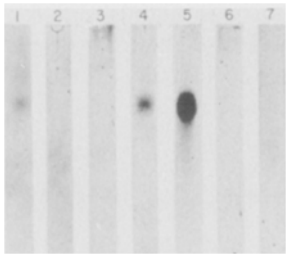


Fig. 6. SDS-PAGE of fractions collected during affinity chromatography on a Ricin I-Sepharose 4B column. PAS staining. Gel *1*, fraction applied; gel *2*, fraction nonretained; gel *3*, elution with 100 mM NaCl, 100 mM glucose; gel *4*, elution with 100 mM NaCl; gel *5*, elution with 10 mM NaCl, 100 mM D(+)galactose; gel *6*, elution with 100 mM NaCl, 100 mM D(+)galactose; gel *7*, elution with 1000 mM NaCl, 100 mM D(+)galactose. There was no strong PAS staining of gel *2* since sialic acid containing glycoproteins represent a small proportion of the total fraction. PAS staining can be demonstrated in overloaded gels

tained on Ricin I and specifically eluted (10/100) was phosphorylated (Fig. 7, Panel *C*). These data corroborate data obtained by Shapiro and Marchesi (1977) using [^{32}P]inorganic phosphate to label the major sialoglycoprotein of the erythrocyte membrane.

Both fractions of Band 3 were phosphorylated (Fig. 7, Panel *D*), but the rela-

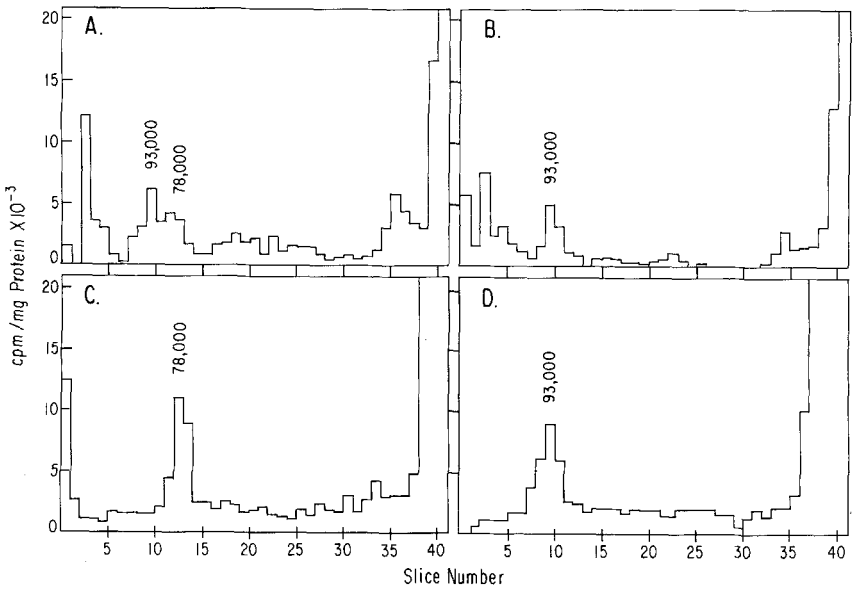


Fig. 7. Radioactivity profile on SDS-PAGE of proteins eluted specifically with D(+)galactose. (*A*): fraction applied on the Ricin I-Sepharose 4B column; (*B*): fraction nonretained; (*C*) elution with 10 mM NaCl, 100 mM D(+)galactose; (*D*): elution with 100 mM NaCl, 100 mM D(+)galactose. Radioactivity at bottom of gels represents phospholipid associated with the intrinsic membrane proteins in Triton X-100, but removed by SDS (Roses *et al.*, 1976)

tive specific phosphorylation was slightly different in the 100/10 and 100/100 fractions. Approximately 5-10% of the total Band 3 radioactivity was associated with the fractions of Band 3 specifically eluted with D-galactose; the remainder was associated with nonretained Band 3 material.

Comparison of Control and MyD Patients

The fractionation of erythrocyte membrane proteins and glycoproteins from patients with MyD was identical in every respect to that of controls, with one exception. All fractions yielded the same protein and sialic acid yields, and identical [^{32}P]phosphoprotein profiles (phosphorylation per milligram). The only difference was the relative specific phosphorylation of the 100/100 Band 3 fractions.

Table 3 reports the relative specific activity of the three glycoprotein fractions isolated by Ricin I affinity chromatography. There were no differences in the glycoprotein yields of any fraction. The 10/100 (major sialoglycoprotein) and 100/10 (Band 3) fractions similarly demonstrated no differences in specific phosphorylation. There was, however, a highly significant decrease in the specific phosphorylation of the 100/100 (Band 3) fraction of MyD patients.

Table 4 reports the data for each of seven consecutive experiments in which seven patients from different pedigrees were matched (age and sex) with seven controls. In each experiment the 100/10 and 100/100 fractions represent sequential elutions of the same membrane material from the same column; thus the 100/10 fractions serve as internal controls for the 100/100 fractions. Phosphorylation of MyD 100/100 Band 3 was approximately half that of controls. These data extend previous experiments (Roses & Appel, 1975) and are discussed below.

Discussion

Previous results from this laboratory provide the rationale for the present series of experiments. We had earlier observed that abnormal phosphorylation might represent a clinical marker, and more importantly, might reflect or be related to to the underlying biochemical defect in MyD. Using lithium diiodosalicylate extraction techniques, we had established a difference in phosphorylation in Band 3 glycoproteins (Roses & Appel, 1975). Although alterations in the enzyme or changes in the three dimensional relationship of Band 3 to the protein kinase enzyme have not been ruled out as explanations, we chose to test the hypothesis that an alteration was present in the substrate. The sequential affinity chromatography experiments described in this manuscript were designed to fractionate Band 3 glycoprotein by taking advantage of its carbohydrate heterogeneity. Our purpose was the isolation of an abnormally phosphorylated Band 3 glycoprotein in MyD. The present studies represent one of a series of experiments using several lectins including Concanavalin A, Ricin II, and wheat germ agglutinin. None of these other lectins yielded a fraction that showed a difference in phosphorylation in MyD membranes as compared to controls (Roses, 1976).

These comparative experiments depended on a complete solubilization of

Table 3. Phosphorylation of glycoprotein fractions retained on Ricin I-Sepharose 4B

Elution mM NaCl, mM D-galactose	Phosphorylation (pmol ^{32}P/mg protein SD)		
	Control	MyD	Ratio[b]
10/100 (major sialoglycoprotein)	3.6 ± 1.3 (5)[a]	4.0 ± 2.0 (5)	0.9
100/10 (93 K glycoprotein)	5.3 ± 3.4 (6)	5.1 ± 3.1 (6)	1.0
100/100 (93 K glycoprotein)	4.4 ± 1.9 (7)[c]	2.2 ± 0.75 (7)[d]	2.0

[a] Number of experiments in parenthesis.

[b] Ratio of $\frac{\text{pmol/mg control}}{\text{pmol/mg MyD}}$.

[c] Mean protein yield = 2.7 ± 1.9% (7).

[d] Mean protein yield = 2.8 ± 1.7% (7).

Band 3 intrinsic membrane glycoproteins and on proof that lectin specificity was unaffected by the concentration and properties of the detergent. As demonstrated in Figs. 2 and 3, several previously reported solubilization techniques were unsuitable for comparative experiments. Ricin I affinity chromatography allowed separation of three phosphorylated glycoprotein fractions: 10/100, the major sialoglycoprotein; 100/10 and 100/100, two Band 3 fractions that could be identical except that the phosphorylation of MyD Band 3 is significantly decreased in only one of them (100/100).

Table 4. Phosphorylation of the Band 3 glycoprotein eluted in each experiment

Experiment Number	Phosphorylation (pmol ^{32}P/mg protein)			
	100/10 Fractions		100/100 Fractions	
	Control	MyD	Control	Myd
1	3.8	1.2	5.8	1.9
2	11.0	7.2	6.6	2.5
3	−[+]	−[+]	4.6	2.5
4	2.2	5.0	2.3	2.0
5	5.9	7.6	2.6	1.6
6	6.7	8.0	6.5	3.6
7	1.9	1.5	2.5	1.3
Mean	5.3	5.1	4.4	2.2
SD	±3.4	±3.1	4.4	2.2
P[a]	NS		<.01	
P[b]	NS		<.001	

[a] Student's t test.

[b] Paired t test.

Tanner and Anstee (1976) demonstrated that a mixture of ^{125}I-Ricin I and ^{125}I-Ricin II bound specifically to the region of migration of Band 3 and the major sialoglycoprotein on SDS-PAGE. The failure of Adair and Kornfeld (1974) to find receptors for Ricin I on the major sialoglycoprotein in their affinity chromatography system has several possible explanations. Our experiments suggest that Adair and Kornfeld's use of relatively high salt concentration to wash the Ricin I column may have eluted the major sialoglycoprotein. Further, borate buffers, also used in their experiments, can form complexes with carbohydrate and thus inhibit the binding of the major sialoglycoprotein to the Ricin I column.

In our experiments the glycoproteins in the 10/100, 100/10, and 100/100 fractions represent only a fraction of the total Band 3 and major sialoglycoprotein present in the erythrocyte membrane. Band 3 proteins as well as large amounts of the major sialoglycoprotein remained in the nonretained fractions. Since almost all of the sialic acid of the erythrocyte membrane is associated with the major sialoglycoprotein (Winzler, 1969), only a fraction of this material is therefore specifically retained on the column. This result cannot be attributed to overloading the Ricin I column since a two- or threefold increase in the total amount of protein applied resulted in a proportional increase in the specifically eluted Band 3 and major sialoglycoprotein.

Such differences in ability to bind to Ricin I may be explained by one of three properties summarized below. The basis for differences in carbohydrate moieties among proteins of Band 3 is not completely understood at this time. One possibility is that Band 3 and the major sialoglycoprotein may exist in an aggregative or conformational state which may not allow the binding to the Ricin I column through their carbohydrate moieties. It is possible that intrinsic membrane proteins, such as Band 3 and the major sialoglycoprotein, may exist in different aggregative or conformational states depending on the conditions used to solubilize them with detergents.

A second possible explanation is that certain Band 3 proteins and the major sialoglycoprotein lack the carbohydrate receptors for Ricin I as a result of glycoprotein carbohydrate heterogeneity. The carbohydrate moieties of the major sialoglycoprotein have been shown to vary, depending on the age of the erythrocyte cells (Marikovsky, Danon & Katchalsky, 1966; Balduini & Ascari, 1974). Carbohydrate heterogeneity may also be an explanation for the Band 3 glycoprotein since affinity chromatography with the lectin, Concanavalin A (Roses, 1976; Findlay, 1974), and the binding of ^{125}I-Concanavalin A to the electrophoretic region of migration of Band 3 (Kawaguchi & Osawa, 1976) have demonstrated that Concanavalin A can interact with only a fraction of Band 3 (approximately 30%). The use of galactose oxidase with tritiated sodium borohydride also indicated a carbohydrate heterogeneity in proteins of Band 3 since the peak of incorporation of tritium in the electrophoretic region of migration of isolated Band 3 lags slightly behind the Coomassie blue peak of Band 3 (Yu & Steck, 1975*a*).

A third explanation for differences in carbohydrate properties is that Band 3 represents a composite of structurally different apoproteins of similar molecular weight but involved in different functions. Some of these may not have carbohydrate receptors for Ricin I or for other particular lectins.

A number of authors have suggested that Band 3 may be composed mainly of a

single type of polypeptide chain (Steck, Fairbanks & Wallach, 1971). Cross-linking studies with bifunctional or oxidizing agents (Wang & Richards, 1974; Yu & Steck, 1975*b*; Steck, Ramos & Strapazon, 1976) as well as fragmentation studies with protease and low molecular weight reagents of proteins (Steck *et al.*, 1971, 1976; Drickamer, 1976; Fukuda, Eshadt & Marchesi, 1977) have supported this view. A number of the reported features of Band 3 need to be explained in light of the suggestion that Band 3 is mainly composed of a single type of polypeptide chain (Steck *et al.*, 1971). Band 3 has been demonstrated to be heterogeneous with respect to its ability to accept the gamma (γ) phosphate group of ATP, as indicated by the relatively low level of phosphorylation in the relatively large fraction of Band 3 retained on a Concanavalin A column (Roses, 1976). Although there are, as yet, no published studies of Band 3 glycoproteins that have been fractionated according to the carbohydrate moiety and analyzed structurally, we have found differences in the peptide maps of isolated Band 3 glycoproteins (P. Wong & A.D. Roses, *unpublished*).

The level of incorporation of the gamma (γ) phosphate of ATP into Band 3 and the major glycoprotein was found to be relatively low. For those fractions retained on the Ricin I column less than 0.1 moles ^{32}P per mole Band 3 were incorporated. This low level of [^{32}P]incorporation may be a function of only a small fraction of sites of Band 3 and the major glycoprotein that are available to receive the radioactive phosphate. Both glycoproteins may exist *in situ* with available sites already phosphorylated. Spectrin has approximately 2 moles of phosphate per mole of spectrin and only accepts less than 0.1 mole of the gamma (γ) phosphate of ATP per mole (Roses & Appel, 1976). The low level of [^{32}P]phosphorylation may also be due to a lack of accessibility of ATP and the protein kinase to portions of Band 3 and the major glycoprotein that are buried within the hydrophobic membrane. The heterogeneity and the level of phosphorylation are important to our learning what the biological functions of Band 3 are, and we certainly need more quantitative data before low levels of membrane protein [^{32}P]phosphorylation, as observed in MyD, can be used to explain membrane characteristics such as shape maintenance and transport functions.

Finally, what is the explanation for the decreased phosphorylation of MyD Band 3 glycoprotein in the 100/100 fraction (Table 4)? If this Band 3 fraction does indeed contain an altered glycoprotein substrate for the endogenous cyclic-AMP independent protein kinase, then there are at least two readily testable explanations. Either (i) the primary sequence of 50% of the molecules is mutated so that a serine or threonine is replaced by another amino acid that cannot be phosphorylated, or (ii) a serine or threonine in Band 3 may exist in an already phosphorylated state in the MyD membrane so that an additional ^{32}P cannot be accepted. These possibilities can be confirmed or rejected in future experiments. The 100/10 Band 3 fraction can serve as an internal control for structural analyses. In addition, questions concerning apoprotein differences in molecules, designated Band 3 by their apparent migration on SDS-PAGE, can be answered. Peptide mapping experiments using the 100/10 and 100/100 Band 3 fractions as well as Concanavalin A fractions are currently in progress in our laboratory (Roses & Wong, 1977; Wong & Roses, 1978, and *unpublished*).

The specificity of this postulated structural mutation of 50% of Band 3 glyco-

proteins eluted with 100/100 in autosomal dominantly inherited MyD can be evaluated in terms of genetic theory as well as biochemically. If a particular sequence abnormality can be demonstrated in an affected patient, the identical alteration *must* be present in *every* affected member of his pedigree. On the other hand several mutations involving the identical molecule may lead to disease. Any particular sequence abnormality must therefore be consistently found in affected individuals within pedigrees, but the abnormality need not be identical in all pedigrees (Roses & Wong, 1977; Wong & Roses, 1978, and *unpublished*). Large numbers of controls must now be examined to determine the range of structural polymorphisms within Band 3 fractions before the specificity of a membrane defect in myotonic dystrophy suggested by these data can be structurally defined.

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