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# Isolation of the Major Intrinsic Transmembrane Protein of the Human Erythrocyte Membrane

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*Summary.* The major intrinsic protein of the human erythrocyte membrane commonly referred to as "Band 3", was isolated by a multi-step procedure. Extraction of ghost membranes in dilute solutions of lithium diiodosalicylate removed most of the proteins considered to be extrinsic to the membrane. The resulting membrane fragments were solubilized in sodium dodecyl sulfate, and the major sialoglycoprotein (glycophorin A) was removed by wheat germ agglutinin-Sepharose affinity chromatography. Gel filtration in sodium dodecyl sulfate was used as the final step to yield the band 3 polypeptide in electrophoretically homogeneous form.

The distinction between extrinsic and intrinsic proteins of cell membranes proposed by Singer (1971) is a convenient and useful way to classify membrane proteins, and it has stimulated many investigators to design new ways to isolate membrane components. Early attempts to isolate membrane proteins en mass were notably unsuccessful, and these negative results led to the widespread feeling that the bulk of the proteins could not be isolated in a water-soluble form. Recently, it has become evident that if suitably prepared red blood cell membranes are exposed to different classes of solvents in a systematic manner, specific sub-groups of proteins can be isolated from the membranes.

Steck and Yu (1973) found that a variety of different solvents resulted in selective extraction of the *extrinsic* membrane proteins of human ghost membranes. Under their conditions the extracted membranes vesiculated, but lipid was not lost. In contrast, they found that nonionic detergents selectively extracted the *intrinsic* proteins and approximately 50% of the membrane phospholipid (Yu, Fischmann & Steck, 1973). This procedure apparently removed lipoprotein complexes from these membranes,

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and these were subsequently identified as band 3 and the sialoglycoproteins as well as a few minor components. Previous studies have shown that these two proteins are glycoproteins which are intimately associated with the lipid bilayer, and probably span the membrane (Bretscher, 1971*a, b*; Hubbard & Cohn, 1972; Gahmberg & Hakomori, 1973; Reichstein & Blostein, 1973; Mueller & Morrison, 1974; Shin & Carraway, 1974; Staros & Richards, 1974; Steck & Dawson, 1974; Whiteley & Berg, 1974; Itaya, Gahmberg & Hakomori, 1975).

The sialoglycopeptides have been characterized extensively (Winzler, 1969; Furthmayr, Tomita & Marchesi, 1975) and sequence data support the idea that they form an intrinsic protein which spans the membrane (Segrest, Kahane, Jackson & Marchesi, 1973; Tomita & Marchesi, 1975). The isolation of the band 3 polypeptide, which is the major intrinsic protein of the human red blood cell membrane on a mass basis, has been attempted previously, but with variable success (Tanner & Boxer, 1972; Ho & Guidotti, 1975). In order to prepare sufficient material for detailed chemical studies we have used a combination of selective extraction, lectin-affinity chromatography, and gel-filtration in SDS.

## Materials and Methods

### *Materials*

Reagents for polyacrylamide gel electrophoresis were those described previously (Furthmayr & Marchesi, 1976). Lithium 3,5-diiodosalicylate (LIS) was prepared from twice recrystallized 3,5-diiodosalicylic acid, purchased from Eastman (Marchesi & Andrews, 1971). Sodium dodecyl sulfate (SDS) was obtained from Alcolac Chemical Company (Baltimore, Maryland), N-acetyl-D-glucosamine from Sigma, Sepharose 4B and 6B from Pharmacia. Wheat germ agglutinin (WGA) and the WGA-Sepharose conjugate were prepared as described (Kahane, Furthmayr & Marchesi, 1976). [$^3$H]$NaBH_4$ was purchased from New England Nuclear.

### *Membrane Preparations*

Hemoglobin-free erythrocyte membranes were prepared from fresh human red blood cells in 5 mM Na phosphate, pH 8 (Fairbanks, Steck & Wallach, 1971; Furthmayr & Marchesi, 1976). The final wash was done in 25 mM Tris-HCl, pH 8. For the preparation of LIS-fragments, one volume of membranes (approximately 5–8 mg protein/ml) was mixed with one volume of 10 mM Tris-HCl, pH 8 and one additional volume of LIS prepared in the same buffer. Incubation was done at ice bath temperature for 20 min on a magnetic stirring apparatus, at final concentrations of LIS indicated under results. The LIS membranes were recovered by centrifugation in a Beckman JA-20 rotor at $48{,}000 \times g$ for one hour and used without further washing.

*WGA-Affinity Chromatography and Gel-Filtration in SDS*

The conditions for solubilization of the membrane fragments and for separation of the sialoglycopeptides on WGA-Sepharose were as reported (Kahane *et al.*, 1976) except that LIS-membranes were used instead of intact membranes. To summarize briefly, pelleted LIS-fragments were dissolved at a concentration of 2–4 mg protein/ml in 1% SDS containing 15 mM sodium phosphate, pH 7.2, 250 mM NaCl and 0.025% sodium azide. The solution was incubated for 15 min at 37 °C and an additional 5 min at 80 °C. After 20-fold dilution with the buffer, which did not contain SDS, the solution was filtered through a WGA-Sepharose column equilibrated with the buffer containing 0.05% SDS as the final concentration. The band 3 material was recovered entirely in the nonbound fraction. The sialoglycoproteins could be eluted by including 0.1 M N-acetyl-D-glucosamine into the buffer after extensive washes of the column to $A_{280} < 0.005$. To prevent crystallization of SDS at this high ionic strength, the buffer had to be kept at or above 25 °C. The nonbound fractions were pooled and dialyzed against distilled water at room temperature for 16 hr and then lyophilized. Gel-filtration was done on Sepharose 6B columns (95 × 2.5 cm) equilibrated at 45 °C in 1% SDS, 10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.05% $\beta$-mercaptoethanol at constant flow rate.

*Analytical Methods*

SDS-polyacrylamide gel electrophoresis and staining methods according to Fairbanks *et al.* (1971) and Maizel (1971) were used as described previously (Furthmayr & Marchesi, 1976). Protein was determined according to the method of Lowry, Rosebrough, Farr and Randall (1951). Sialic acid was assayed according to Warren (1959), hexoses according to Dubois, Gilles, Hamilton, Rebers and Smith (1956) and phospholipid phosphorus as described by Ames (1966). Since LIS interfered with some of these assays, all samples were dialyzed against 0.1% Triton X-100 prior to analysis. Amino acid and carbohydrate analysis was done as described by Tomita and Marchesi (1975). Carbohydrates were derivatized to trimethyl silyl methyl-glucoside (TMS) or analyzed by the alditol acetate method (Laine, Esselman & Sweeley, 1972; Reinhold, 1972) on a Hewlett-Packard 5710A gas chromatograph.

*Labeling Methods*

Incorporation of [$^3$H] into sialic acid of intact cells after mild periodate oxidation and reduction with [$^3$H] $NaBH_4$ was done according to established procedures as described (Blumenfeld, Gallop & Liao, 1972; Furthmayr & Marchesi, 1976). The radioactive samples were counted in a Beckman scintillation spectrophotometer Model LS-230 using scintillation liquids based on Triton X-100, Toluene and liquifluor (New England Nuclear).

*Electron-Microscopy*

Membrane pellets were fixed in 1% glutaraldehyde in 0.05 M phosphate, pH 7.0, for 30 min, then washed, post-fixed in 2% osmium tetroxide in 10 mM Na phosphate, pH 7.0, embedded in Araldite and processed according to standard procedures. The sections taken on a Sorvall MT-2 microtome were stained with uranyl acetate and lead citrate (Reynolds, 1963). Thin sections were examined and photographed in a Phillips 300 electron-microscope.

*Nomenclature*

The numerical designation for the individual bands on SDS-gels suggested by Fairbanks *et al.* (1971) was used in this paper.

## Results

The general procedure for the isolation of the major intrinsic protein (band 3 polypeptides) is outlined in Fig. 1.

### *Extraction of Erythrocyte Membranes with LIS*

Extraction of freshly prepared membranes with increasing concentrations of LIS differentially releases membrane components (Fig. 2). At 0.1 M LIS 75–80% of the protein and sialic acid is released into the supernatant, but only 5% of the membrane phospholipids and 30% of the neutral sugars are solubilized.

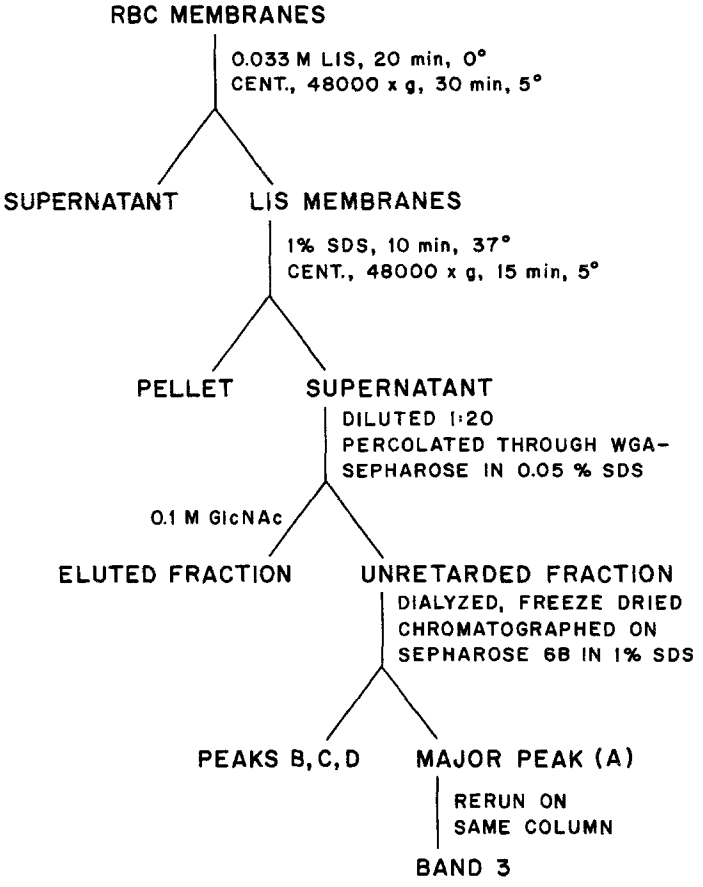


Fig. 1. Flow sheet for the isolation of the major intrinsic proteins of the human erythrocyte membrane

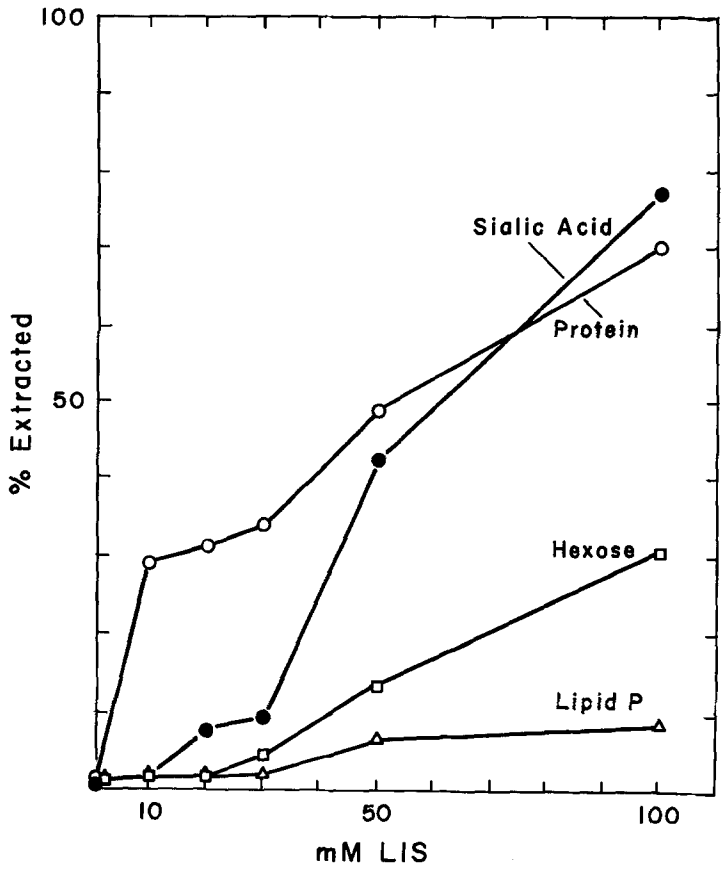


Fig. 2. Extraction of human erythrocyte membranes with lithium 3,5-diiodosalicylate (LIS) for 20 min at 0 °C. The extracts and the pellets obtained after centrifugation for one hour at 27,000 rpm in a Beckman SW-27 rotor, were analyzed after dialysis overnight at 4 °C against 0.1% Triton X-100, 0.1 M Na phosphate, pH 8, for sialic acid (●—●), hexoses (□—□), protein (○—○), and phospholipid phosphorus (△—△). The data are expressed as % from total, extracted at various concentrations of LIS

When lower concentrations of LIS are used (approximately 30 mM) less than 10% of the sialic acid and 2–3% of the neutral sugar are found in the extract but approximately 35% of the total membrane protein is still released from the fragmented membranes. The polypeptides which remained associated with the membrane fragments (Fig. 3), were found to be bands 3, 7, the polypeptides termed 4.5, and all the sialoglycopeptides (PAS 1, 1a, 2, 3). Similar results have been reported by Steck and Yu (1973).

The membrane fragments produced by incubating ghosts in 33 mM LIS still retain the typical "unit membrane" image when examined by

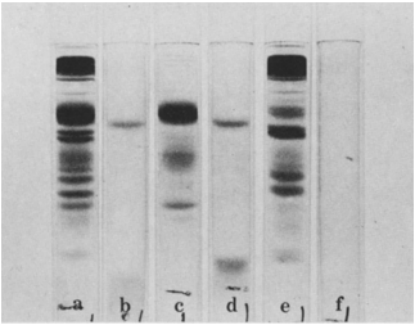


Fig. 3. Analysis on SDS-polyacrylamide gels (0.1% SDS, phosphate buffer system of Maizel, 1971) of total membranes (*a, b*), LIS-pellet (*c, d*) and LIS-supernate (*e, f*) obtained after extraction of membranes at 33 mM LIS in 10 mM Tris-HCl, pH 8.0. Aliquots of the pellet and the supernate were incubated for 30 min at 37 °C with equal volumes of 2% SDS, containing 100 mM phosphate, pH 7.2, and 1% 2-mercaptoethanol. Gels *a, c, e* are stained with Coomassie brilliant blue; gels *b, d, f* are stained with periodic acid-Schiff's reagent

electron-microscopy (Fig. 4). These membranes differ from vesicles prepared by physical disruption by having "free ends" as opposed to the "closed vesicle" form. Fragments prepared in 33 mM LIS also contain significant numbers of intramembranous particles when examined by the freeze-cleavage technique. Such particles are not seen in membranes suspended in 100 mM LIS.

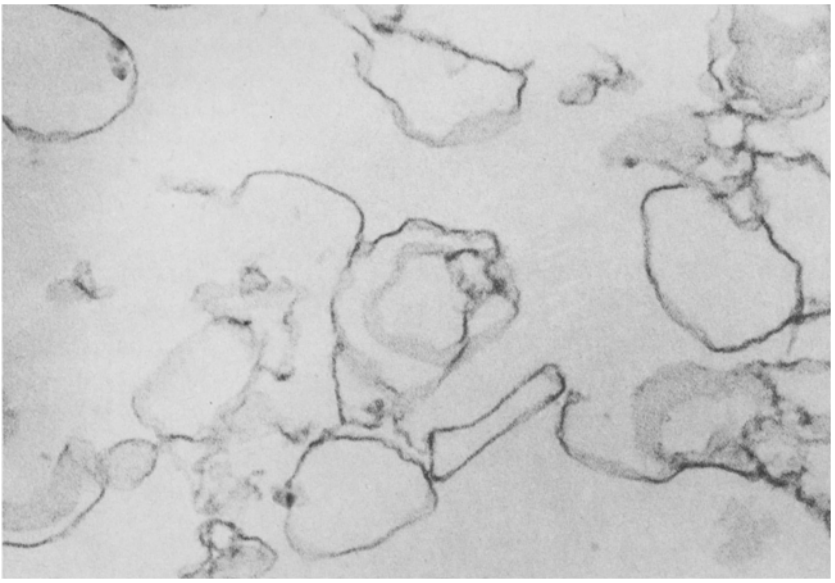

Fig. 4. Electron-micrographs of LIS membranes, obtained at 33 mM LIS. (Mag. 89,000×)

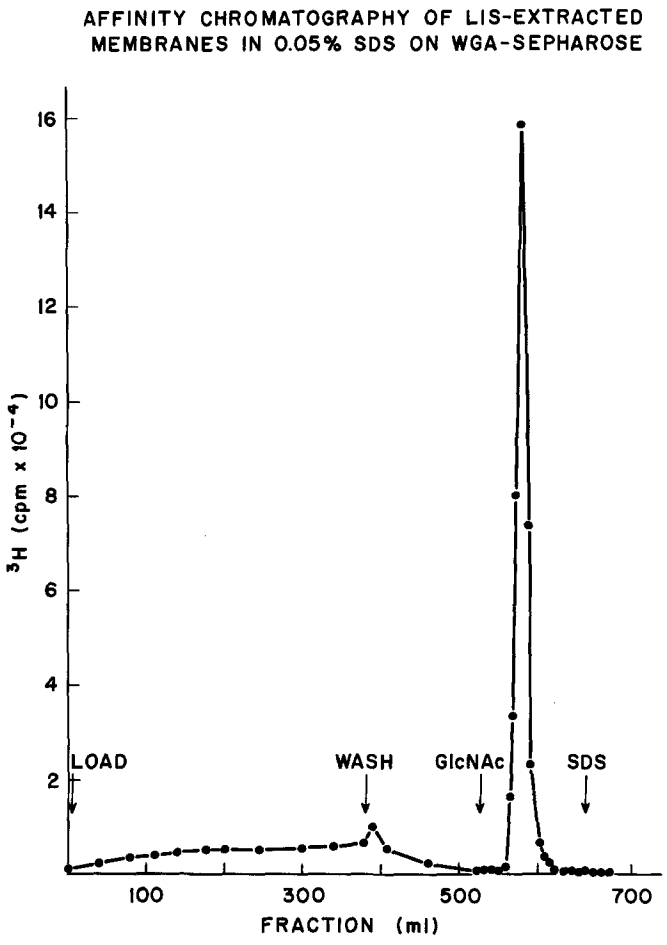


Fig. 5. Separation of [$^3$H] sialoglycopeptides from other unlabeled intrinsic membrane proteins, contained in LIS membranes (33 mM LIS) by WGA affinity chromatography in SDS. Elution was done with 0.1 M N-acetyl-D-glucosamine (GlcNAc) after loading of 375 ml of sample in 0.05% SDS, 15 mM Na phosphate, 250 mM NaCl, pH 7.2, and washing of the column with 200 ml of the same buffer. After elution of the [$^3$H]-sialoglycopeptides, a 2% SDS solution was applied, which did not elute any more counts. The nonbound fraction which contained the band 3 polypeptide, was pooled (approximately 500 ml), dialyzed against distilled water and concentrated by freeze-drying. For further analysis of this fraction *see* Figs. 6 and 7

## *Separation of the Sialoglycopeptides from Solubilized LIS-Membranes by Lectin-Affinity Chromatography*

The major sialoglycopeptides were separated from the other proteins associated with the LIS fragments by affinity chromatography in SDS with a WGA-Sepharose system (Fig. 5). This step was found to be crucial

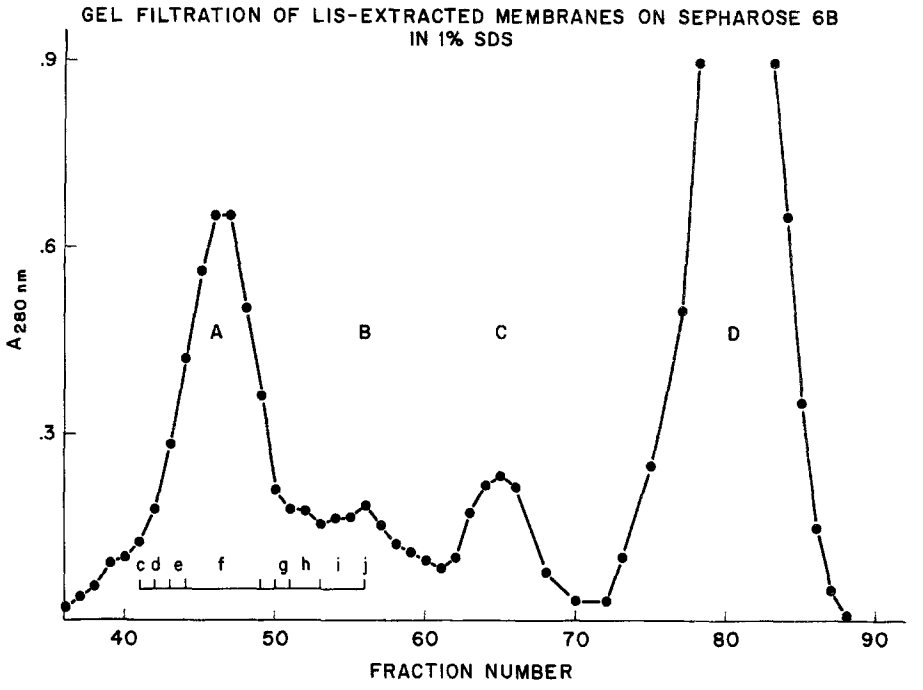


Fig. 6. Gel filtration on Sepharose 6B (95 × 2.5 cm) in 1% SDS, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.02% $NaN_3$, 0.05% 2-mercaptoethanol, at 45 °C (5 ml/fraction). *A–D* indicate pooled fractions and *c–j* indicate single or pooled fractions analyzed by SDS-gel electrophoresis (*see* Fig. 7)

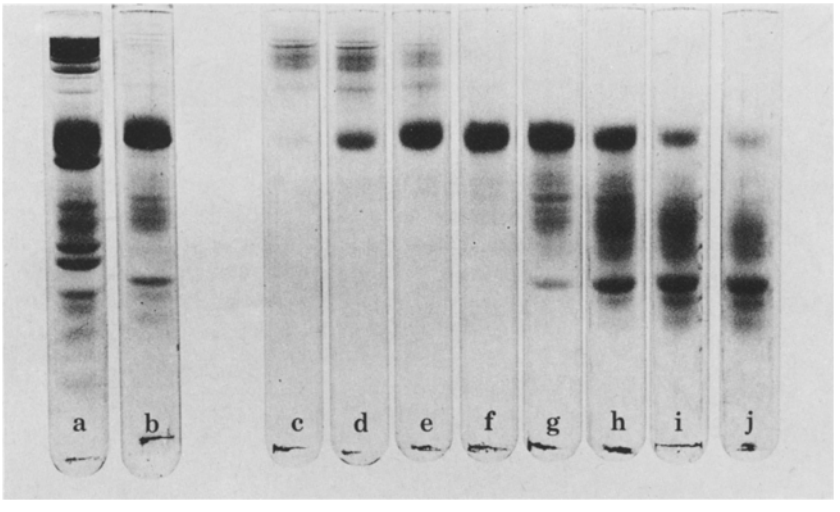


Fig. 7. SDS-gel electrophoresis of erythrocyte membrane proteins (Fairbanks *et al.*, 1971). The gels were stained with Coomassie blue. (*a*) Intact ghost membranes; (*b*) LIS membrane fragments; (*c–j*) individual or pooled fractions from chromatogram in Fig. 6. 100 μg aliquots of these fractions were mixed with 10 μliters of 10 M urea containing 10% 2-mercaptoethanol and were directly applied to the gels for electrophoresis

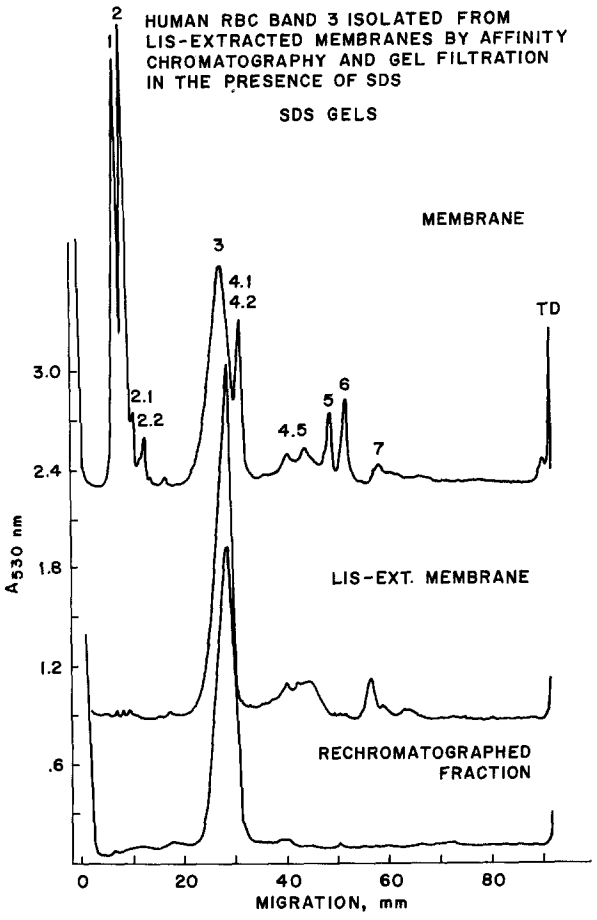


Fig. 8. SDS-gel electrophoretic pattern obtained by scanning at 550 nm on a Gilford Model 2400-S spectrophotometer and scanning device, of Coomassie blue stained gels (Fairbanks *et al.*, 1971), of total membrane proteins (top), LIS membrane fragments before or after WGA-affinity chromatography (middle), and purified band 3 polypeptide (lower). PAS staining of parallel gels (not included in Figure) gave the usual sialoglycopeptide pattern for the top and middle gels (*cf.* Kahane *et al.*, 1976). No PAS-stainable bands were seen in the purified band 3 preparations, unless the gels were overloaded (approximately 300 μg of protein/gel). At this protein concentration band 3 was stained with the PAS reagent

for this isolation procedure of band 3, since the sialoglycopeptides cannot be separated by gel filtration in SDS (Ho & Guidotti, 1975). Since residual LIS bound to the membrane fragments has a high absorbance in the 280 nm range, the results of this separation are described in terms of the removal of tritiated sialoglycopeptides, labeled according to the procedure of Blumenfeld *et al.* (1972). Little label was extracted with 33 mM LIS and most of the [$^3$H] counts associated with the LIS fragments were subsequently bound to WGA-Sepharose and could be specifically

Table 1. Protein recovery during fractionation of the intrinsic proteins of human erythrocyte membranes from one unit of blood[a]

| Purification step | Fraction | Protein in fraction | | |
|---|---|---|---|---|
| | | Lowry | | Absorbance at 280 nm |
| | | mg | % | $A_{280}$ units |
| | membranes | 357 | 100 | 363 |
| LIS extraction | LIS fragments | 214 | 60 | —[b] |
| WGA-affinity chromatography | nonbound | 149 | 41.8 | 187 |
| | eluted | 22.1[c] | 6.2 | 11.7 |
| Gel filtration | peak A | 58.8 | 16.5 | 67.6 |
| | peak B | 10.9 | 3.1 | 13.1 |
| Gel filtration (re-chromatography) | peak A | 35.9 | 10.1 | 47.6 |

[a] These values represent approximations and are obtained from one preparation.
[b] Data not included because of the high absorbance of LIS in this wavelength range.
[c] Glycophorin A, corrected for carbohydrate content.

Table 2. Amino acid and carbohydrate composition of band 3

| Amino acid | moles/1,000 residues | Carbohydrate | moles/100,000 g | |
|---|---|---|---|---|
| | | | TMS | Alditol acetate |
| Asp | 63.9 | Fucose | 0.81 | 0 |
| Thr | 46.5 | Mannose | 3.67 | 3.28 |
| Ser | 55.3 | Galactose | 5.56 | 7.22 |
| Glu | 119.1 | Glucose | 0.83 | 0.22 |
| Pro | 64.0 | GalNAc | 0.93 | 0.10 |
| Gly | 73.8 | GlcNAc | 5.88 | 5.43 |
| Ala | 82.6 | Sialic acid | 0.93 | —[b] |
| Cys | 7.1[a] | | | |
| Val | 76.4 | | | |
| Met | 27.2 | | | |
| Ile | 49.8 | | | |
| Leu | 138.4 | | | |
| Tyr | 26.0 | | | |
| Phe | 62.9 | | | |
| His | 20.7 | | | |
| Lys | 34.1 | | | |
| Arg | 52.2 | | | |

[a] Estimated as cysteic acid.
[b] Could not be estimated by this method.

eluted with 0.1 M N-acetyl-D-glucosamine (Fig. 5), as has also been shown previously for whole membranes (Kahane *et al.*, 1976). The peptides which did not bind to this column (not seen in Fig. 5) contained all the Coomassie blue staining polypeptides found to be associated with the LIS fragments before affinity chromatography (Fig. 7*b*, 8), and included the band 3 peptide as the major component.

### *Purification of the Major Integral Protein (Band 3)*

The separation of the remaining membrane proteins of the LIS-fragments after removal of the sialoglycopeptides (nonbound fraction in Fig. 5) is shown in Fig. 6. Peaks *A* and *B* were found to contain protein, peak *C* corresponded to the included volume and the retarded peak *D* contained residual LIS and β-mercaptoethanol. The material isolated from half a unit of blood (approximately 250 ml) could be separated on this chromatography system in a single run with satisfactory resolution, as indicated in Fig. 7. Rechromatography of the pooled fractions *e*, *f* and *g* yielded a preparation, which was electrophoretically homogeneous (Fig. 8) and which accounted for approximately 10–15% of the original membrane proteins. In a typical preparation, the yield was 40–50 mg of purified band 3 polypeptides per unit of blood (Table 1). The amino acid and carbohydrate compositions are given in Table 2.

## Discussion

The major intrinsic membrane protein of the human erythrocyte, commonly referred to as band 3 (Fairbanks *et al.*, 1971) is a polypeptide of about 90,000 daltons molecular weight. Based on quantitation of its stainability on SDS gels, this polypeptide constitutes about 30–35% of the total protein mass and may represent approximately $10^6$ copies/cell.

It has been suggested that this protein may be involved in several distinct functions including anion permeability (Cabantchik & Rothstein, 1974; Ho & Guidotti, 1975) and sugar transport (Lin & Spudich, 1974). Recently, it has also been suggested that this molecule might carry a receptor for Concanavalin A (Findlay, 1974).

Most of the above considerations are based upon selective labeling of the band 3 polypeptide with group-specific reagents or association of radio-label with the inhibition of specific functions. However, so little

data exists concerning the structure of this molecule that it is still not possible to determine how many different polypeptide chains contribute to these functions.

Studies on the disposition of band 3 within the membrane using selective labeling techniques and/or proteolytic digestion experiments provide strong evidence that some part of these polypeptides extend through the membrane (Bretscher, 1971*a*, *b*; Hubbard & Cohn, 1972; Steck, 1972*b*; Reichstein & Blostein, 1973; Mueller & Morrison, 1974; Shin & Carraway, 1974; Whiteley & Berg, 1974). This conclusion implies that band 3 is an integral protein, intimately associated with lipid molecules (Singer & Nicolson, 1972) and this is consistent with the solubilization results (Yu *et al.*, 1973). Labeling methods, which introduce [$^3$H] or [$^{35}$S] specifically into galactose residues available on the external surface of the intact red cell, indicate that band 3 polypeptides are also glycopeptides (Gahmberg & Hakomori, 1973; Steck & Dawson, 1974; Itaya *et al.*, 1975).

In freeze-fracture studies on enzymatically modified membranes, Tillack, Scott, and Marchesi (1972) suggested that a link may exist between the intramembraneous particles and the major sialoglycoprotein (glycophorin). Membrane vesicles prepared by treatment with NaOH (Steck & Yu, 1973) still contain intramembraneous particles (D. Branton, *personal communication*), as do LIS-fragments (this paper). The major protein components in the LIS-membrane fragments are the band 3 polypeptides and the sialoglycopeptides, as shown by SDS-electrophoresis (*cf.* Fig. 3). Thus, it is not clear which protein is actually responsible for the structure seen on freeze-fracture. In studies employing cross-linking reagents (Steck, 1972*a*; Wang & Richards, 1974) no association between the band 3 polypeptides and the sialoglycopeptides was demonstrable. However, these studies did suggest that the band 3 polypeptide is a subunit of a larger protein of a molecular weight of 165,000 daltons. Thus it remains to be determined, which of the various integral proteins can ultimately be correlated with the morphological structure of the intramembraneous particles.

The isolation of the band 3 polypeptide has been attempted previously using lectin-affinity systems (Adair & Kornfeld, 1974; Findlay, 1974), gel-filtration on Sepharose 4B columns in SDS (Ho & Guidotti, 1975), or sequential extraction methods followed by SDS-gel chromatography and preparative SDS-gel electrophoresis (Tanner & Boxer, 1972). Affinity chromatography in both cases, gave poor yields and did not result in the isolation of an electrophoretically pure band 3 polypeptide. Tanner and Boxer (1972) attempted the separation of band 3 from the band 4.1 and 4.2 polypeptides by preparative SDS-gel electrophoresis after sequen-

tial extraction followed by gel filtration. The analytical data presented by these investigators are different from our data for both the band 3 polypeptide, which they call protein E, and the sialoglycoprotein with regard to amino acid and carbohydrate analysis (*cf.* Tomita & Marchesi, 1975; Furthmayr *et al.*, 1975). Some of these differences may be accounted for by difficulties encountered in the analysis of small amounts of material and related problems. Contrary to their results we have found that the sialoglycoproteins do not have a blocked N terminal (Tomita & Marchesi, 1975), but we were also unable to identify the N terminus of the band 3 polypeptide by dansylation, Edman degradation or cleavage with pyrrolidonecarboxylyl peptidase[1] (Kwiatkowska, Torain & Glenner, 1974), in agreement with results obtained in other laboratories (Tanner & Boxer, 1972; Ho & Guidotti, 1975).

The largest differences were found in data on the carbohydrate composition. The band 3 polypeptide isolated by the procedure described here contains approximately 18 moles of carbohydrate as compared to 46 moles (Ho & Guidotti, 1975) and 40–80 moles (Tanner & Boxer, 1972) per mole of protein assuming a molecular weight of 100,000 daltons. The significance of these data has to await further studies, but contamination with sialoglycopeptides can probably be excluded to explain these differences.

A more stringent question relates to the problem of homogeneity of the isolated band 3 polypeptide. It is still uncertain whether band 3 is composed of a single polypeptide chain or a mixture of several closely related species as has been suggested recently (Knüfermann, Bhakdi, Schmidt-Ullrich & Wallach, 1973).

It is also worth pointing out that we do not have a clear idea as to how the band 3 components are arranged in the intact membranes. Recent studies on the molecular anatomy of the major sialoglycopeptide show that this molecule is composed of three distinct molecular domains each of which seems well-suited to interact with a specific region of the membrane (Tomita & Marchesi, 1975). Thus, although the data supporting a transmembrane orientation for the band 3 material seem firm, the precise way by which this molecule achieves this state will require more detailed information on its primary structure.

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1 The enzyme treatment was kindly performed by Dr. R. Timpl, Max-Planck-Institute for Biochemistry, Munich.

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