MEMBRANE PROTEINS SYNTHESIZED
BY RABBIT RETICULOCYTES

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ABSTRACT
Intact rabbit reticulocyte cells synthesize two predominant species of polypeptides which are components of the cell plasma membrane. Previous work (Lodish, H. F. 1973. *Proc. Natl. Acad. Sci. U. S. A.* 70:1526–1530.) showed that these proteins were synthesized by polyribosomes not attached to membranes. We show here that both polypeptides are confined to the cytoplasmic surface of the cell membrane. These studies utilized iodination of whole cells and of membranes with lactoperoxidase, and digestion of whole cells and membranes with chymotrypsin. One of these proteins is synthesized as a precursor, and about 20–40 amino acids are removed after it is incorporated into the membrane. We discuss the probable sites of synthesis of these and other classes of membrane proteins.

Although a great deal is known of the protein composition of mammalian erythrocyte membranes, and of the asymmetric distribution of these proteins within the membrane, little is known about the biosynthesis of erythrocyte membrane proteins. The literature concerning the proteins of erythrocyte membranes has been reviewed several times (1–4). As resolved by sodium dodecyl sulfate (SDS) gel electrophoresis, all mammalian erythrocyte membranes contain about seven to nine principal protein components, although the detailed pattern of membrane proteins shows some species differences (1–11). The major polypeptides common to all mammalian erythrocytes include two polypeptides of molecular weight greater than 200,000 (7) and one of 43,000 (the three spectrin polypeptides) (1, 3, 4, 12), a protein of molecular weight 90,000, and a sialoylglycoprotein which in the human contains the MN, A, and B blood group antigens (11). The latter two species are the only membrane proteins found on the cell surface; recent work suggests that they penetrate to the interior surface of the membrane (references 6, 13–20, reviewed in reference 4). Most membrane proteins are confined to the cytoplasmic surface of the membrane; glyceraldehyde-3-phosphate dehydrogenase, for instance, is found only on the inner surface of the human erythrocyte membrane, and the isolated enzyme forms stable interactions with high affinity for a limited number of sites on the cytoplasmic face of the membrane (21).

Rabbit reticulocytes contain, with possibly one or two exceptions (22), the same proteins in their membranes as do rabbit erythrocytes. We recently showed that rabbit reticulocytes synthesize only two major and two to three minor membrane proteins; most of the membrane proteins are no longer being synthesized in significant amounts at this stage (23). We also showed that these two proteins as well as all other proteins made by reticulocytes are synthesized on polyribosomes which are not attached to membranes (23, 24). A small fraction of reticulocyte polysomes are apparently bound to the cell membrane. Woodward et
al. (25) have shown, in contrast to earlier work (26), that these polyribosomes synthesize globin almost exclusively. In this paper we show that, by several criteria, the two major membrane proteins synthesized by rabbit reticulocytes are components of the cell plasma membrane; neither protein penetrates to the outer surface of the membrane and both appear to be localized on the cytoplasmic face. One of these proteins is synthesized as a precursor, and is modified by loss of 20-40 amino acids after it is incorporated into the membrane. On the basis of these and other considerations, we present a model for the synthesis of different classes of membrane proteins.

MATERIALS AND METHODS

Materials

Acetylphenylhydrazine was purchased from Sigma Chemical Co., St. Louis, Mo., and [35S]methionine (100,000 Ci/mol) from New England Nuclear, Boston, Mass. α-Chymotrypsin and pronase were purchased from Worthington Biochemical Corp., Freehold, N. J., and Streptomyces griseus protease and papain from Sigma Chemical Co. Sources of all other chemicals have been detailed previously (23, 24, 27). 5P8 is 0.005 M sodium phosphate, pH 8.0.

Reticulocytes

Rabbits were made anemic as described (28), except that acetylphenylhydrazine was used in place of phenylhydrazine. Blood was taken from the ear, and the cells were washed by centrifugation four times, removing theuffy coat of white cells. In some experiments the top 25% of the packed red cells was removed after each of the first two washes, in order to insure that no white cells contaminated the preparations (28). In one study, we compared directly the properties of reticulocyte preparations in which the top 25% of the cells was or was not removed. No difference was found either in the rate of [35S]methionine uptake into protein, or in the pattern of labeled or Coomassie blue-stained membrane proteins. As determined by staining of cells with methylene blue, our preparations always contained over 90% reticulocytes and fewer than 0.1% nucleated cells.

Labeling of Whole Cells

0.5 ml packed reticulocytes was resuspended in 4.5 ml of buffer A (0.005 M KCl, 0.12 M NaCl, 0.001 M CaCl₂, 0.002 M MgSO₄, 0.020 M sodium phosphate, pH 7.4) which also contained 19 nonradioactive amino acids (2.5 x 10⁻³ M each), 90 μCi/ml [35S]methionine, and 2 mg/ml glucose. Incubation was carried out at 30°C for 40 min. Incorporation of radioactivity into protein was linear with time during this period; per milliliter, there were 2-4 x 10⁵ dpm incorporated into protein. The cell suspension was poured into 15 ml of ice-cold buffer A; the cells were recovered by centrifugation (6,000 g, 10 min), washed twice in buffer A, then lysed in 15-30 ml of cold 5P8. The suspension was centrifuged at 20,000 g for 15 min, and the supernate was removed.

Preparation of Membranes. Crude Particulate Fraction

The pellet from the lysed cells was washed by centrifugation (20,000 g, 15 min) four times with 10-30 ml of 5P8 (7), at which point it was yellow-brown in color.

"Partially Purified" Membranes

To the pellet from the lysed cells were added about 2 cm³ 5P8, and the tube was gently swirled. This frees the white topmost membrane fraction of the pellet from the hard, deep yellow button which consists primarily of mitochondria and unlysed cells. The released membrane fraction was placed in another tube containing 10 ml 5P8 and the contents of the tube were mixed vigorously for 1 min. The particulate fraction was recovered by centrifugation and the above process was repeated twice more. The resulting membrane preparation was white and opalescent. When ¹²⁵I-labeled cells were used, this fraction routinely contained not less than 50% of the acid-precipitable ¹²⁵I radioactivity originally bound to the cells. When this partially purified membrane fraction (from unlabeled cells) was centrifuged to equilibrium in a sucrose gradient (see below), the only light-scattering band was at the interface between the 30% and 40% sucrose layers.

Equilibrium Sucrose Gradient Centrifugation

All sucrose solutions were made in 5P8. To a crude particulate fraction or a partially purified membrane fraction from 0.5 ml packed reticulocytes which had been labeled with [35S]methionine were added 7 ml 60% sucrose (wt/vol). The particulates were thoroughly resuspended by 10 strokes of a tight-fitting Dounce homogenizer. This suspension was placed in a centrifuge tube for the Beckman SW27 rotor and overlaid with the following solutions: 6 ml 50% sucrose, 6 ml 40% sucrose, 6 ml 30% sucrose, 6 ml 15% sucrose, and 7 ml 5P8. Centrifugation was carried out at 25,000 rpm for 18 h at 4°C. The white, opalescent membrane band at the interface between 30% and 40% sucrose layers was collected with a syringe, diluted fivefold with 5P8, and recovered by centrifugation for 60 min at 25,000 rpm in the Beckman 30 rotor.

Iodination of Cells

0.2 ml packed cells was resuspended in 1.8 ml buffer A. Iodination with 50 μCi [¹²⁵I]NaI was performed as...
detailed by Sefton et al. (29) for Sindbis virus-infected cells, except that iodination was performed in buffer A. The cells were recovered by centrifugation, and washed four times by centrifugation in buffer A. Between 5 x 10^4 and 2.4 x 10^4 dpm ^125I were recovered in membrane proteins.

**Iodination of Membranes**

Membranes from 0.2 ml packed cells were resuspended in 2.0 ml buffer A and iodinated exactly as described in the previous paragraph (identical results were obtained if the membranes were resuspended and iodinated in SP8). Membranes were diluted twofold with SP8, recovered by centrifugation, and washed four times by centrifugation in SP8. Recovered in the washed membranes were about 4 x 10^7 cpm.

**Digestion of Cells with Chymotrypsin**

0.03 ml packed cells was resuspended in 0.3 ml buffer A containing 2 mg/ml glucose. 400 μg α-chymotrypsin were added, and the suspension was incubated with gentle shaking at 37°C for 1 h. The cells were cooled, and 3 cm³ of buffer B (buffer A containing 0.015 M EDTA) were added. The cells were recovered by centrifugation, washed once in buffer B, and twice in buffer A. Membranes were prepared as detailed above.

**Digestion of Membranes with α-Chymotrypsin**

Membranes from 0.03 ml packed cells were resuspended in 0.2 ml buffer A. Chymotrypsin (0.4 mg) was added and the suspension incubated at 37°C for 1 h. Identical results were obtained if the digestion was performed in SP8. Membranes were recovered by centrifugation and washed by centrifugation three times in SP8.

**Preparation of Membranes for Gel Electrophoresis**

The procedure of Bender et al. (13) was followed. Membranes from 0.03 ml packed cells were resuspended in 0.05 ml SP8. To this suspension was added 0.1 ml of SDS solution (5%, in 0.05 M potassium phthalate, pH 4.0) which had been preheated to 70°C, and the membranes were immediately incubated for 10 min at 70°C. 15 μl were used for one gel analysis.

**Polyacrylamide Gel Electrophoresis**

Two gel systems were used. Urea SDS gels contained 7.5% acrylamide, 0.1% SDS, and 6 M urea, and have been described in detail (24); these were used for all experiments except those in Fig. 9. The experiment in Fig. 9 used the gel system described in detail by Weber and Osborn (30), except that 5% acrylamide gels were used. Conditions for staining the gels and for autoradiography have been detailed previously (24). Gels and autoradiograms were scanned in a Gilford 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 560 nm for Coomassie blue stain, and at 600 nm for the autoradiograms.

**RESULTS**

**Isolation of Reticulocyte Plasma Membrane**

Rabbit reticulocytes contain no nucleus and no endoplasmic reticulum. The only intracellular organelle which contains membranes is the mitochondrion. We developed two procedures which completely separated the reticulocyte plasma membranes from the mitochondria.

The first of these involves centrifugation to equilibrium in a sucrose density gradient. In the experiment depicted in Fig. 1, a crude particulate fraction (see Materials and Methods) from erythrocytes and reticulocytes was layered atop a continuous 30%-60% sucrose gradient and centrifuged to equilibrium. Erythrocytes yielded only one light-scattering band—the ghost or plasma membrane fraction—at 1.13 g/cm³. The crude particulate fraction from reticulocytes yielded three light-scattering bands: a white, opalescent band at 1.14 g/cm³, and two yellow-brown bands at 1.18 and 1.20 g/cm³. These latter bands are the mitochondria; they contained all of the succinic dehydrogenase activity (a typical mitochondrial membrane-bounded enzyme) found in the gradient (Table I) and they contained no radioactivity when the particulate fraction from ^125I-labeled cells (see below) was analyzed (Table I). The band at 1.14 g/cm³ is the reticulocyte plasma membrane. It contained all of the radioactivity when the particulate fraction from ^125I-labeled cells was analyzed; as judged by the activity of succinic dehydrogenase (Table I), it is essentially free of mitochondria. The difference in density of 0.01 g/cm³ between reticulocyte and erythrocyte plasma membranes was obtained in three separate experiments. Routinely, plasma membranes were isolated from a discontinuous sucrose gradient, as detailed in Materials and Methods.

During low speed centrifugation of the crude particulate fraction of reticulocytes, the plasma membranes formed a loose white pellet above the tighter, yellow-brown mitochondrial pellet. It was possible to exploit this difference to isolate the
plasma membrane fraction essentially free of mitochondria (partially purified membranes—see Materials and Methods). When banded in an equilibrium sucrose density gradient, this fraction yielded a single light-scattering band, at density 1.14 g/cm$^3$. As shown in Fig. 2, the proteins in the partially purified membrane preparation yielded a gel profile identical to that obtained from plasma membranes purified by equilibrium sucrose gradient centrifugation.

**Protein Composition of Reticulocyte Membranes**

The proteins present in partially purified membrane preparations from human erythrocytes, rabbit reticulocytes, and rabbit erythrocytes were resolved by electrophoresis in polyacrylamide gels containing urea and SDS, and visualized by staining with Coomassie blue (Fig. 3a, b, e). The numbering of the human membrane components follows that of reference 4. Many of the principal species (bands 1, 2, 3, 4.1, 4.2, 5, and 6) are common to both human and rabbit erythrocyte membranes. In the human membrane, bands 1, 2, and 5 correspond to the actomyosin-like spectrins (4), and 6 is glyceraldehyde-3-phosphate dehydrogenase (21). Membranes from rabbit reticulocytes contain all of the species found in the erythrocyte membranes, although one protein (labeled 4.8) is present in the reticulocyte membrane but undetectable in membrane preparations from erythrocytes. Protein 4.8 is also present in reticulocyte plasma membrane preparations purified by sucrose gradient centrifugation (Fig. 2). As determined by its position in the gel profiles, relative to that of marker proteins, protein 4.8 appears to have a molecular weight of 45,000 ± 3,000. Koch et al. (22) report that only one protein, with an estimated molecular weight of 33,000, is present in membranes of rabbit reticulocytes but is absent in erythrocyte membranes. Our preparations do not show this difference. Incubation of human erythrocyte membranes in solutions of low ionic strength (37°C for 20 min in 10^{-4} M or 10^{-5} M EDTA, pH 8.0) has been reported to elute quantitatively the three spectrin polypeptides (bands 1, 2, and 5) (4, 7). We have repeated these results in several experiments gradient-purified rabbit erythrocyte or reticulocyte membranes were incubated under these conditions. Membranes were recovered by centrifugation and analyzed by gel electrophoresis. There was loss only of 40% of spectrin bands 1, 2, and 5 and no detectable loss of any other polypeptide. Incubation of human erythrocyte ghosts in solutions of high ionic strength (37°C for 20 min in 0.2 M NaCl or 3 M NaCl, pH 8.0) elutes specifically band 6, glyceraldehyde-3-phosphate dehydrogenase (4, 7, 21). We have also repeated this result. By contrast, treatment of purified rabbit reticulocyte or erythrocyte membranes under these conditions results in no significant loss of any polypeptides from the membrane (data not shown). These results suggest that proteins are attached to rabbit erythrocyte or reticulocyte membranes.
TABLE I
Purification of Reticulocyte Plasma Membranes by Equilibrium Sucrose Gradient Centrifugation

<table>
<thead>
<tr>
<th>Band</th>
<th>Density (g/cm³)</th>
<th>Protein (band density)</th>
<th>Succinic dehydrogenase activity</th>
<th>[³⁵S]Methionine radioactivity</th>
<th>[¹²⁵I]Iodine radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma mem-</td>
<td>1.14</td>
<td>17</td>
<td>4</td>
<td>77</td>
<td>96</td>
</tr>
<tr>
<td>brane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.18</td>
<td>58</td>
<td>63</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>1.20</td>
<td>25</td>
<td>33</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

Membranes were prepared from 1.0 ml packed reticulocytes labeled with [³⁵S]methionine (columns 3–5) or [¹²⁵I]iodine (column 6) and centrifuged to equilibrium in a discontinuous sucrose density gradient. The three light-scattering bands were removed, diluted with SP8, recovered by centrifugation, and resuspended in SP8. Protein was determined by the Lowry procedure and succinic dehydrogenase activity by the method of Ziegler and Rieske (38).

membranes in a more stable linkage than in human erythrocytes.

One way of labeling the membrane proteins which are exposed to the external surface is to react the intact cells with radioactive iodine, lactoperoxidase, and a source of hydrogen peroxide (9, 15, 18). Fig. 3 d and g show scans of autoradiograms of polyacrylamide gel analyses of membranes of iodinated rabbit reticulocytes and erythrocytes. Both contain the same major species of apparent molecular weight 90,000–100,000, and one minor species of lower molecular weight. Both principal iodinated species comigrate with a major band (number 3) visualized by Coomassie blue staining. Since control experiments showed that hemoglobin and other cytoplasmic proteins were not iodinated by this procedure, we presume that at least parts of the two iodinated membrane proteins are exposed to the external surface, and that these proteins are found on the surface of both reticulocytes and erythrocytes. One minor iodinated species apparently is different in the two membrane preparations.

Membrane Proteins Synthesized by Rabbit Reticulocytes

Previous work showed that rabbit reticulocytes synthesized two major and two to three minor species of proteins present in a partially purified membrane preparation. This can be seen in Fig. 3 c; each of the two major labeled proteins (called for historical reasons B2, molecular weight 55,000; and E, molecular weight 36,000 [ref. 24]) comigrates with a protein visualized by the Coomassie blue stain. As expected, much smaller amounts of membrane proteins are synthesized by blood from a nonanemic rabbit (Fig. 3 f).

By the following criteria the labeled species B2 and E are considered to be authentic membrane proteins: (a) when a crude particulate fraction from [³⁵S]methionine-labeled reticulocytes is centrifuged to equilibrium in a sucrose density gradient, most of the [³⁵S] radioactivity bands at the position of plasma membranes (1.14 g/cm³, Fig. 1). The polyacrylamide gel studies in Fig. 2 show that these purified membranes contain the same amount of radioactive B2 and E polypeptides as does the partially purified membrane fraction; (b) labeled proteins the size of B2 and E are not found in the membrane-free cytoplasm (Fig. 4, see also reference 23). Conversely, none of the labeled cytoplasmic proteins are found in appreciable amounts in the partially purified membrane fraction (Fig. 4). In particular, less than 0.5% of radioactive globin is recovered in this membrane fraction; (c) the labeled proteins are not removed from membranes when a preparation of membranes is incubated at 37°C for 20 min in any of the solutions of low or high ionic strength mentioned previously (Fig. 5).

A Precursor of Membrane Protein B2

Several experiments demonstrated that protein B2 is synthesized as a precursor containing an additional 20–40 amino acids; these are removed...
FIGURE 2 Polyacrylamide gel analysis of crude and purified membranes from rabbit reticulocytes. Partially purified (see Materials and Methods) membranes were isolated from rabbit reticulocytes which had incorporated $[^{35}S]$methionine; a part of the membranes were purified by equilibrium sucrose gradient centrifugation. (a) and (c) are scans of the gels stained with Coomassie blue, and (b) and (d) are scans of the autoradiograms of the dried gels. (a) and (b) gel analysis of gradient purified reticulocyte membranes. (c) and (d) gel analysis of partially purified reticulocyte membrane preparations.

after the protein is incorporated into the cell membrane. Previous work indicated that a cell-free (and membrane-free) extract of rabbit reticulocytes synthesizes a protein (B1) which migrates on a polyacrylamide gel as if it is about 2,000 daltons larger than species B2 isolated from membranes, yet which yields a pattern of tryptic peptides very similar to that of B2 (23). Figs. 4 and 6 show that membranes isolated from cells labeled for 15 min contain, in addition to labeled species B2 and E, a labeled protein (B1) which comigrates with polypeptide B1 synthesized by reticulocyte lysates (23). It has not been possible to prepare sufficient labeled B1 from reticulocyte membranes to compare its fingerprint with that of B2.

When cells are labeled in the presence of the chymotrypsin inhibitor tosyl-L-phenylalanyl-chloromethane, species B1 (and E) are labeled, but not B2 (Fig. 6 a, b). Neither the trypsin inhibitor tosyl-L-lysyl-chloromethane (Fig. 6 c) nor methanol (the solvent for the two compounds) has any effect on the pattern of membrane proteins produced. (66 μg of L-(tosylamido 2-phenyl)ethyl chloromethyl ketone (TPCK) per milliliter inhibits overall reticulocyte protein synthesis by 20%, and 100 μg/ml inhibits by about 50%.) This experiment suggests that the unprocessed precursor B1 can be incorporated into reticulocyte membranes, and that the conversion of B1 to B2 is mediated by a chymotrypsin-like enzyme.

That B1 has the kinetic properties of a precursor to B2 is shown by the experiment in Fig. 7. After addition of $[^{35}S]$methionine to reticulocytes, radioactivity appears in membrane proteins B1 and E with a lag of only about 2 min. By contrast, species B2 is labeled with a lag of about 6 min, and the time when the rate of labeling of B2 becomes constant (about 8 min) corresponds to the time when the total amount of radioactivity in species B1 becomes constant.

Localization of Polypeptides B2 and E within the Reticulocyte Membrane

Several experiments showed that species B2 and E are not exposed to the external surface of the membrane, and that most, if not all, of these labeled proteins are localized on the cytoplasmic side of the membrane. First, iodination of whole cells with lactoperoxidase does not label membrane species which comigrate with B2 or E, but does label three other proteins (Fig. 3); by contrast, iodination of isolated membranes results in labeling of all Coomassie blue-stained membrane proteins, including polypeptides (nos. 4.5 and 6) which comigrate with B2 and E (Fig. 8). Since we have only suggestive evidence that labeled species B2 and E are, in fact, identical to the major stained proteins 4.5 and 6, this experiment cannot show unambiguously that the labeled proteins B2 and E are not exposed on the external surface of the cell.

That this conclusion is correct is supported by studies in which the intact cell is treated with
G3PD is glyceraldehyde-3-phosphate dehydrogenase run in a parallel gel. (a) Membranes from human erythrocytes stained with Coomassie blue. (b) Partially purified membranes from rabbit reticulocytes stained with Coomassie blue. (c) Autoradiogram of membranes from rabbit reticulocytes which had been labeled in vitro with [35S]methionine. (d) Autoradiograms of membranes from rabbit reticulocytes which had been labeled with [125I]iodine and lactoperoxidase. (e) Partially purified membranes from rabbit erythrocytes stained with Coomassie blue. (f) Autoradiogram of membranes from rabbit erythrocytes which had been labeled in vitro with [35S]methionine. (g) Autoradiograms of membranes from rabbit erythrocytes which had been labeled with [125I]iodine and lactoperoxidase.
conclude that [35S]-labeled species B2 and E, and Coomassie blue-staining polypeptides 1, 2, 4.3, 4.5, 4.8, 5, and 6 are not simply resistant to chymotrypsin digestion; rather, in whole cells they are localized in the membrane in such a way as to be inaccessible to the enzyme in the external medium. Since chymotrypsin does destroy 35S species B2 and E in isolated membranes, it appears that at least some part of each B2 and E polypeptide must be exposed to the cytoplasmic surface of the membrane. The same conclusion can be reached for polypeptides 1, 2, 4.1, 4.5, 4.8, 5, and 6. Whether parts of each of these molecules are buried within the lipid bilayer cannot be determined from these experiments.

Results identical to those obtained with α-chymotrypsin were obtained with three other proteolytic enzymes: pronase (1 mg/ml and 2.5 mg/ml); Streptomyces griseus protease (1 mg/ml and 2.5 mg/ml); and papain (1.0 mg/ml and 2.5 mg/ml); except for the enzyme concentrations, the protocols were exactly as outlined in the legend to Fig. 9 (data not shown). This adds support to our conclusions on the localization of 35S-labeled species B2 and E.

DISCUSSION

Asymmetric Distribution of Membrane Proteins

Of the major species of polypeptides in the human erythrocyte membrane, only two-component 3 and the major glycoprotein PAS-I (which stains for carbohydrate with the PAS reagent but which stains poorly with Coomassie blue) are present at the outer surface of the membrane. The other major species components 1, 2, 2.1, 4.1, 4.2, 5, 6, and 7 are confined to the cytoplasmic surface (reviewed in references 1-4). These conclusions were derived from studies in which intact human red cells were exposed to enzymes such as proteases or lactoperoxidase plus 125I and H2O2, or to presumably nonpenetrating covalent ligands such as [35S]diazonium benzene sulfonate, [35S]formyl-

Shown is the scan of an autoradiogram of the dried gel. Tracings for the main part of the figure were done from film which had been exposed for 96 h, while the tracing of the globin region in (a) and (b) utilized a film exposed for 4 h.
FIGURE 5 Treatment of membranes from rabbit reticulocytes with solutions of different salts. Gradient-purified membranes from rabbit reticulocytes which had incorporated [35S]methionine in vitro were diluted 50-fold in the following solutions, and incubated for 10 min at 37°C. Membranes were recovered by centrifugation, washed by centrifugation in SP8, and then analyzed by polyacrylamide gel electrophoresis. Shown are autoradiograms of the dried gels. A, Membranes incubated in 10^-5 M EDTA, pH 7.0. B, Membranes incubated in 10^-4 M EDTA, pH 7.0. C, Membranes incubated in 5 mM sodium phosphate, pH 8.0. D, Membranes incubated in 0.2 M NaCl, 5 mM sodium phosphate, pH 8.0. E, Membranes incubated in 3 M NaCl, 10 mM sodium phosphate, pH 8.0.

methionyl sulfate methylphosphate, and trinitrobenzene sulfonate. This work was reviewed extensively (1-4).

The protein composition of the rabbit erythrocyte and reticulocyte membranes, and the distribution of the proteins within the membranes, appear very similar to those of the human erythrocyte membrane. As determined by lactoperoxidase iodination of whole cells and isolated membranes (Fig. 3) and by chymotrypsin digestion of whole cells and membranes (Fig. 9), species 1, 2, 4.1, 4.2, 4.5, 4.8, 5, and 6 are confined to the cytoplasmic surface. Rabbit reticulocytes and erythrocytes appear to contain only one principal protein species—component 3—which is exposed to the external surface. As judged by the reaction of polyacrylamide gels of rabbit reticulocyte or erythrocyte membranes with the PAS reagent, these membranes contain only about 0.1 the amount of surface carbohydrate of an equivalent amount of human erythrocyte membranes, and the small amount of staining material comigrates with band 3 (data not shown). When whole rabbit reticulocytes are iodinated and the membranes analyzed on gels containing 5, 8, and 12% acrylamide, only one predominant labeled band is observed and this comigrates on all gel systems studied with Coomassie blue component no. 3 (see Figs. 3 and 9).

Synthesis of Reticulocyte Membrane Proteins

Rabbit reticulocyte membranes contain essentially the same protein components as erythrocytes. Reticulocyte membranes contain one polypeptide (4.8) which is not found in erythrocytes. The function of this protein is obscure. Reproducibly, plasma membranes from reticulocytes banded in an equilibrium sucrose gradient at a density 0.01 g/cm³ heavier than that of erythrocyte membranes. We have at present no explanation for this.

Rabbit reticulocytes incorporate radioactive amino acids into two predominant membrane species, B2 and E. These labeled species comigrate with Coomassie blue-stained components nos. 4.5 and 6 on 5% (Fig. 9), 8%, and 12% (data not shown) polyacrylamide gels containing SDS, and on 7% polyacrylamide gels containing urea and SDS (Figs. 2, 3). With all these gel systems, component 6 and labeled species E comigrate with authentic rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Figs. 3, 9). In the human erythrocyte membrane, band 6 and glyceraldehyde-3-phosphate dehydrogenase are identical (21). Although our gel results are suggestive, we do not have as yet direct evidence to determine whether labeled species E in the rabbit reticulocyte is identical to component 6 or to glyceraldehyde-3-phosphate dehydrogenase, or whether species B2 is identical to stained species 4.5 (Figs. 2, 3, 9).

The most important result of this paper is that labeled species B2 and E are components of the reticulocyte membrane and are confined to the cytoplasmic surface. This was shown in two ways. First, iodination of whole cells with lactoperoxidase did not label polypeptides which comigrate with B2 or E, while when isolated membranes were iodinated these species were labeled. Second, digestion of whole reticulocytes, previously labeled in vitro with [35S]methionine, with α-chymotrypsin, pronase, Streptomyces griseus protease, or papain degraded only Coomassie blue-staining...
polypeptide 3; $^{35}$S-labeled species B2 and E were unaffected. By contrast, if isolated membranes were digested with any of these enzymes, all of the Coomassie blue-staining polypeptides and all $^{35}$S species were destroyed. Also consistent with these enzyme studies is the possibility that either or both B2 and E are completely buried within the lipid bilayer, but that somehow during isolation of the membranes part of each molecule is exposed on one membrane surface.

Previous work showed that both polypeptides B2 and E are synthesized exclusively on polysomes which are not attached to membranes (23, 25). A repeat of this experiment is shown in Fig. 5: a membrane-free cytoplasmic extract from reticulocytes synthesizes two proteins (B1 and E) which comigrate with authentic labeled B1 and E isolated from membranes of $[^{35}$S]methionine-labeled reticulocytes. The cell-free extract also synthesizes all cytoplasmic proteins which are produced by intact reticulocytes (compare panels A and B). About 10% of the ribosomes in reticulocytes are loosely attached to membranes. Woodward et al. (25) showed that over 95% of the protein made by these ribosomes is globin, and we have confirmed that result (unpublished data).

We propose the following simple model for incorporation of labeled proteins B2 and E into the reticulocyte membrane. Protein B1 is synthesized as precursor containing an additional 20–40 amino acids (Figs. 4, 6, and 7, and reference 23); there is no evidence for a protein precursor of E. Both B1 and E are released from the ribosome into the cytoplasm, and then bind to specific sites on the cytoplasmic surface of the plasma membrane. In agreement with this notion, Kant and Steck (21) have shown that glyceraldehyde-3-phosphate dehy-
drogenase, which appears similar to labeled species E, forms stable interactions with high affinity for a limited number of sites on the cytoplasmic face of the human erythrocyte membrane, and that the enzyme is bound to band 3, the predominant membrane protein. Soon after binding to the membrane, about 20–40 amino acids are removed from species B1 by an enzyme which has certain of the properties of chymotrypsin (Fig. 6). Whether this protease is bound to membranes and whether attachment of B1 to the membrane is a prerequisite for the specific proteolytic cleavage remain for future work.

Role of Membrane-Bound Polyribosomes and Synthesis of Membrane Proteins

In mammalian cells which secrete a significant fraction of newly synthesized protein, such as liver cells, plasma, or myeloma cells, and pancreatic acinar cells, a significant fraction of the polyribosomes are firmly attached to membranes; these are the site of synthesis of most, if not all, proteins that are eventually transported out of the cell, such as albumin, trypsinogen, and immunoglobulins (31–36). It is not at all clear that this is the sole function of these polyribosomes.

In mammalian cells which are not secreting any significant amount of protein, such as reticulocytes (25) and HeLa cells (37), there are also some polyribosomes which copurify with membrane fractions during most fractionation procedures. Some of these polysomes can be removed from the membrane by treatment with 0.5 M KCl or 0.5 M NaCl, while an appreciable fraction cannot. The function of these polysomes is obscure. As noted above, Woodward et al. (25) showed that membrane-bound polysomes in reticulocytes synthesize globin almost exclusively, in contrast to earlier work which claimed that they synthesize predominantly nonglobin proteins (26). It is not clear, however, how tightly bound are these reticulocyte polysomes to the cell membrane. We showed that a reticulocyte lysate, free of membranes, will synthesize precisely the same globin and nonglobin proteins—including the two membrane proteins—as are made by the intact cell (23, 24) (see Fig. 4).

Consistent with our work and that discussed above is the notion that two classes of proteins are made on what might be called “true” membrane-attached polysomes, polysomes which remain bound to the membrane in 0.5 M salt solutions; proteins which are excreted from the cell, and membrane proteins which are localized—at least in part—on the external surface of the membranes. Included in this class would be proteins on the inside surface of cytoplasmic vesicles, topologically equivalent to the outside of the cell membrane. Also in this class would be the erythrocyte glycoproteins and presumably other surface glycoproteins. The attachment of the ribosome to a specific site in the membrane would be a prerequisite for the vectorial transport of at least part of the protein through the membrane, as has been shown clearly for proteins which are excreted from the cell (31–36).

We postulate that all cytoplasmic proteins, and all membrane proteins localized at the cytoplasmic surface of the membrane, would be synthesized on free polysomes. In this class would be the two reticulocyte proteins studied in this work. Such membrane proteins would bind to specific receptor sites on the cytoplasmic surface of the membrane such as has been demonstrated for glyceraldehyde-3-phosphate dehydrogenase (21).

A critical test of our model would be, first, to identify the cells which synthesize the erythrocyte...
FIGURE 9  Chymotrypsin digestion of intact reticulocytes and of isolated membranes. 5% polyacrylamide gels containing SDS but not urea were used. (a–c) Control membranes. (d–f) Membranes from cells which had been treated with chymotrypsin. (g–i) Digestion of isolated membranes with chymotrypsin. (a, d, g) Scan of gels stained with Coomassie blue. (b, e, h) Scan of autoradiogram of dried gels. Reticulocytes were labeled in vitro with [35S]methionine before digestion with enzyme or isolation of membranes. (c, f, i) Scan of autoradiograms of dried gels. Reticulocytes were iodinated with [125I] and lactoperoxidase before digestion with chymotrypsin or isolation of membranes. G3PD denotes the position of glyceraldehyde-3-phosphate dehydrogenase run in the same gel as (c).
glycoproteins and spectrin, and second, to determine the types of polyribosomes which synthesize these two proteins.

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