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Asynchronous synthesis of erythrocyte membrane proteins

(erythropoiesis/spectrin/actin/glycoprotein)

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ABSTRACT **The synthesis of membrane proteins of the mature mouse erythrocyte is asynchronous. During erythropoiesis, synthesis of the bulk of the spectrin and actin polypeptides is completed before that of the major transmembrane glycoprotein. Synthesis of the glycoprotein ceases before that of several minor proteins found on the inner surface of the red cell membrane, and one of these minor proteins is made predominantly by reticulocytes. These findings were the result of experiments in which a normal mouse was given a single injection of [^{35}S]methionine. The appearance of radioactivity in the membrane proteins of circulating mature erythrocytes was followed. The earliest labeled proteins to emerge into the blood represent those synthesized at the last stages of erythropoiesis.**

Since the first description of the erythrocyte membrane as a lipid bilayer containing proteins (1), much information has accumulated in recent years about its structure (2–4). In particular, the protein moiety, which provides much of the structural and functional specificity of the cell, is becoming well characterized (5, 6). The human erythrocyte membrane contains 7 to 10 major protein components separable by sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide gel electrophoresis; the predominant species include two polypeptides of molecular weight greater than 200,000 (spectrins), one of molecular weight 90,000, and a sialoglycoprotein (glycophorin) containing blood group antigens. All four appear to be common to mammalian erythrocytes; the first two are found on the interior surface, whereas the latter two span the membrane (7).

Biosynthetic studies with rabbit reticulocytes have shown that two nonglobin proteins of 33,000 and 56,000 daltons (8) are made by the free polyribosomes in the cytoplasm and then attach to the inner surface of the membrane (9). The latter protein loses about 30 amino acids with incorporation into the membrane. Degradative enzymes may result in the loss of specific cytoplasmic proteins during erythroid maturation (10). Since some membrane proteins are lost during red cell development (11–13), it is likely that a degradative process may play a role here as well.

Prior studies of membrane biosynthesis have been carried out under artificial conditions, and it is important to be certain that incubations *in vitro*, treatment with erythropoietin in culture (14), or treatment with phenylhydrazine to produce reticulocytes (12) do not significantly alter the metabolism of the cell to affect translation or degradation. Accordingly, an experiment was designed to investigate membrane protein biosynthesis *in vivo* by the red cells of the normal mouse. The results confirm the observation that membrane protein synthesis is asynchronous. Synthesis of spectrins appears to diminish earlier in erythropoiesis than does that of the 90,000 molecular weight glycoprotein. Confirming earlier work with the rabbit, we show that the only membrane proteins whose synthesis persists until the reticulocyte stage are specific small polypeptides.

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

MATERIALS AND METHODS

Injection of Mice. High specific activity [^{35}S]methionine in an aqueous solution of 10 mM 2-mercaptoethanol (New England Nuclear, Boston, Mass.) was prepared to a concentration of 3 mCi/ml, 200 μCi of which were injected into the medial thigh muscle of each 6-week-old female CD-1 mouse (Charles River Breeding Laboratories, Wilmington, Mass.). For comparison with studies *in vitro*, blood was collected from mice that had been treated with 0.1 ml 0.8% (vol/vol) 1-acetyl-2-phenylhydrazine solution (Sigma, St. Louis, Mo.), pH 7, intramuscularly for 5 days (about 70% reticulocyte count). In addition, nonradioactive normal red cells were collected for external ^{125}I (New England Nuclear) labeling by the method below.

Collection of Blood and Preparation of Membranes. Each mouse was etherized and bled by cutting off a segment of tail, allowing about 0.1 ml of blood to drip into 10 ml of heparinized cold Krebs-Ringer phosphate buffer (15). Although there are bones in the tail, only peripheral blood, but not nucleated marrow cells, was seen after staining with Wright's Stain. Similarly, incubation with new methylene blue revealed only a 2–3% reticulocytosis after the successive episodes of bleeding. These were at 6, 12, 24, 36, 48, 60, 72, and 96 hr, and several days later, at which time a presumed "steady state" had been reached. The cells were pelleted by centrifugation, and the diluted supernatant plasma was saved for analysis of non-trichloroacetic acid precipitable radioactivity as an indication of remaining free [^{35}S]methionine. The cells were washed three times with cold Krebs-Ringer buffer and then lysed by rapid addition of 10 ml of cold 5 mM phosphate buffer, pH 7.4 (16). After the membranes had been pelleted at 20,000 $\times$ *g* for 10 min, the hemolysate was saved for specific activity determination of hemoglobin using acid acetone precipitation and Lowry (17) assay on globin. The membranes were washed three times in the 5 mM phosphate buffer, and the creamy surface of the pellet was solubilized in 5% $NaDodSO_4$ (Matheson, Coleman, and Bell, Norwood, Ohio), 10 μM dithiothreitol (Calbiochem, San Diego, Calif.) by boiling for 5 min. The intact cells or membranes were never stored or frozen during preparation but were kept as close to 4° as possible. The solubilized samples in $NaDodSO_4$, however, were frozen at −20° until use.

Labeling *In Vitro*, Enzyme Digestion, and Elution Studies. Reticulocytes from anemic acetylphenylhydrazine-treated mice were labeled *in vitro* as described (9).

Proteins on the external red cell surface were iodinated by the lactoperoxidase method (18), using $Na^{125}I$.

Red cells that had reached a "steady state" of radioactive labeling about 10 days after the injection of isotope were treated with α-chymotrypsin to digest the proteins on their external

surface (9). One modification was used, however; after removing much of the enzyme by washing the cells with Krebs-Ringer buffer containing 0.015 M EDTA, a specific inhibitor of α-chymotrypsin, tosylphenylalanyl chloromethylketone (TPCK, Calbiochem) (19), 100 μg in 30 μl of methanol, was added with mixing on a Vortex to each milliliter of resuspended red cells, which were then incubated for an additional 30 min at 37°. The cells were again washed in Krebs-Ringer buffer, and membranes were prepared by hypotonic lysis. Band 5 was identified by elution from ghosts incubated at low ionic strength in 0.1 mM EDTA (pH 8) at 37° for 15 min (20).

Polyacrylamide Gel Electrophoresis. The gel system of Fairbanks *et al.* (20) was used. Because the samples of membrane protein were still of low specific activity, 1/8 inch (0.32 cm) thick slab gels were used. Staining with Coomassie blue and destaining were done as in ref. 20. The bands were then scanned on a Joyce-Loebl model MK III C microdensitometer (Joyce-Loebl & Co., Princesway, England).

Autoradiofluorography. In order to enhance the detection of ^{35}S in each band, we permeated the gels with a scintillant, 20% (wt/vol) 2,5-diphenyloxazole (PPO, Interex, Natick, Mass.) in dimethylsulfoxide (Matheson, Coleman, and Bell) (21), but the time for soaking was reduced to 2 hr for slab gels. The dried gel was then placed in contact with a sheet of RP Royal X-Omat film (Kodak, Rochester, N.Y.) that had been exposed to a background optical density of 0.1–0.2 with a strobe flash unit. This precaution was necessary to preserve the linearity of the film's response to samples with low radioactivity (22). The gel-film folder was incubated at −70° for several days to 1 week.

Calculations. After development, the autoradiographs were scanned on the microdensitometer. The area under each peak was measured with a planimeter (model 620015, Keuffel and Esser Co., Morristown, N.J.) and both the percentage of the total area and the ratio of one peak to another were calculated. These values were graphed with respect to changes over the times of bleeding.

RESULTS

Mouse Erythrocyte Membrane Proteins. The human erythrocyte membrane has 7 to 10 major components resolved by the Fairbanks gel system (see numbered bands, Fig. 1A). Comparison of human and mouse membrane preparations by scans of the gels stained with Coomassie blue reveals basic similarities in their banding patterns (Fig. 1A and B), in particular, bands 1 and 2 (the spectrins) and 3 to 5. Bands 6 (glyceraldehyde 3-phosphate dehydrogenase) and 7 are present in human red cells, but they are absent from CD-1 mouse erythrocyte preparations. Instead, a smaller component in mice (band 7.1) is prominent. Fig. 1C shows the autoradiographic pattern of membrane proteins from a mouse labeled for 10 days, in which the bone marrow is no longer incorporating significant amounts of isotope and the circulation contains a stable complement of red cells not yet senescent. All polypeptides visualized with Coomassie blue are evident, although the relative heights of the peaks (intensity of the bands) on the autoradiogram differ somewhat. This may be due to variations in methionine content or Coomassie blue staining of the proteins, or to the presence of glycoproteins, labeled with [^{35}S]methionine, which stain poorly. That all of these proteins are from red cell and not white cell or platelet membranes was confirmed by removal of neutrophils and platelets by filtration of blood through glass wool followed by separation of lymphocytes by sedimentation through a solution of Ficoll-metrizoate

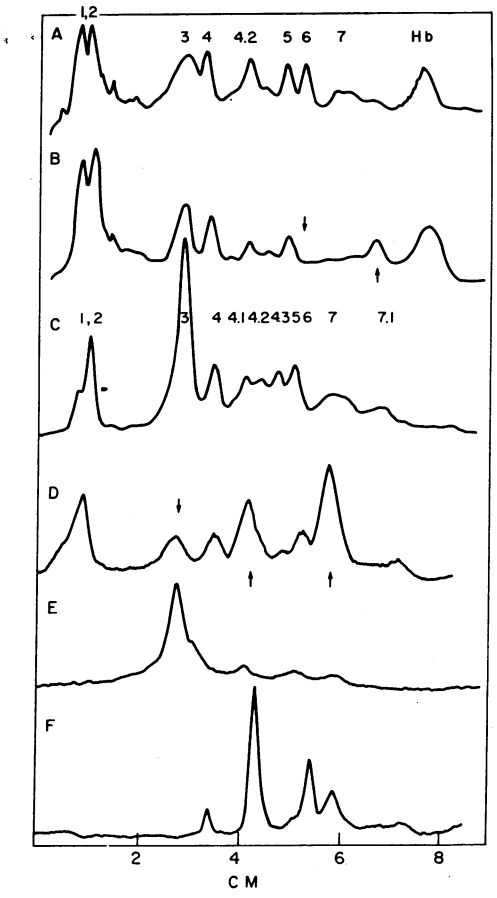

FIG. 1. Polypeptide composition of erythrocyte membrane proteins. Shown are scans of gels stained with Coomassie blue (A and B) and scans of autoradiograms from fixed, dried gels (C–F). (A) Coomassie blue stain of human erythrocyte membrane. Hb, hemoglobin. (B) Coomassie blue stain of mouse erythrocyte membrane. (C) Autoradiogram of mouse erythrocyte membrane 10 days after labeling with [^{35}S]methionine. (D) Autoradiogram of membranes of ^{35}S-labeled mouse erythrocytes (panel C) after digestion of the intact cells with α-chymotrypsin (see *Materials and Methods*). (E) Autoradiogram of mouse erythrocyte membranes after labeling of whole cells with ^{125}I. (F) Autoradiogram of membranes from reticulocytes incubated *in vitro* with [^{35}S]methionine.

("Lymphoprep," Nyegaard, Oslo, Norway) before hemolysis (data not shown).

Several studies showed that, as in the rabbit erythrocyte membrane, only band 3 is exposed on the outer surface. Chymotrypsin digestion of intact cells labeled with [^{35}S]methionine results in reduction of the amount of radioactive peak 3; no other membrane components are diminished. Two new polypeptides (molecular weights 50,000 and 25,000; Fig. 1D, right-hand arrows) are evident; presumably these are derived from fragments of the band 3 polypeptide imbedded within or internal to the lipid layer. Additionally, when intact red cells are reacted with Na^{125}I and lactoperoxidase, only band 3 is labeled (Fig. 1E). These studies show that at least a part of polypeptide 3 is localized to the exterior surface of the plasma membrane; it appears analogous with the two glycoproteins that span the human red cell membrane. The identity of band 5 (actin) was established by its elution from red cell ghosts incubated with EDTA (23) and by its mobility in NaDodSO$_4$ gels with respect to authentic mammalian actin (data not shown).

Synthesis of Membrane Proteins by Reticulocytes. Previous

Table 1. Decline in radioactivity of non-trichloroacetic acid-precipitable ^{35}S with time in the plasma of a mouse after intramuscular administration of radioactive methionine

Time after injection	cpm × 10^{-6}/ml of plasma
15 min	7.1
1 hr	3.8
3 hr	1.7
7 hr	1.1
12 hr	0.7
24 hr	0.6

work showed that rabbit reticulocytes make only two membrane proteins; neither spectrins nor the major glycoprotein is synthesized by these cells, or they are synthesized much more slowly. Fig. 1F shows that a similar situation obtains with mouse reticulocytes found in the peripheral blood after phenylhydrazine injection. The predominant species of membrane protein produced by these cells comigrates with band 4.2; no detectable amount of polypeptide 1, 2, or 3 is made by these cells.

Rationale of the Experiments. Erythrocyte maturation is an orderly sequence of events progressing from the basophilic erythroblast to the adult red cell over the course of several days, but only the reticulocyte and the erythrocyte are found in the peripheral blood. Because of its small size, the mouse can readily be labeled with an injection of [^{35}S]methionine. The isotope is incorporated into protein by the mixed population of cells in the bone marrow. As the erythroid cells in the marrow successively mature, they enter the peripheral circulation as red cells. Marrow reticulocytes are the first to complete the maturation process, and the resultant red cells are sampled at the end of the first 12-hr period. Cells at an earlier stage of development contain a complement of radioactive membrane proteins synthesized at that stage, but they do not appear as erythrocytes until a later collection period. The length of this interval between labeling and sampling reflects the time required for cellular maturation to occur; it is chronologically shorter for those cells closest to maturity. The membrane proteins synthesized by the most immature cells will appear in the peripheral blood only at the longest interval of several days.

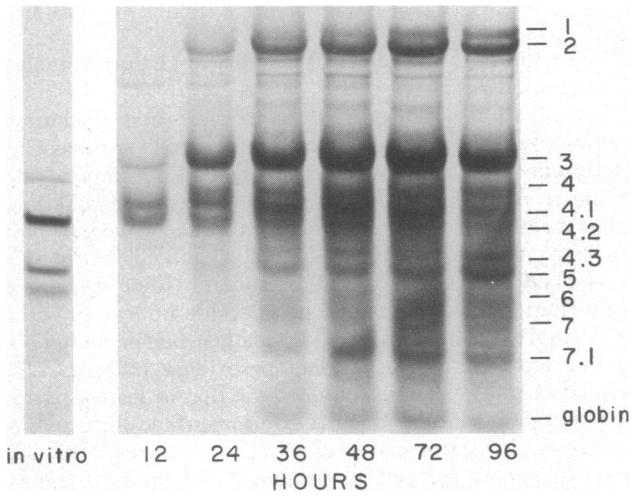

FIG. 2. Autoradiogram of a NaDodSO$_4$-polyacrylamide gel showing membrane preparations taken at various time intervals with label *in vitro*. (Left) Membrane sample from reticulocytes incubated with [^{35}S]methionine *in vitro*. (Right) Membrane samples from a normal mouse labeled *in vivo* (times of collection shown).

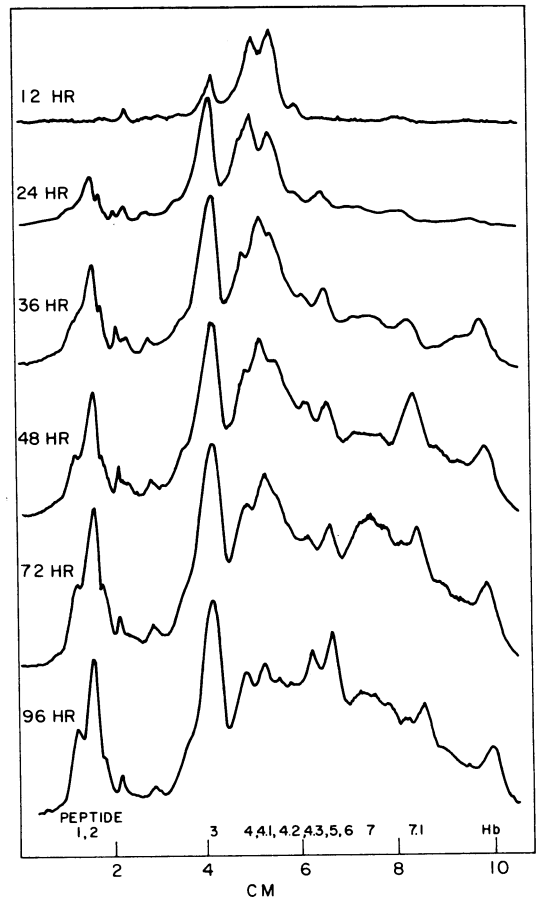

FIG. 3. Scans of autoradiograms in Fig. 2.

Label Description. The time course of radioactivity after the injection of isotope was followed in the serum (Table 1). At 15 min, a large amount of free [^{35}S]methionine is present, but with a half-life of about 1.5 hr; it becomes metabolized, excreted, or bound onto trichloroacetic acid-precipitable plasma protein, so that relatively small amounts of nonprecipitable radioactivity are left by the end of 12 hr. We assume, therefore, that incorporation of ^{35}S radioactivity into marrow proteins occurs predominantly within 6 hr of injection. Similar results were obtained by the intravenous and intramuscular routes, so the latter was chosen for convenience.

Synthesis of Erythrocyte Membrane Proteins. Shown in Fig. 2 is an autoradiogram of a polyacrylamide gel analysis of membrane proteins prepared from peripheral red cells at successive intervals after injection of [^{35}S]methionine. Scans of these gels are in Fig. 3, and Fig. 4 depicts the relative amount of labeled species at the different times after injection. In membranes from red cells collected 12 hr after injection of [^{35}S]methionine, the predominant labeled species were polypeptides 4.1 and 4.2 (in most experiments these peptides migrated together on gels); this correlates well with the pattern of membrane proteins synthesized by reticulocytes *in vitro*. At 12 hr there is little label in band 3 and none in the spectrin region (bands 1 and 2) or actin region (band 5). At 24 hr after injection, labeling of band 3 has almost reached the maximum steady state level, and there are small amounts of radioactivity in the spectrin region and in band 5, which appears in parallel. At later times the amounts of radioactivity in the spectrin and actin polypeptides (bands 1, 2, and 5) increase considerably.

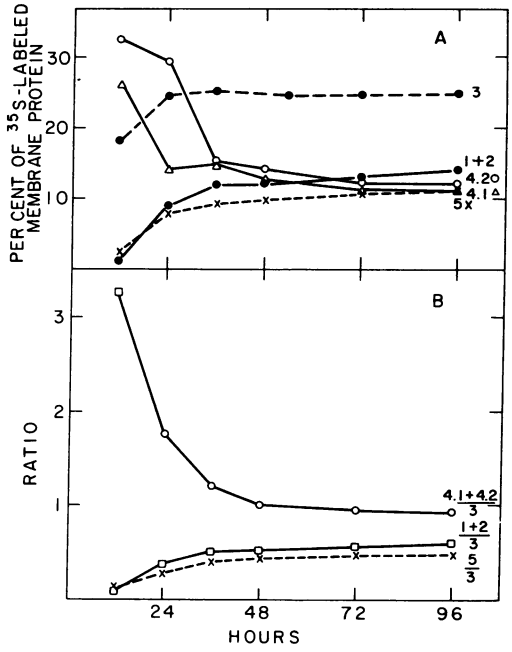

FIG. 4. (A) Variation in percentage of radioactive membrane components compared to total membrane proteins over time. (B) Variation with time in radioactivity in bands 1 + 2, 4.1 + 4.2, and 5 compared to that in band 3.

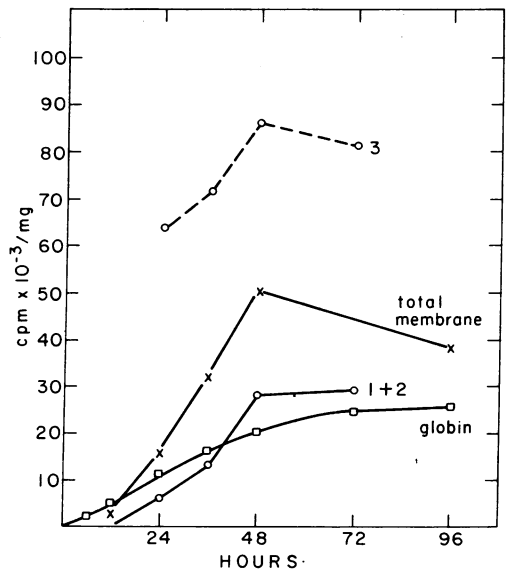

FIG. 5. Changes in specific activity of bands 1 + 2, band 3, and total membrane protein relative to specific activity of hemoglobin over time. The higher specific activity of band 3 may be due to a carbohydrate moiety, which leads to decreased staining by Coomassie blue and, hence, underestimation of protein in the band.

Changes in the pattern of labeled membrane proteins occur early, after only four episodes of bleeding; compensatory erythropoiesis is not marked. One study showed that indeed the successive bleedings did not significantly influence the orderly, sequential release of cells from the marrow. Different mice were bled for the first time at 12, 24, 36, 48, and 60 hr after injection of [^{35}S]methionine; the profile of labeled erythrocyte membrane protein was essentially the same as depicted in Fig. 2 (data not shown).

Fig. 4B shows that the ratio of radioactivity in bands 4.1 plus 4.2 to band 3 decreases over 3-fold from 12 to 48 hr. The ratio of radioactivity in bands 1 plus 2 to that in band 3 and also of band 5 to band 3 increases over 5-fold during the same period. We conclude that synthesis of red cell membrane proteins occurs asynchronously during erythropoiesis; chronologically, production of the spectrins is completed first, followed by the glycoprotein (band 3). Synthesis of polypeptides 4.1 and 4.2 finishes last; both the *in vitro* (Fig. 1F) and *in vivo* (Figs. 2–4) mouse experiments suggest that reticulocytes make these proteins predominantly.

Finally, the specific activity of the membrane polypeptides was compared to that of the hemoglobin in the red cells at each given point in time as a means of assessing relative rates of synthesis (Fig. 5). Appearance of radioactivity in membrane polypeptide 3 parallels that of hemoglobin, a result suggesting that the two proteins are made in the same cell types. Since radioactivity in the red cells 12 hr after injection of [^{35}S]methionine is a result of synthesis by reticulocytes predominantly, Fig. 5 confirms the studies *in vitro* that reticulocytes synthesize hemoglobin and polypeptides 4.1 and 4.2, but little of polypeptides 1, 2, or 3.

DISCUSSION

In this experiment a mouse is given a single injection of [^{35}S] methionine, and incorporation of radioactivity into the membrane proteins of peripheral red cells is followed. It is important to note that this type of study measures the synthesis of only those proteins that are found in the mature erythrocyte. Consistent with our results is the possibility, for instance, that polypeptides 4.1 and 4.2 are synthesized at the same level throughout erythropoiesis, but that the protein is degraded rapidly in the immature cells but not in reticulocytes. A critical test of such alternative explanations will require studies on isolated populations of bone marrow precursor cells.

Studies by Morrison *et al.* (24) indicate that at least the externally iodinated membrane proteins are all degraded at the same rate during membrane remodeling, although in contrast, preferential destruction of the larger proteins on the cytoplasmic face is suggested by the work of Lodish *et al.* (10). Furthermore, since only the appearance of proteins in the membrane was studied, differential rates of transport or incorporation of these proteins into the membrane cannot be excluded. It is possible that smaller molecules such as in bands 4.1 or 4.2 arrive at the membrane more quickly than spectrin, but these differences should be small, not over the course of hours as in this experiment. From other work in our laboratory (25), it would appear that transmembrane glycoproteins similar to that in band 3 may be synthesized *in situ* by membrane-bound ribosomes, followed by attachment of inner surface proteins that are synthesized by ribosomes free in the cytoplasm, which is not the sequence found in this experiment. Thus, the possibility that band 4.1 and 4.2 protein is synthesized but hindered cytoplasmically from reaching the membrane in the immature cells but not in the reticulocyte, cannot be excluded.

Assuming that there is no change in the turnover or transport of specific red cell membrane proteins during erythropoiesis, our results show that synthesis of red cell membrane proteins is asynchronous and that different polypeptides are made by precursor cells at different stages of development. The last membrane proteins made in erythropoiesis are found in polypeptides 4.1 and 4.2, which are similar to membrane polypeptide synthesized *in vitro* by mouse reticulocytes (Fig. 2). Synthesis of polypeptide 3 occurs earlier in erythropoiesis than that of bands 4.1 and 4.2 and finishes later than that of spectrin. Since spectrin is thought to be associated as a complex with band

5, it is not surprising that they appear together in peripheral red cells, beginning at 24 hr after injection and reaching a maximum at 48 hr. Since the total process of erythroid maturation encompasses 3 to 4 days, synthesis of all the major membrane proteins occurs in the last half of red cell development, from normoblast to reticulocyte. Spectrin production begins to decline before release of the cell from the marrow, but band 3 continues at least for a while in parallel with hemoglobin synthesis.

Obviously band 3 is not made by reticulocytes *in vitro* (Figs. 1F and 2), although hemoglobin is, and it would probably be correct to conclude that membrane protein synthesis is completed earlier; however, it remains to be proven that the apparent absence of synthesis of peptide 3 by reticulocytes is not due to incomplete glycosylation *in vitro*, altering the protein's characteristic electrophoretic mobility.

Finally, it has been noted that much of the variability in pattern of membrane proteins between mammalian erythrocytes occurs in the lower molecular weight regions (bands 4–7). Although it would be convenient to impute species specificity to these differences, many of the smaller proteins lie on the internal side of the membrane, and hence might be contaminated with cytoplasmic proteins. It is of interest, though, that the proteins that are the most variable among mammals are among the last to be made in erythropoiesis. The larger proteins (bands 1, 2, and 3) and actin (band 5) most likely provide a basic structure for all mammalian red cells upon which modifications (such as glycosylation) unique to a species are superimposed. Further characterization of membrane protein synthesis using fractionated erythroid precursors will be necessary to delineate more precisely such developmental changes.

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