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BIOGENESIS OF ERYTHROCYTE MEMBRANE PROTEINS IN VIVO STUDIES IN ANEMIC RABBITS

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SUMMARY

To study the process of red cell membrane protein synthesis we have followed the time course of [^{3}H]leucine appearance in total protein and individual peptides of the erythrocyte membrane following injection of the amino acid into phenylhydrazine-anemic rabbits. Multiple peripheral blood samples were taken from single animals over a 5-week period. Erythrocyte membrane proteins were separated by polyacrylamide gel electrophoresis in sodium dodecylsulfate and dithiothreitol; incorporation of radioactivity was determined by gel slicing and liquid scintillation spectrometry. Appearance of [^{3}H]leucine in circulating erythrocytes reached a peak at 1–3 days, with a steady decline thereafter. The radioactive amino acid appeared first in the lowest molecular weight peptides and last in the largest peptides; at the earliest time point (8 h), little radioactivity was observed in any of the four largest peptides present in the membranes (bands A, 1, 2 and 3). Certain smaller peptides (bands 4, 5 and 9) were the predominant species labeled at this time. By 24 h all peptides showed significant incorporation. With maturation of the red cells, label largely disappeared from bands A, 9 and several smaller peptides; this was confirmed by finding that the peptides are virtually absent from mature circulating erythrocytes. These data are interpreted as showing that red cell membrane proteins are synthesized asynchronously during the life cycle of the erythrocyte; the largest peptides are made predominantly in the earlier marrow stages of development, while certain of the smaller peptides are still being synthesized in the reticulocyte stage. Several membrane proteins appear to be specific to the reticulocyte and are lost during the process of cell maturation in the circulation.

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INTRODUCTION

Mammalian red cell membranes have been extensively studied because of their physiologic importance and ready availability. They contain between 6 and 12 major protein constituents [1, 2] which can be divided into two groups. The first consists of proteins intimately associated with the lipid "core" of the membrane [3, 4]. In human red cell membranes there appear to be only two major proteins in this class: the predominant glycoprotein, which contains many of the antigenic determinants of the cell surface, and a peptide of molecular weight 90 000–100 000 which is the most abundant species present on a molar basis [5, 6]. Evidence from labeling and enzymatic studies suggests that these two major proteins span the width of the membrane and are exposed at the inner as well as the outer surface [7–9].

The remaining peptide species are consistently isolated with hemoglobin-free membranes but are more loosely associated with the lipid "core" and can be extracted by a variety of mild buffer and salt solutions [1]. They appear to be exposed only at the inner surface of the membrane [10]. These peptides include the largest red cell membrane protein, "spectrin" [11], with a molecular weight of 200 000–250 000, and several smaller peptides; one of the latter has recently been purified and identified as a subunit of glyceraldehyde-3-phosphate dehydrogenase [12, 13]. The other proteins have not as yet been associated with specific membrane functions.

Despite these advances, little is known of the biosynthesis of red cell membrane proteins. Bulova and Burka [14] have studied the synthesis, by intact reticulocytes and isolated polysomes, of "non-globin" protein; they have concluded that the bulk of "non-globin" protein is made by membrane-bound polysomes. In contrast, recent evidence [15, 16] has been presented that membrane-bound and free polysomes synthesize the same protein species. In studies with reticulocytes in vitro, Lodish [17] reported that only two minor species of membrane proteins were actively synthesized by reticulocytes from acetylphenylhydrazine-treated rabbits.

In view of the paucity of information on red cell membrane biogenesis, we have examined the process in detail both in vivo and in vitro. We report here the results of in vivo studies in anemic rabbits.

MATERIALS AND METHODS

Materials

Phenylhydrazine was obtained from Sigma Chemical Company; [^{3}H]leucine, with a specific activity of 33 Ci/mmol, was purchased from New England Nuclear; NCS Tissue Solubilizer is an Amersham-Searle product. All other materials were reagent grade.

Preparation of rabbits and collection of samples

Male white rabbits weighing approximately 2 kg were rendered anemic by five daily subcutaneous injections of 2.5 % phenylhydrazine, neutralized to pH 7.0 with NaOH prior to injection, at a dose of 6 mg per kg. 48 h after the last injection, 500–1000 μCi of [^{3}H]leucine was given by intracardiac injection. Subsequent blood samples were obtained at specified intervals (see Results) by intracardiac puncture, without the use of anesthesia. Whole blood was collected in EDTA and rapidly chilled;

the volume of blood drawn varied between 10–20 ml depending on the hematocrit of the rabbit at the time, so that 3–5 ml of washed red cells were obtained for study. All subsequent steps were carried out at 4 °C. Crosstransfusion experiments were carried out in five rabbits (see Results).

Preparation of samples

Whole blood was centrifuged for 10 min in a clinical centrifuge, and the plasma removed and saved. The red cells were washed three times in 0.154 M NaCl/5 mM sodium phosphate, pH 8.0; at each step red cells were freely sacrificed to insure complete removal of the "buffy coat". Ghosts were prepared by hypotonic lysis in 20 volumes of 5 mM sodium phosphate, pH 8.0 (Buffer A) according to Dodge et al. [18], followed by 3–4 washes in the same buffer. The original supernatant, and that from the first wash, were combined for hemoglobin studies. In general, ghosts prepared from red cells with high reticulocyte counts (i.e. the first 5 days after leucine injection) always contained some residual hemoglobin despite exhaustive washing; as reticulocytosis decreased, completely "white" membranes could be routinely prepared. The final washed membrane preparation was suspended in Buffer A in a volume equal to that of the initial packed red cells; protein concentrations were routinely 2–4 mg per ml. To determine total membrane protein specific activity, an aliquot (usually 0.5 ml) of the membranes was precipitated with 9 volumes of 7 % trichloroacetic acid, the pellet washed twice by resuspension in 5 % trichloroacetic acid, and finally dissolved in 1 ml of NCS Tissue Solubilizer. Radioactivity was determined by liquid scintillation counting in a toluene-based mixture (42 ml Liquifluor (New England Nuclear) per l of toluene) in a Packard TriCarb scintillation counter; correction for quenching was made by use of an external standard.

To determine the specific activity of "hemoglobin", globin and other soluble proteins were precipitated from solution by addition to 10 volumes of acetone containing 1.5 % HCl, washed twice in the same solution, once in 5 % trichloroacetic acid, dissolved and radioactivity determined as above. This procedure effectively removed all heme and left a colorless precipitate. Since greater than 90 % of the soluble protein of red cells is hemoglobin, the radioactivity in this precipitate was taken to represent incorporation of leucine into "hemoglobin" without further correction.

Aliquots of plasma were prepared for counting as described for ghost membranes.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of membrane proteins in sodium dodecylsulfate and dithiothreitol was carried out as described by Fairbanks et al. [1]. To determine protein patterns, aliquots of dissolved membranes containing approximately 50 μg of protein were electrophoresed on 6 × 85 mm gels and stained with Coomassie blue. For determination of radioactivity, up to 4 mg of protein were applied to 19 × 90 mm gels and subjected to electrophoresis overnight at a current of 8–12 mA per gel; the tracking dye was allowed to migrate a distance of 75 mm. Two separate techniques for slicing the 19 mm gels were used. In our initial studies (Method A, Figs 3 and 4), the gels were removed and frozen at −20 °C. They were then allowed to shaw slightly for 1–2 min at room temperature and were sliced into approximately 45 equal sections by an array of razor blades spaced at 1.7–1.8 mm intervals, clamped

in place on a frame of three brass rods. The individual slices were placed in counting vials and air-dried for 24–48 h; 0.5 ml of 30 % H_2O_2 was added to each dried slice [19], the vials tightly capped and incubated in a metabolic shaker at 40 °C until dissolved, usually after 24–48 h. Two ml of NCS Tissue Solubilizer and 10 ml of toluene scintillation solution were added in that order, and radioactivity determined. With this method, [^{3}H]leucine can be counted with 15–20 % efficiency. Correction for quenching was made using an external standard.

Careful evaluation of the pattern of labeling seen in the large gels sliced by this means indicated that clear separation of closely spaced peptide bands, especially in the low molecular weight region, was not achieved. For this reason peptides were combined into three readily separable groups, A, B and C, based on molecular weight, and total leucine incorporation into these three groups was determined as the sum of the counts contained in each group (see Results).

To examine in more detail the changes in specific peptide bands, a recently described technique [20] was utilized which permits reproducible slicing of large gels into 1 mm sections (Method B); the gels are not frozen in this method, avoiding the distortion inherent in the freezing process. To insure exact identification of individual peptide bands, the large gels were marked at 1 cm intervals prior to slicing, and the location of the 1 cm markers noted at the time of slicing. With this technique, peaks of [^{3}H]leucine incorporation exactly corresponded to individual peptide bands as determined on scans of Coomassie blue stained gels (see Results).

By prolonged staining of representative large (19 mm) gels, we determined that R_F values for individual peptide bands were similar on the large and small gels. Individual bands may be slightly wider on the 19 mm gel than on the usual analytical (6 mm) gel. Because of their size, stain penetrates poorly to the center of the 19 mm gels; to be certain that "streaming" of protein bands during electrophoresis was not a problem, we sectioned several gels longitudinally into quarters and stained these sections. The bands were horizontal and showed no distortion at the center of the gel. Once we had demonstrated the equivalence of electrophoretic patterns on the 19 mm and 6 mm gels, the larger gels were routinely cut without prior staining.

Protein was determined by the method of Lowry et al. [21] using bovine serum albumin as standard. Hemoglobin was determined by the cyanmethemoglobin method [22]. Reticulocyte counts were performed using New Methylene Blue as the stain; 500 cells were counted to obtain the percentage.

RESULTS

Time course of [^{3}H]*leucine incorporation*

At the time of [^{3}H]leucine injection, all rabbits were significantly anemic (Hb 7.2±2.8 g %) with high reticulocyte counts (83 and 90 % in the "high reticulocyte" animals and 62 and 51 % in the "low reticulocyte" animals). Over the 3-week-course of the experiment, hemoglobin rose to a final level of 11.0±0.8 g % and reticulocyte counts dropped to a mean of 7±3 % despite repeated blood sampling. The time course of appearance and disappearance of label in the circulation of typical "high" and "low" reticulocyte" animals is shown in Fig. 1a. Specific activity of total plasma protein declined logarithmically over the 3-week period. The specific activity of globin was generally somewhat higher than that of the total red cell membrane

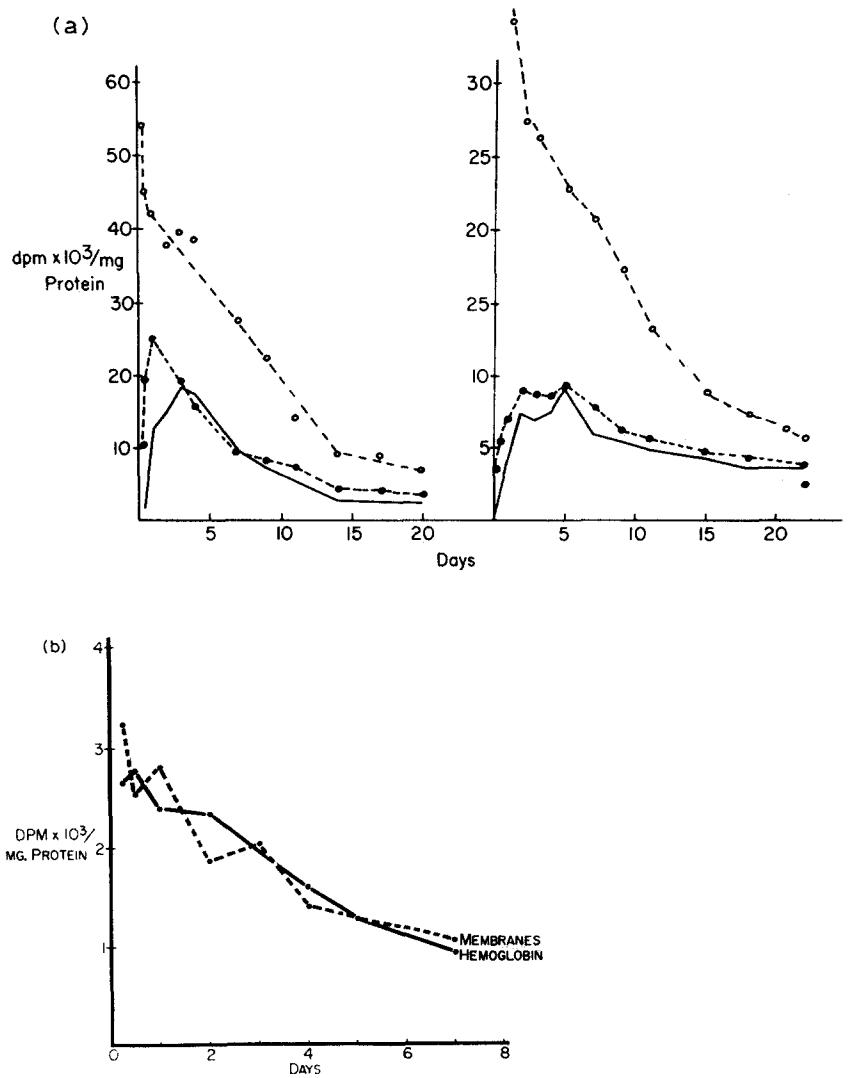

Fig. 1. (a) Time course of [^{3}H]leucine incorporation into total plasma proteins (○- - -○), hemoglobin (●- - -●), and erythrocyte membrane proteins (●—●) in phenylhydrazine-treated rabbits. For details of procedure see Materials and Methods. The figure on the left represents a typical "high reticulocyte" rabbit and that on the right a "low reticulocyte" rabbit. (b) Changes in specific activity of hemoglobin and red cell membrane proteins after transfusion of labeled reticulocytes into normal recipients.

protein and peaked earlier. There was considerable fluctuation in these two specific activities for the first few days, probably due to sequestration and release by the reticuloendothelial system of newly-released red cells, but from Day 3 on specific activity of globin and membrane protein declined in parallel. The specific activities in "high reticulocyte" animals were higher than those in "low reticulocyte" animals, as expected, but generally followed similar patterns.

To ensure that the time course of disappearance of labeled cells in vivo was not influenced unduly by a prolonged effect of phenylhydrazine within the circulation, crosstransfusion experiments were carried out in five rabbits. Reticulocytosis was induced in donor rabbits using phenylhydrazine as described above (see Materials and Methods). 24 h after the last injection, the donor rabbits received 2 mCi of [^{3}H]leucine by intracardiac injection. Blood was obtained 48 h later by cardiac puncture, collected in acid citrate dextrose solution, and crosstransfused into non-anemic recipients via a marginal ear vein. An identical volume of blood was drawn from the recipient immediately prior to the transfusion. Samples from the recipients were then obtained at specified intervals by cardiac puncture and collected in EDTA as described above (see Materials and Methods). The results from a typical experiment are shown in Fig. 1b. The parallel decrease in the specific activity of globin and membrane protein is similar to that seen in the previously described non-crosstransfused animals. The $T_{\frac{1}{2}}$ for hemoglobin was 4.8$\pm$0.4 days, and for membrane proteins 4.7$\pm$0.3 days.

Polyacrylamide gel patterns of red cell membrane proteins

Fig. 2a shows a typical pattern of rabbit red cell (reticulocyte-rich) membrane proteins as separated on polyacrylamide gel in comparison with human red cell

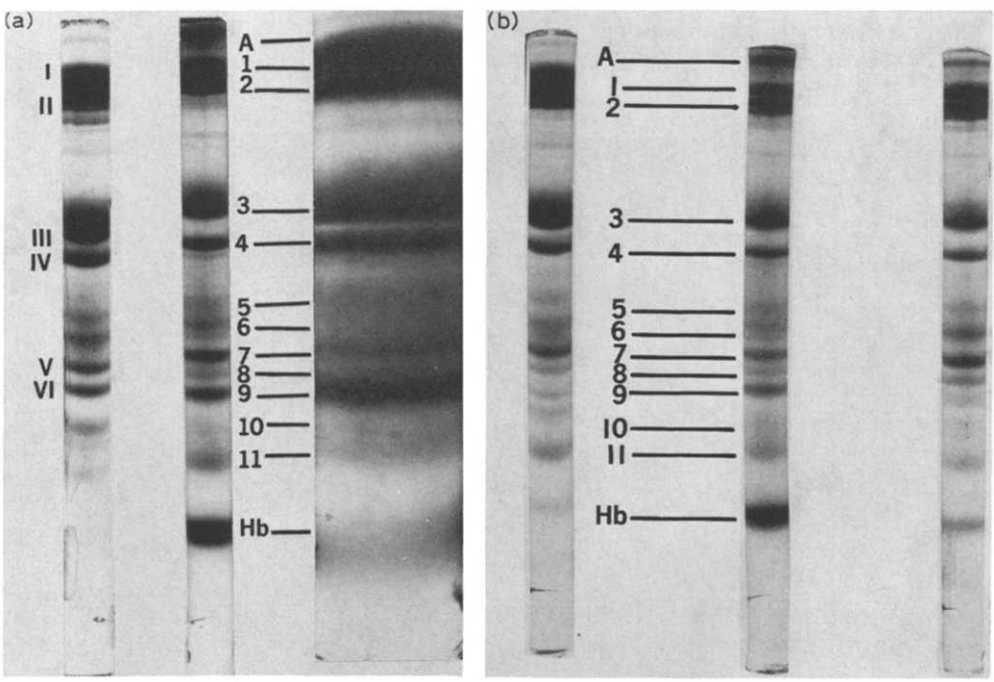

Fig. 2. Red cell membrane proteins examined by polyacrylamide gel electrophoresis. (a) The gel on the left shows human red cell membranes with the numbering system of Fairbanks et al. [1]. The gel in the center shows the pattern seen with rabbit reticulocyte membranes. On the right is a 19 mm gel of the same membrane preparation showing the resolution achieved with the large gels. (b) The gel on the left shows the pattern seen with normal red cells from an untreated rabbit. In the center is the pattern seen following the induction of reticulocytosis by phenylhydrazine. The gel on the right shows the pattern seen with cells from the same rabbit obtained 5 weeks following phenylhydrazine treatment.

membranes; they show similar peptide patterns with only minor exceptions. For comparison, a large (19 mm) gel of rabbit membranes is included; resolution is similar to that on the smaller gel. The major peptide bands of the rabbit red cell membranes are numbered strictly in sequence beginning with the double band at 200 000–250 000 molecular weight, to conform with the numbering system for human red cell ghosts used by Fairbanks et al. [1] which is also shown. A large peptide, seen primarily in rabbit reticulocytes and to a much lesser extent in mature red cells, is labeled band A. Another peptide observed in rabbit red cell membranes prepared from reticulocyte-rich blood, band 9, is virtually absent from mature circulating (i.e. reticulocyte-poor) rabbit red cells [23].

Fig. 2b shows a comparison of polyacrylamide gel electrophoresis patterns of rabbit red cell membranes from an untreated rabbit, a rabbit with a high reticulocyte count at 4 h following injection of [^{3}H]leucine, and at 5 weeks following the injection of the label. The untreated erythrocytes and those obtained 5 weeks later show similar patterns. The middle gel in the picture shows the pattern obtained in a rabbit with a reticulocytosis showing the increased prominence of bands A and 9, and the increased hemoglobin present which could not be washed free from the membranes. The remainder of the gel pattern is identical.

Radioactive labeling of membrane proteins — Method A

Fig. 3 shows the pattern of incorporation of [^{3}H]leucine seen when large gels

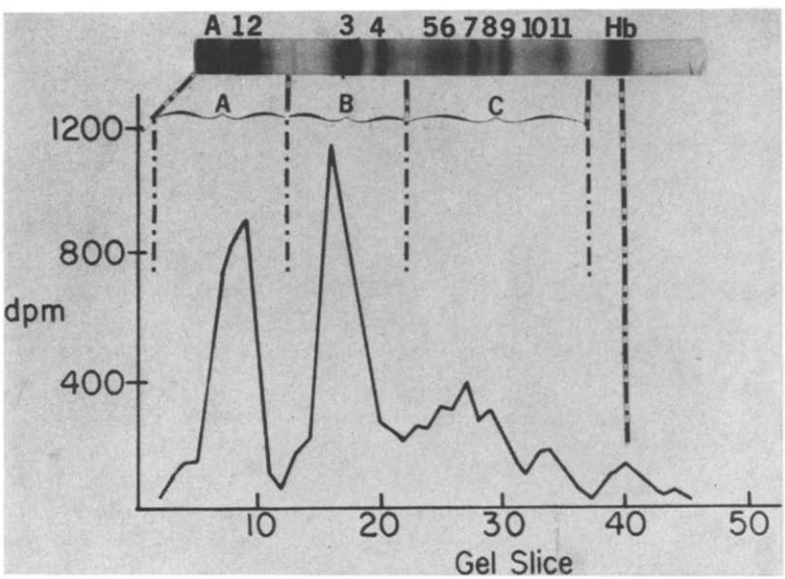

Fig. 3. [^{3}H]Leucine labeling of red cell membrane peptides. The graph shows a typical pattern of incorporation into membrane proteins when separated on a large (19 mm) polyacrylamide gel, sliced by Method A (see Materials and Methods). For comparison, the pattern seen in the Coomassie blue stained analytical gel of the same sample is shown above, with the arbitrary division of the peptides into three groups of differing molecular weights (see Results). As will be noted, separations of radioactivity in Group C on the large gel are not sufficiently clear to give a one-to-one correspondence between radioactive peaks and stained bands on the small gel. The small peak of radioactivity at gel slice 40 is hemoglobin.

were sliced by Method A, compared with a small stained gel. Separation of label into the three major classes as shown was readily achieved, but distinct separation of radioactivity in individual peptide bands in Group C was not possible.

[^{3}H]leucine appeared with a different time course in each of the three major groups of peptides. Absolute rates of incorporation varied considerably from animal to animal, with peak specific activities reached at 3–5 days (Fig. 1a). To achieve comparability, the data are expressed (Fig. 4) as the fraction of the total incorporated

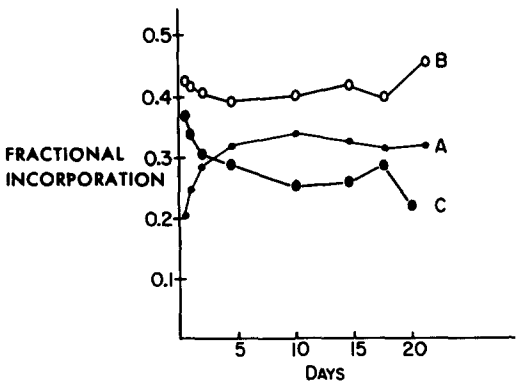

Fig. 4. Incorporation of [^{3}H]leucine into major peptide groups with time; slicing Method A (see Materials and Methods). The amount of radioactivity in each group at each time point was determined from a graph similar to Fig. 3; the major groups were separated by visual inspection of the patterns following staining, since R_F values for individual bands differed by one or two slices from gel to gel. The radioactivity in each group is the sum of the counts in the slices within the group and is shown as a fraction of the total label applied to the gel.

[^{3}H]leucine present in each of the three major peptide subgroups, described in Fig. 3, at various time points from 12 h to 20 days after injection. Only samples with sufficient radioactivity present so that 5000 dpm could be applied to the gel were used. We noted consistently that the lowest molecular weight peptides (Group C) contained a relatively high proportion of the total [^{3}H]leucine incorporated at the early time points, but then represented a progressively declining fraction of total radioactivity from Day 1 to Day 10, remaining stable thereafter up to 3 weeks. Group B contained a relatively constant fraction of the total radioactivity throughout. Group A (the higher molecular weight peptides) showed the lowest relative incorporation initially, but represented a progressively increasing fraction of the total [^{3}H]leucine incorporation over 8 days, and then remained stable for the subsequent 10 days.

It should be emphasized that polyacrylamide gel patterns of membrane proteins do not change over the first 5–7 days of the experiment, the period during which greatest change in fractional incorporation is seen. This indicates that changes in fractional incorporation must reflect a change in the specific activities of peptides within a particular fraction, and not a change in total protein content. This is not true for changes seen later in the time course (see below).

Radioactive labeling of membrane proteins — Method B

To demonstrate in more detail the pattern of [^{3}H]leucine incorporation into specific peptide bands, we utilized gel slicing Method B (see Materials and Methods).

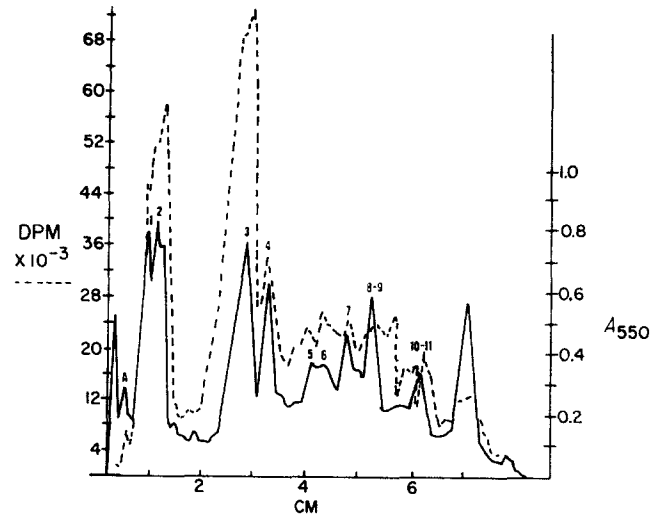

Fig. 5. [^{3}H]Leucine labeling of membrane proteins using slicing Method B (see Materials and Methods). The horizontal axis represents distance in cm along the large (19 mm) gel. Dpm of radioactivity in individual slices of the gel (- - -) is compared with the densitometric scan (—) of the Coomassie blue stained analytical gel of the same membrane preparation, obtained from a rabbit 48 h after [^{3}H]leucine injection. The greater resolution of peptide bands using this method is evident. The individual bands on the densitometric scan are numbered A to 11, and show clear correspondence with the peaks of radioactivity. The sharp peak at 7 cm is hemoglobin.

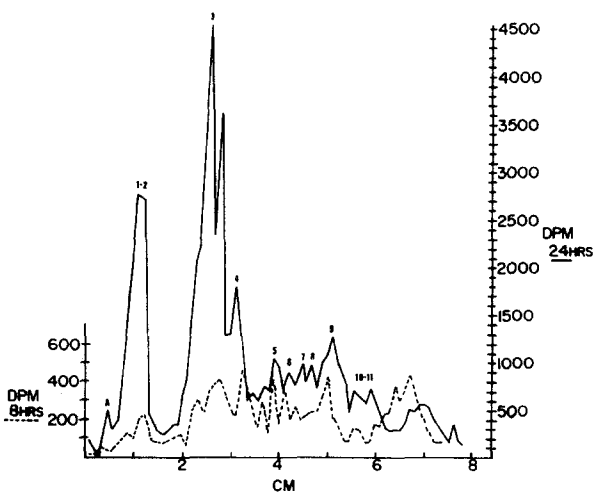

Fig. 6. [^{3}H]Leucine incorporation at early time points — Method B (see Materials and Methods). The incorporation of radioactivity into individual peptides at 8 h (- - -) and 24 h (—) after [^{3}H]leucine injection in red cell membranes from the same rabbit are superimposed; the peaks are numbered as in Fig. 2. The horizontal axis represents distance in cm along the gels.

That individual peptide bands can be adequately resolved on 19 mm gels is shown in Fig. 5; the pattern of radioactivity observed when a large gel is sliced by this method is compared with a densitometric tracing of the same sample taken from a 6 mm stained gel. The membranes used were obtained 48 h after [^{3}H]leucine injection into an anemic rabbit. Peaks of radioactivity exactly correspond with the stained peptide bands, with the exception of a small peak of radioactivity occurring after band 9. Whether this represents a minor peptide of high specific activity between bands 9 and

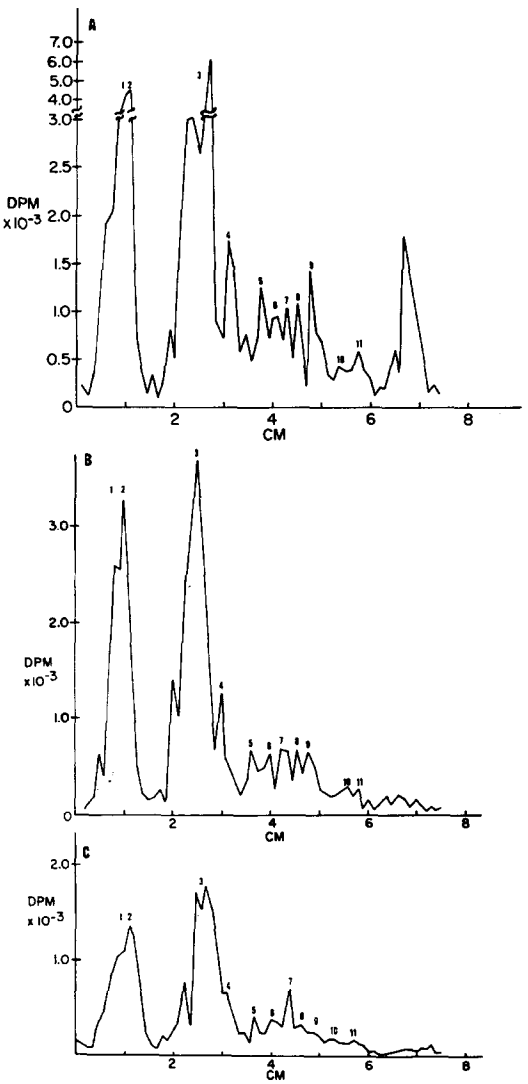

Fig. 7. Changes in [^{3}H]leucine label in red cell membranes at late time points. The pattern of radioactivity is gels sliced by Method B (see Materials and Methods) of red cell membranes obtained from the same rabbit at 1 week (A), 3 weeks (B) and 5 weeks (C) is compared. The peak of hemoglobin is prominent at 1 week at 7 cm on the horizontal axis and virtually absent by 5 weeks.

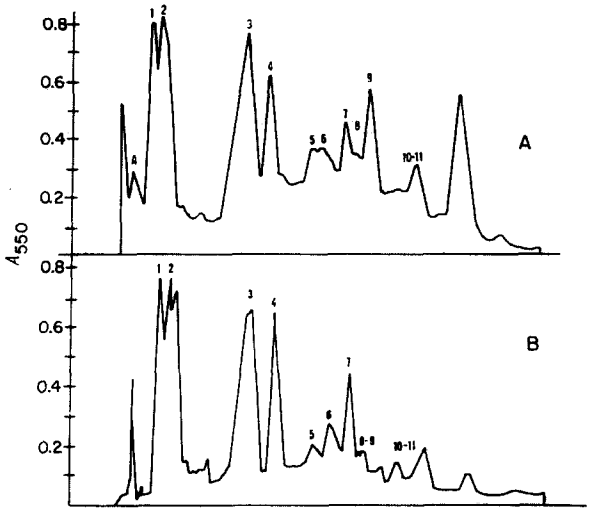

Fig. 8. Densitometric scans of Coomassie blue stained analytical gels — changes with time. The scans of stained gel patterns of membrane preparations from the same rabbit obtained at 1 week (A) and 5 weeks (B) are compared. The scans show changes similar to those seen with [^{3}H]leucine labeling in Fig. 7.

10 or is an artifact of slicing is not clear, but it is not present as a major peak of radioactivity at later time points.

Fig. 6 shows a typical experiment in which labeled red cell membranes were prepared from a single rabbit 8 and 24 h after [^{3}H]leucine injection and were examined by the above technique. The differences in the pattern of incorporation are striking; similar changes were seen in two other animals studied. At 8 h there is no label in band A, and minimal amounts in bands 1 and 2. Relative to the total amount of protein present in band 3, there is also little incorporation of the amino acid into this peptide. All of the smaller peptides from band 4 on show significant incorporation; at 8 h band 9 is particularly prominent. By 24 h a striking increase in incorporation into band 1–3 is evident, and there is some [^{3}H]leucine present in band A. Among the smaller peptides, bands 4 and 9 show the greatest incorporation; band 7 still shows only a small peak. These changes persist through 48 h; the data in Fig. 5 represent the pattern of incorporation seen in the same rabbit at 48 h. Of interest is the small amount of labeled leucine present in the hemoglobin which remains bound to the membrane; this is in marked contrast to the pattern seen when reticulocytes are studied in vitro [24].

To follow the disappearance of labeled red cell membranes from the circulation, we sampled rabbits at 1–5 week time points following injection (Fig. 7). It became clear that there was a not symmetric loss of radioactivity from the various peptide bands. In particular, [^{3}H]leucine in bands A and 9 virtually disappeared, and band 7 became relatively more prominent among the lower molecular weight group.

Densitometric scans (Fig. 8) of membrane proteins taken at early and late time points confirmed the changes seen in the disappearance of [^{3}H]leucine. Three major differences are seen between the protein patterns of membranes prepared from

reticulocyte-rich (early) and mature (late) red blood cells. Bands A and 9 almost disappear during the process of cell maturation. Furthermore, there is a marked decrease in a number of minor peptides which are scattered throughout the lower molecular weight range. While these do not appear as separate peaks on the scan, they can be seen by direct observation of the stained gel, and their loss is reflected in the marked lowering of the "baseline" of the scan in the region from band 4 to hemoglobin. These changes exactly parallel those seen in the labeling experiment (Fig. 7). The scan patterns also show the consistent presence of significant amounts of hemoglobin bound to reticulocyte membranes despite exhaustive washing, whereas mature cells can be washed free of virtually all traces of hemoglobin.

DISCUSSION

In interpreting the results of studies using in vivo labeling of red cells, it is essential to keep in mind the unique life cycle of this cell. Erythrocytes are thought to begin in the marrow as undifferentiated "stem" cells, to undergo several maturation divisions, then a "normoblast" stage in which the nucleus becomes progressively smaller, more densely stained (pyknotic), and is finally extruded from the cell. It is during this latter normoblast stage that hemoglobin synthesis occurs to a major extent [25]. When entering the circulation from the marrow, the young red cell or reticulocyte is devoid of nucleus but contains single ribosomes, polysomes, and a few mitochondria. The reticulocyte is unable to synthesize DNA or RNA [26] but maintains the capacity to synthesize protein, predominately hemoglobin [15], for 1–2 days before losing the last of its intracellular organelles [27, 28]. For the remainder of the life span of the cell, about 120 days in humans and 50 days in the rabbit [29], no new protein is synthesized; there is evidence for some progressive loss of intracellular protein (hemoglobin) and cell water during this period [30].

In the present experiments, red cell proteins were pulse-labeled by a single injection of [^{3}H]leucine. Neglecting the possibility of "recycling" of the amino acid the majority of the leucine is incorporated into protein within a few hours of injection. Cells at all stages of maturation, from the earliest marrow stem cell to the circulating reticulocyte, will incorporate the label into newly synthesized proteins, both hemoglobin and membrane proteins. The continued increase in total radioactivity seen in the peripheral red cells over the first 24–72 h (Fig. 1a) thus represents the influx into the circulation of cells labeled in and subsequently released from the marrow. In general, [^{3}H]leucine will appear first in proteins synthesized by the circulating reticulocyte or marrow cells at the last stages of maturation. Radioactivity appearing in the circulation at later time points will represent proteins labeled at earlier stages of the red cell life cycle. The appearance of increased amounts of radioactive leucine in the circulation over 24–72 h correlates well with the estimated marrow maturation time of 3–5 days [31]. In most animals, peak specific activities were reached at 24 h; this is not unexpected, since in severely anemic animals marrow transit time has been shown to be decreased [32, 33]. In these experiments, we have no way of determining whether turnover of membrane proteins occurs while the cell is still in the marrow; thus it is possible that some proteins labeled early in the cell life cycle could be replaced by unlabeled newly-synthesized protein before release into the circulation. However, this does not invalidate the general assumption that the early appearance

of label in an individual peptide species in the circulating red cell indicates that this peptide was made late in the cell cycle.

Since red cells beyond the reticulocyte stage do not synthesize new proteins, the decline in overall specific activity observed after 2–3 days can be attributed either to dilution of the cells by newly appearing, unlabeled red cells or by loss from the circulation of the labeled cells; our experiments do not distinguish between these possibilities. However, selective loss of radioactivity from a specific peptide species must reflect loss of that peptide from the membrane of still-circulating cells.

The present studies show clear differences between the time course of appearance of [^{3}H]leucine in membrane proteins of the circulating red cell when these proteins are divided into broad classes based on molecular weight. Peptide subunits with molecular weights below 60 000 (Group C) show the earliest labeling and contain a relatively high percentage of the total [^{3}H]leucine radioactivity in the first 1–2 days; this proportion declines steadily over the next 4–8 days. The largest peptides (Group A), with molecular weights in the range of 200 000–250 000, show the lowest labeling; the amount of [^{3}H]leucine in this group increases relative to both Groups B and C for 5–10 days after the injection of the amino acid, remaining stable thereafter. It thus appears that, at the time a red cell appears in the circulation as a reticulocyte, the proteins present in the membrane will have been incorporated at different times in the cell's development; on the average, the larger the peptide subunit the earlier in the maturation process it will have been made.

If individual peptide bands are evaluated for the time of appearance of labeled leucine following a pulse dose, it is evident that some incorporation is seen in all of the major peptides at the earliest time point (8 h). However, three peptides in particular, bands 4, 5 and 9, show a higher incorporation than the rest. At later time points the remaining peptides show increasing incorporation, indicating that individual peptides are indeed being synthesized at different times in the cell cycle.

Lodish has reported [17] that reticulocytes in vitro synthesize predominantly two peptide species, with molecular weights of 53 000 and 33 000. These peptides would fall in our Group C and most closely correspond to bands 5 and 9 in our nomenclature. The early labeling seen in these peptides is consistent with his observation, since any proteins made by reticulocytes would be among the first to show incorporation in vivo. The sharp early peak of radioactivity in band 4 in our experiments suggests that more than two peptides may be made by reticulocytes in vivo. We have subsequently carried out experiments in vitro [24] and have confirmed that considerable synthesis of peptide 4 can be seen in reticulocytes in vitro.

As the fate of the labeled peptide bands in the red cell membrane is followed later in the time course, we have noted the significant decline, relative to the other bands, in the amount of [^{3}H]leucine in bands A and 9. Densitometric scans of stained gels confirm the disappearance from the membrane of these peptides. The population of cells being sampled at later times has very few reticulocytes compared to the earlier samples taken shortly after phenylhydrazine administration; it appears that the loss of these bands as the reticulocytes mature reflects a change in the membrane proteins with aging.

A clear distinction should be made between the changes in [^{3}H]leucine incorporation seen in early and late time points. The former must represent changes in specific activity of individual peptides, since no change in peptide pattern is observed.

Changes seen at later time points appear to reflect actual loss of certain peptide species with aging and not a change in specific activity of these peptides.

It is clear from the present experiments that red cell membranes are similar in some respects to other mammalian membranes, despite the unique life cycle of this cell. Different membrane proteins are made at varying rates and probably at different times in the cell cycle. In general, larger proteins are inserted into the membrane earlier in the maturation process and are no longer made at the last (reticulocyte) stage of the cell capable of protein synthesis. Smaller peptides may be made at all stages of maturation but are still being actively synthesized in the reticulocyte, so that they represent the most recently synthesized proteins in the cell membrane at the time of entrance into the circulation. Even among peptides of relatively similar size there are significant differences in rates of synthesis and/or turnover. It is evident that red cell membranes are not laid down as single "units" at one stage in cell maturation. This striking heterogeneity is well known in other cell membranes such as those of rat liver [34–36]. The exact details of the dynamic processes of membrane synthesis and turnover in the red cell will require more direct studies of bone marrow cells.

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