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BIOGENESIS OF ERYTHROCYTE MEMBRANE PROTEINS IN VITRO STUDIES WITH RABBIT RETICULOCYTES

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SUMMARY

The capability of rabbit reticulocytes to synthesize red cell membrane proteins has been tested in vitro. Reticulocyte-rich blood from phenylhydrazine-treated rabbits was incubated in vitro in a complete amino acid medium containing ferrous salts, glucose, rabbit plasma and [^{3}H]leucine. Red cell ghost membranes were prepared by hypotonic lysis and leucine incorporation into hemoglobin and total membrane proteins determined. The pattern of incorporation into individual peptides was determined by polyacrylamide gel electrophoresis of labeled membranes on large (19 mm) gels which were then sliced into 1 mm sections; radioactivity was compared with densitometric tracings of Coomassie blue stained analytical (6 mm) gels. Incorporation of [^{3}H]leucine into both hemoglobin and membrane protein was linear over 1 h. Gel analysis of labeled membranes revealed that the amino acid was primarily incorporated into peptides with molecular weights of 90 000 or less; three peptides of molecular weights 90 000, 60 000 and 33 000 showed the highest specific activity. Synthesis of the four largest peptide species was negligible. Removal of ferrous salts inhibited synthesis of both globin and membrane protein equally (approx. 50 %). However, puromycin and cycloheximide preferentially inhibited the synthesis of globin as compared to membrane proteins. Reticulocytes remain capable of synthesizing a number of membrane proteins; these results are consistent with studies of red cell membrane synthesis in anemic rabbits in vivo.

INTRODUCTION

Rabbit reticulocytes have been shown to carry on active protein synthesis both in vivo and in vitro [1, 2]. The red cell at this stage of maturation can no longer

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synthesize DNA or RNA but contains ribosomes which remain functional in protein synthesis for several days [3, 4]. During this stage, 70–90 % of the protein produced is globin [5]. This system has served as a useful model for the study of mechanisms of protein synthesis.

Recently, attention has been focused on the synthesis by reticulocytes of proteins other than globin. Bulova and Burka have reported that "non-globin protein" is the major product of membrane-bound ribosomes [6] while globin synthesis occurs predominantly on free ribosomes. This has been disputed by Woodward et al. [7] and by Lodish [8], who showed that free and membrane-bound ribosomes produced essentially the same proteins. Two of the non-globin proteins synthesized in vitro were shown by Lodish to be membrane proteins [8].

Previous studies from our laboratory [9], done in phenylhydrazine-anemic rabbits, showed that red cell membrane proteins are synthesized asynchronously; high molecular weight peptides appear to be made, in general, earlier in the cell cycle, while smaller peptides are still being synthesized at the later stages of cell maturation, presumably in reticulocytes. These data are compatible with Lodish's demonstration [8] that reticulocytes in vitro make only two of the smaller red cell membrane proteins. We report here our own studies on erythrocyte membrane synthesis in vitro by rabbit reticulocytes.

MATERIALS AND METHODS

[^{3}H]Leucine, with a specific activity of 33 Ci per mM, and Liquifluor were obtained from New England Nuclear. Phenylhydrazine and L-amino acids were obtained from Sigma Chemical Company. NCS Tissue Solubilizer was from Amersham-Searle. Puromycin was a product of Nutritional Biochemical Company, and cycloheximide of Searle. All other materials were reagent grade.

Preparation of rabbit reticulocytes. Male white rabbits weighing 2–3 kg were made anemic by five daily subcutaneous injections of phenylhydrazine (neutralized to a pH of 7.0 with NaOH) at a dose of 6 mg/kg. Two days following the last injection, blood was obtained by intracardiac puncture and collected in EDTA. At this time, reticulocyte counts were between 65 and 90 %. All subsequent steps, except for the incubations, were carried out at 4 °C.

Red cells were washed three times in cold 0.154 M NaCl/5 mM sodium phosphate, pH 8.0, with careful aspiration of the buffy coat after each wash. The plasma was saved for later use in the incubation mixture.

Incubations of intact reticulocytes. Incubations were carried out in 50 ml siliconized glass flasks in a Dubnoff metabolic shaker at 37 °C under a constant gas flow of 95 % O_2/5 % CO_2. The complete amino acid mixture used for incubation was modified from that of Borsook et al. [10] and was sterilized prior to use by passage through a Millipore filter (0.45 micron pore size). The amino acids were dissolved in modified Ringer-bicarbonate solution [11] prepared without the addition of calcium [10]; final pH of the medium was 7.4.

Incubation flasks contained packed, washed red blood cells suspended in 3 volumes of amino acid mixture to which was added glucose in a final concentration of 1 mg/ml and 5 μg/ml of ferrous ammonium sulfate; rabbit plasma (0.05 ml/ml of incubation mixture) was added as a source of transferrin. The final incubation mixture

was equilibrated under 95 % O_2/5 % CO_2 for 5 min at 37 °C prior to the addition of [^{3}H]leucine. In experiments with inhibitors, these were added in appropriate concentration to the flask prior to the addition of the red cells.

The reaction was stopped at specified time points by adding aliquots of the cell suspension to 3 volumes of cold modified Ringer-bicarbonate which contained 10 mM unlabeled leucine. The cells were then washed four times in 0.154 M NaCl/5 mM sodium phosphate prior to preparation of ghost membranes.

Determinations of [^{3}H]leucine incorporation into reticulocyte proteins. Ghost membranes were prepared by the method of Dodge et al. [12] by hypotonic lysis in 20 vols of 5 mM sodium phosphate, pH 8.0, collected by centrifugation for 15 min at 27 000 × *g*, and were washed 3–4 times in the same solution. Despite exhaustive washing, small amounts of hemoglobin remained visible in most preparations; we have consistently observed that completely hemoglobin-free ghosts cannot be prepared by this method from reticulocyte-rich blood, in contrast to the "white" ghosts that can be readily prepared from mature red cells. The supernatant from the first centrifugation of the lysate was saved for determination of hemoglobin concentration and [^{3}H]leucine incorporation. Ghost membrane protein was determined by the method of Lowry et al. [13]. Hemoglobin contamination of ghosts was checked by the benzidine method [14] and hemoglobin concentration in the supernatant was determined by the cyanmethemoglobin method [15].

To determine specific activity of total membrane proteins, aliquots of ghost membranes were precipitated in 7 % trichloroacetic acid at 0 °C, washed twice with 5 % trichloroacetic acid, dissolved in NCS and radioactivity determined in a toluene/Liquifluor mixture. To determine hemoglobin-specific activity, aliquots of the supernatant from the reticulocyte lysate were precipitated in 9 volumes of 1.5 % HCl in acetone, and the white globin precipitate was then washed once in HCl/acetone, once in 5 % trichloroacetic acid and then dissolved in NCS and counted as above. For the purpose of these studies, we did not correct for the small incorporation into other soluble proteins (see Fig. 6).

Polyacrylamide gel electrophoresis was carried out by the method of Fairbanks et al. [16] using 6.5 % acrylamide gels. Ghost membranes or hemoglobin supernatant were dissolved at 23 °C in 1 % sodium dodecylsulfate containing 40 mM dithiothreitol and were electrophoresed at the same temperature, either on 6 mm × 85 mm gels or on large 19 mm × 90 mm gels, at 5–8 or 8–12 mA/gel, respectively, until the tracker dye had migrated a distance of 75 mm. 50–100 μg and 2–5 mg of protein were electrophoresed on the small and large gels, respectively.

The gels were stained and decolorized by the method of Fairbanks et al. [16] except that the large gels required a longer time in each solution: 2 days in both the first and second bath and 3–4 days to decolorize.

We have shown [9] that peptide separation by this method is equivalent on the large and small gels except for slightly greater band widths on the large gels. The 6-mm gels were scanned in a Gilford spectrophotometer at 550 nm. The large gels were hand sliced at 1 mm intervals as previously described [9]; radioactivity in each slice was determined by scintillation counting after solution in 30 % H_2O_2 [17] and the sequential addition of 2 ml of NCS and 10 ml of toluene/Liquifluor.

RESULTS

[^{3}H]Leucine incorporation in vitro. Reticulocyte-rich red blood cells incubated as described showed an essentially linear incorporation of radioactivity into protein over a 1 h incubation period (Fig. 1). The specific activity of hemoglobin was approximately four times that of membrane proteins, with considerable variation between experiments. Preliminary experiments carried out over a 4 h period showed initial rapid incorporation of [^{3}H]leucine into protein in the first h with a progressive decline in the rate of incorporation over the next 3 h. Subsequent experiments were conducted over 1 h.

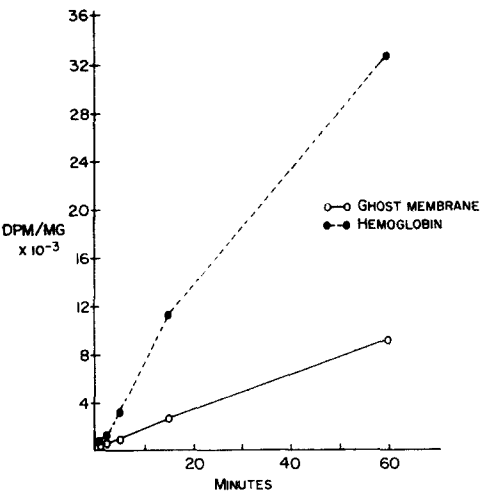

Fig. 1. Time course of [^{3}H]leucine incorporation into reticulocyte proteins in vitro. Incubation conditions and determination of the specific activities of hemoglobin and total membrane protein are described in Materials and Methods.

Effect of inhibitors of protein synthesis on [^{3}H]leucine incorporation. Omission of ferrous salts from the medium decreased the incorporation of [^{3}H]leucine into both membrane protein and hemoglobin to an equal extent, approximately 50 % of the control value.

Fig. 2 shows the effects of varying concentrations of puromycin and cycloheximide on the incorporation of [^{3}H]leucine into hemoglobin and membrane protein. Puromycin at $1 \cdot 10^{-4}$ M maximally inhibited incorporation of [^{3}H]leucine into hemoglobin, while a 10-fold greater concentration of the inhibitor was required for maximal inhibition of membrane protein synthesis. For cycloheximide, concentrations of $0.5 \cdot 10^{-4}$ M and $1 \cdot 10^{-4}$ M were required for maximal inhibition of [^{3}H]-leucine incorporation into hemoglobin and membrane proteins, respectively. At each submaximal concentration of the inhibitors tested, synthesis of hemoglobin was affected more than that of membrane proteins.

Pattern of [^{3}H]leucine incorporation into red cell membrane proteins. To determine which proteins were being actively synthesized by intact reticulocytes, [^{3}H]-leucine labeled membranes prepared from in vitro incubations were subjected to

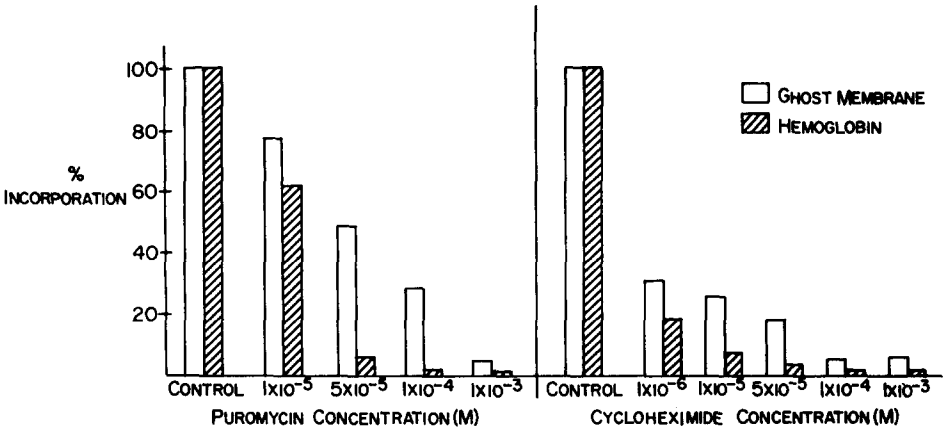

Fig. 2. Effect of inhibitors of protein synthesis on [^{3}H]leucine incorporation into membrane protein and hemoglobin in vitro. The incorporation of [^{3}H]leucine at varying concentrations of the inhibitors is shown as a percentage of the control value. Incubations were carried out for 1 h. (see Materials and Methods).

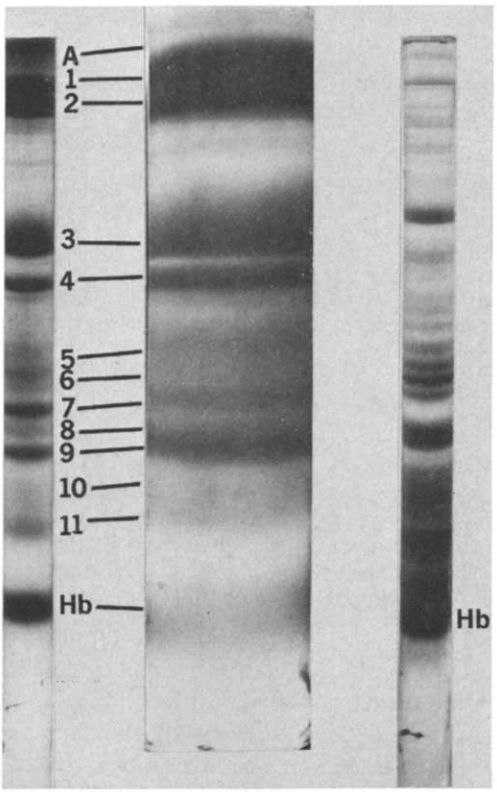

Fig. 3. Polyacrylamide gel pattern of rabbit reticulocyte membrane proteins. The small (6 mm) analytical gel on the left and the large (19 mm) gel in the center are from the same preparation of reticulocyte membranes. The peptide bands are numbered in sequence from A to 11. The gel on the right is of a preparation of reticulocyte ribosomes, obtained by centrifugation at 140 000 $\times g$ for 90 min of the post-membrane supernatant. The ribosome button was resuspended and washed before solubilization in dithiothreitol and sodium dodecylsulfate and application to the gel.

polyacrylamide gel electrophoresis in sodium dodecylsulfate and dithiothreitol. This method reproducibly separates membrane proteins into a small number of peptide bands based on molecular weight (Fig. 3) [17].

Although gel pattern of ghost membranes prepared from reticulocyte-rich blood are similar in most respects to those of mature circulating erythrocytes, several differences are noted [9]. At least two peptide bands are present in reticulocyte membranes which diminish markedly with maturation: a high molecular weight peptide (> 250 000) that appears above band 1 on the gel, and a smaller peptide, band 9, with a molecular weight of about 33 000 [19]. In addition, there are a number of minor peptides present mainly in the region of the lower molecular weight proteins that do not appear as distinct bands on the scan. That these are not simply ribosomal proteins is shown in Fig. 3, where the protein pattern of reticulocyte membranes is compared with that of sedimented ribosomes. A 19 mm (large) gel of reticulocyte membranes is also shown for comparison.

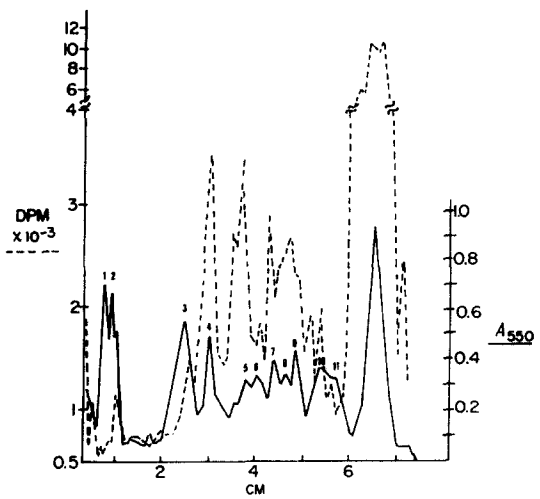

Fig. 4. Incorporation of [^{3}H]leucine into membrane peptides in vitro. Washed labeled membranes from a 60-min in vitro incubation similar to Fig. 1 were subjected to polyacrylamide gel electrophoresis on both 19 and 6 mm gels. Radioactivity (- - -), expressed as dpm per slice of the large gel (see Materials and Methods), is superimposed on a densitometric scan of the stained 6 mm gel (—). The peptide bands on the scan pattern are numbered as shown in Fig. 3. The horizontal axis represents distance in cm along the gel. The sharp peak at 6.5 cm is hemoglobin.

Fig. 4 shows the pattern of [^{3}H]leucine incorporation into reticulocyte membrane proteins. Radioactivity determined from slicing a 19 mm gel into 1 mm sections is superimposed on a densitometric tracing (6 mm gel) of an aliquot of the same sample. Little radioactivity appears in any of the highest molecular weight peptides, bands 1 and 2, or the dominant peptide subunit, band 3, with a molecular weight of 100 000. Several of the smaller peptides show peaks of radioactive leucine, notably bands 4 (90 000 daltons), 5 (60 000 daltons), 7 (41 000 daltons) and 9 (33 000 daltons). There is some incorporation into all of the smaller peptide bands, although to a lesser extent than these three. The relative proportion of [^{3}H]leucine seen in each band varied

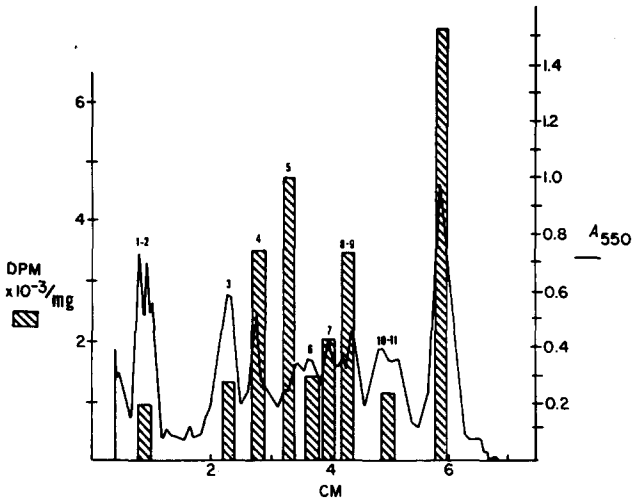

Fig. 5. Specific activity of [^{3}H]leucine incorporation in vitro into individual membrane peptides. Data are taken from an experiment similar to Fig. 4. The amount of protein in each major peak was determined from integration of a densitometric scan of the Coomassie blue stained gel; the [^{3}H]-leucine incorporated was calculated by summing the dpm in the slices representing the same peak. The specific activity for each major peptide is shown as a shaded bar superimposed upon the densitometric scan of the gel.

considerably between preparations, especially in band 4. However, in none of our experiments did we see major incorporation into the larger peptide groups.

Specific activities of the major peptide bands were determined as follows: the amount of protein in each peak was determined from the scan of the Coomassie blue stained gel, and the counts in the slices of the large gel coinciding with each peak were summed. Two pairs of bands, 8 and 9 and 10 and 11, run so closely together that they could not be effectively separated on the densitometric scan, so they were treated as combined peaks. As shown in Fig. 5, there is incorporation of [^{3}H]leucine into all of the peptide bands, with the highest specific activity being seen in hemoglobin. The membrane peptides which show the highest specific activity are bands 4, 5 and 8–9.

Pulse and chase experiments. To determine whether turnover of membrane proteins occurred during the incubations, we performed "pulse and chase" experiments in which cells were exposed for 15 min in [^{3}H]leucine, followed by the addition of a 10-fold excess of unlabeled leucine and a further incubation for 1 h. During the period of the chase there was an overall decline of 14% in specific activity of membrane protein with little change in specific activity of hemoglobin. Analysis of 15- and 75-min samples by gel indicated that the decline in activity was seen in all fractions, and was not specific for any particular peptide species.

Analysis of [^{3}H]leucine incorporation into soluble protein. To be certain that label in "membrane" proteins did not include trapped or adherent "soluble" proteins other than hemoglobin, we subjected the labeled supernate, obtained after lysis and centrifugation of ghost membranes, to polyacrylamide gel electrophoresis (Fig. 6). [^{3}H]Leucine activity, as expected, was predominantly in the band corresponding

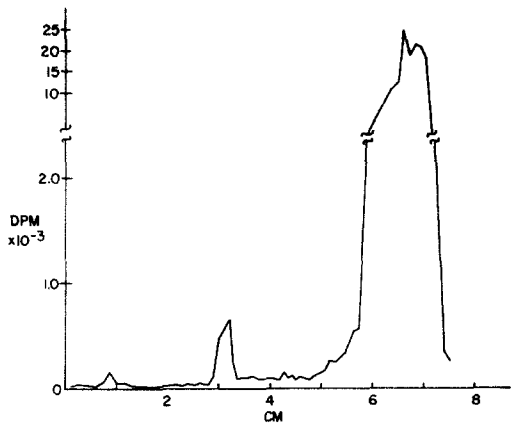

Fig. 6. [^{3}H]Leucine incorporation into soluble protein of the reticulocyte in vitro. Reticulocytes were incubated in vitro, lysed and the supernatant obtained after centrifugation of the whole lysate. Protein was precipitated, washed free of [^{3}H]leucine, dissolved and subjected to polyacrylamide gel electrophoresis on a 19 mm gel as described (see Materials and Methods). The large peak of radioactivity at 6.5 cm is [^{3}H]leucine in globin subunits and contains 96.2 % of all the radioactivity incorporated into soluble protein.

to globin. The counts obtained in the other slices were minimal, and together represented only 3.8 % of the total radioactivity present. Furthermore, with one exception there were no significant peaks corresponding to any of the major bands of membrane protein. A single peak of radioactivity in the soluble fraction at 3 cm corresponds closely with band 4 of the membrane peptides. Whether these are in fact identical proteins in soluble and membrane-bound form or simply represent two separate proteins of similar molecular weight is not clear. Based on the amount of hemoglobin bound to the membranes (see Fig. 4), the radioactivity seen in the higher molecular weight membrane peptides could not reflect trapped soluble proteins.

DISCUSSION

Most studies on the synthesis of protein by reticulocytes have focused on hemoglobin production and the factors involved in its control. The present studies clearly demonstrate synthesis of other proteins, both membrane-bound (Fig. 4) and soluble (Fig. 6) although, as expected, globin makes up greater than 90 % of the total protein synthesized. In our experiments, hemoglobin was synthesized with 2–5 times the specific activity of membrane protein.

Lodish [8] has correctly pointed out that the definition of a "membrane" protein is largely a functional one; in the human red cell membrane, only the predominant glycoprotein and the major peptide of molecular weight 100 000, both of which have been shown [20–22] to span the width of the membrane and to be exposed at outer and inner surfaces, can be considered an integral part of the membrane. The other peptides, which adhere more or less vigorously [16. 20] to the inner surface, are "membrane" proteins only in the sense that they are reproducibly isolated in the ghost fraction in procedures which effectively remove all or most of the cell hemo-

globin. Within this limitation, reticulocytes seem capable of synthesizing a number of these proteins, albeit at considerably different rates.

Two of the membrane proteins made in the greatest amounts (Fig. 4) with estimated molecular weights of 60 000 (band 5) and 33 000 (band 9) closely correspond to the two peptides B and E described by Lodish [8] as being synthesized by intact rabbit reticulocytes and membrane-free polyribosomes, with molecular weights of 53 000 and 33 000 in his studies. Other peptides show more variable incorporation Peptides 4 and 7 showed relatively high specific activities in several but not all incubations, while 6, 10 and 11 generally showed less incorporation. As noted above, peptides 1, 2 and 3, the last of particular interest because of its relative abundance and exposure at both surfaces of the membrane, were synthesized to a very minor extent in all of our preparations.

These data correlate well with our in vivo studies [9] showing that, as a group, the highest molecular weight proteins of the red cell membrane appaer to be made predominantly if not exclusively in the earlier stages of red cell maturation, i.e. while still in the bone marrow. When anemic rabbits are given [^{3}H]leucine in vivo, samples drawn at early time points (8 h) show a pattern of labeling similar to that seen in vitro (Fig. 4), while later time points show incorporation in all peptide bands. In the circulation, label thus first appears in peripheral reticulocytes, which are still capable of synthesizing certain membrane proteins; later, cells appeared which had incorporated the amino acid into cellular membranes at an earlier stage in the maturation cycle, while still in the marrow. Clearly the pattern of membrane proteins synthesized by the red cell varies according to its state of maturity.

Since our results differ somewhat from those of Lodish [8], who reported only two membrane peptide species synthesized by reticulocytes in vitro, it was important to determine whether soluble proteins could have been included in our ghost preparations. This was particularly important since, in our experiments, ghosts prepared from reticulocyte-rich blood always contain some hemoglobin (Fig. 4) despite exhaustive washing. There are three facts that make it unlikely that label in "membrane" proteins represents trapped soluble proteins. First, peaks of [^{3}H]leucine correspond exactly with the major peptide bands seen on stained gels of ghost membranes, and do not correspond to any significant bands seen in an analytical gel of the hemoglobin supernate. Second, in the supernate such a small proportion of the total radioactivity is present as peptides other than globin that it could not account for the incorporation seen in membrane proteins. Last, as noted above, roughly similar patterns of membrane protein synthesis are observed in vivo shortly after labeled leucine is given to anemic rabbits. It seems clear, then, that reticulocytes are capable of making and incorporating into the cell membrane a number of its characteristic protein species.

It is not clear why our reticulocyte preparations show synthesis of more membrane peptides than those of Lodish [8]. This may be a reflection of a greater sensitivity of our method of gel slicing as compared to radioautography, the larger amounts of protein that can be electrophoresed on 19 mm gels, or may reflect the loss of some of these peptides in his preparations during washing procedures. Nevertheless, we agree that the two peptides made to the greatest extent, both in terms of absolute [^{3}H]leucine incorporation and specific activity (Fig. 5), correspond to the 53 000 and 33 000 molecular weight peptides he described.

In the pulse and chase experiment, we observed a small but significant decline

in the specific activity of membrane proteins as a whole; this appeared to affect all fractions equally. This suggests that there is turnover of newly synthesized membrane proteins in vitro, but it appears to be quantitatively insignificant. Both the peptides B1 and B2 described by Lodish [8] are probably included in our band 5 and shifts from one to the other, as he described, would not be observed with the present methods.

The experiments on the inhibition of protein synthesis provide interesting results. Removal of ferrous salts from the medium resulted in a 50 % decline in the synthesis of both hemoglobin and membrane proteins. However, both cycloheximide, an inhibitor of chain initiation and elongation [23], and puromycin, a premature chain terminator [24], caused a disproportionate decrease in hemoglobin synthesis as compared to membrane proteins, although at sufficiently high concentrations of the inhibitors synthesis of all protein could be suppressed (Fig. 2). There continues to be a controversy as to whether "membrane-bound" and "free" ribosomes synthesize the same or different protein species in reticulocytes. The studies of Bulova and Burka [24], claiming that quite different synthesis patterns were observed, have recently been contradicted by Lodish and Desalu [5] and Woodward et al. [7]. Since we have, to date, studied synthesis only in intact reticulocytes, our results do not contribute directly to this argument. We find, however, that hemoglobin synthesis is more readily inhibited than that of membrane protein. In the case of cycloheximide, particularly at lower concentrations which inhibit chain initiation more than elongation [23], this result is entirely expected. All of the major membrane proteins have molecular weights, and hence mRNAs, larger than globin; therefore, they would be expected to have more nascent peptide chains at any one time and to produce more completed protein molecules after inhibition of new chain initiation. The results with puromycin are less predictable. One of two possibilities exists: either synthesis of membrane proteins is relatively more resistant to this antibiotic than is synthesis of globin, or the released peptidyl puromycins are still capable of incorporation into the membrane despite premature termination. Of these, the former possibility seems more likely, but a final decision must await studies on the process by which newly released "membrane" proteins become incorporated into the existing membrane structure.

Of particular interest to us is the smaller of the two peptides made in the largest amount, band 9. We have shown that this peptide is made by reticulocytes in vivo [19] but that it disappears from the red cell membrane during the process of reticulocyte maturation in the circulation. We have subsequently noted a similar loss with maturation of a minor band of the high molecular weight ($> 250\,000$) [9]. Whether these represent two of the many enzymes known to decline during red cell aging [25–27] or whether they are involved in specific membrane structural changes associated with the maturation process is unknown.

The present studies clearly show that reticulocytes maintain their ability to synthesize certain membrane proteins after becoming enucleate. These proteins are not synthesized, however, in proportion to their relative abundance in the membrane; certain of the predominant peptides, especially those with higher molecular weights, are hardly synthesized at all in this stage of cell maturation. It is of considerable interest that the one major protein which completely spans the width of the membrane, band 3, is not synthesized to any significant extent by the reticulocyte. Presumably the peptides which are made reside on the inner surface of the membrane, if they are analogous to similar peptides in human red cells [28]. It may be that different

modes of insertion of proteins into the membrane are involved. However, it should be noted that "spectrin", which is thought [28] also to reside on the inner surface of the human red cell membrane, is also not synthesized in reticulocytes. Studies of the factors involved in the synthesis of these latter proteins will require experiments with nucleated marrow precursors of the red cell.

ACKNOWLEDGEMENT

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