EVIDENCE FOR SOLUBLE α CHAINS AS INTERMEDIATES IN HEMOGLOBIN SYNTHESIS IN THE RABBIT RETICULOCYTE[1]

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The hemoglobin molecule is composed of two α peptide chains, two β peptide chains, and four heme groups. In the rabbit reticulocyte the peptide chains are synthesized on the polyribosomes. The mechanism of assembly of the final molecule from the component polypeptide chains and heme groups is of current interest in several laboratories. One particular problem is to determine the nature of the soluble chain intermediates in hemoglobin completion. The current report documents evidence for a soluble fraction in rabbit reticulocytes containing newly synthesized α chains which appear to be intermediates in hemoglobin construction.

EXPERIMENTAL: Reticulocytes were obtained from the blood of phenylhydrazine treated rabbits and washed once with a modified saline solution (Allen and Schweet, 1962). In order to study the soluble polypeptide products in hemoglobin synthesis the reticulocytes were pulse-labeled with ^{3}H leucine. The washed cells were suspended in a solution having the following composition; 0.5 μmole each of 19 amino acids except leucine, 50 μmoles Tris·HCl pH 7.5, 0.4 μmole $Fe(NH_4)_2(SO_4)_2$, 273 μmoles NaCl, 10.5 μmoles KCl, 16 μmoles $MgCl_2$, 0.25 ml of packed cells, and 0.025 μmole L-leucine-4,5-^{3}H (5000 μcuries/μmole) in a total volume of 2.9 ml. The mixture was incubated for 5 min at 37°.

The labeled cells were centrifuged and lysed as previously described (Allen and Schweet, 1962). The ribosomes were removed from the lysate (90 min at 105,000 X g). In order to determine the electrophoretic nature of the

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newly synthesized protein, 0.02 ml of the resulting soluble phase was diluted with 0.115 ml of 0.01 M $PO_4(K^+)$ buffer pH 7.1; 5 µl of this dilution were electrophoresed on a cellulose acetate strip (Shaeffer et al., 1967). The strip was stained with Ponceau S in 5% trichloroacetic acid (TCA), dried, and cut up at 0.20 cm intervals for analysis of ^3H protein radioactivity in a liquid scintillation system (Shaeffer et al., 1967 and Shaeffer et al., 1964). The applied protein radioactivity was quantitatively recovered in the sections.

Aliquots of the soluble phase were also incubated with isolated human hemoglobin α or β chains (Bucci and Fronticelli, 1965) in order to assay for the presence of complementary ^3H rabbit β or α chains, respectively, by formation of ^3H rabbit-human hybrid hemoglobins. For this "complementary chain assay" (Shaeffer et al., 1967) 0.02 ml of labeled soluble phase was mixed with 0.10 ml PO_4 buffer containing 0.49 mg of human α or β chains. The solutions were incubated for 10 min at 37° and chilled. To each assay was added 0.015 ml of a carrier rabbit-human hybrid hemoglobin mixture (150 mg/ml) prepared according to Itano and Singer (1958). The solutions (5 µl) were electrophoresed as above to separate the hybrid and rabbit hemoglobins; the strips were sectioned for counting.

It was of interest to determine the distribution of radioactivity between the rabbit α and β chains of certain of the electrophoretically separated hemoglobin bands. Radioactive protein was eluted from these bands on unstained cellulose acetate strips and mixed with carrier rabbit globin (Shaeffer et al., 1967). The globin chains were separated on a carboxymethylcellulose (CMC) column (Dintzis, 1961). The fractions were precipitated with 5% TCA and washed onto cellulose nitrate membranes for counting.

It was desirable to find out if the soluble ^3H rabbit α chains detected after a 5 min pulse of ^3H leucine (see Results section) could be converted to completed hemoglobin. The mixture for pulse-labeling the cells was doubled. At the end of a 5 min incubation with ^3H leucine one-half of the labeled cells were removed and analyzed as above. The remaining cells were centrifuged and

resuspended in a normal incubation mixture with the omission of ^{3}H leucine. In addition 0.5 μmole of non-radioactive leucine, 3.0 mg of glucose, and 0.20 ml of fresh plasma were added. The mixture was incubated for 90 min at 37° ("chase") before lysis and analysis of the soluble ^{3}H protein.

RESULTS AND DISCUSSION: At the end of a 5 min pulse with ^{3}H leucine, the soluble phase of the clarified cell lysate contained 89% and the ribosomal

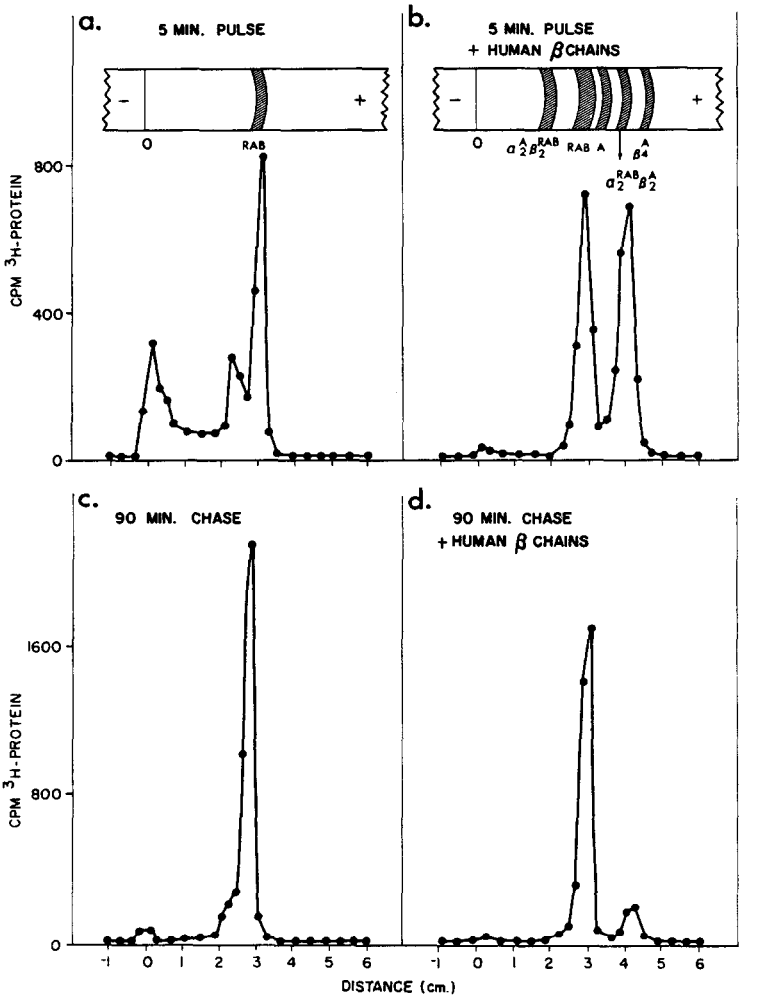

Fig. 1. Radioactive protein patterns after electrophoresis of reticulocyte soluble phases on cellulose acetate strips. The cells were pulse-labeled with ^{3}H leucine for 5 min (a and b). Some of these cells were incubated for 90 additional minutes with non-radioactive amino acids (c and d). The isolated soluble phases were either diluted with PO_4 buffer (a and c) or incubated with added human β chains (b and d) before electrophoresis.

fraction only 11% of the protein radioactivity. Fig. 1a shows the electrophoretic pattern of the soluble protein radioactivity. The rabbit hemoglobin band contained 45% of this radioactivity; the remainder traveled as a disperse component between the line of sample application and the hemoglobin band. Occasionally, as shown in Fig. 1a, there was a small peak of radioactive protein behind the main hemoglobin peak.

Protein from the rabbit hemoglobin band was eluted from several strips, and the globin chains were separated on a CMC column (see Experimental section). Fig. 2b shows that 94% of the hemoglobin radioactivity was in newly synthesized β chains. These results suggest that labeled β chains combined largely with endogenous unlabeled α chains to form completed hemoglobin during a brief incubation with ^{3}H leucine. Other workers have also found that β chains were labeled earlier than α chains in pulse-labeling of reticulocytes (Baglioni and Colombo, 1964).

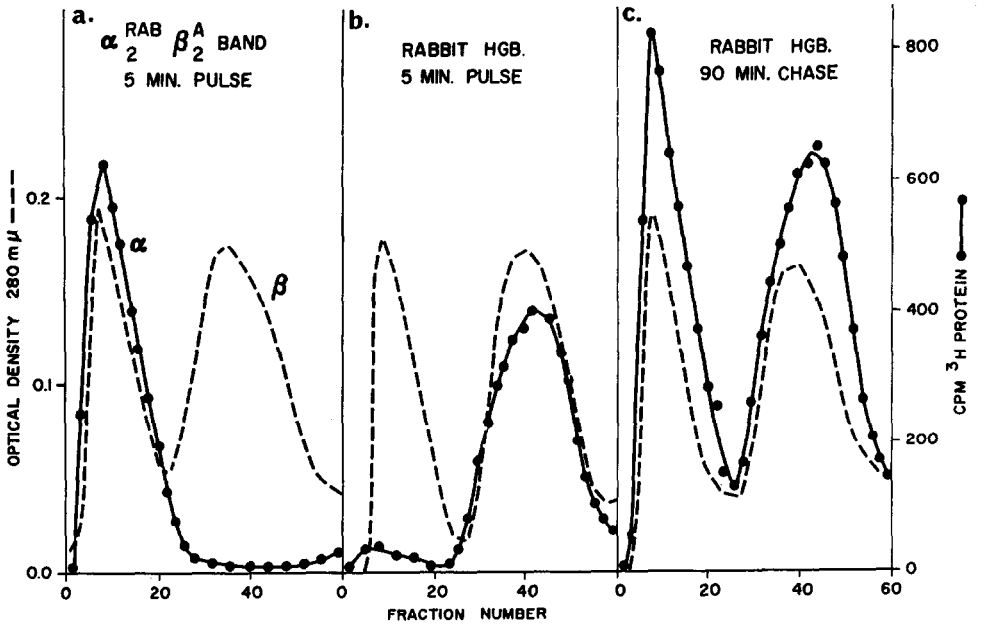

Fig. 2. Chain separation of labeled globin fractions on CMC columns. The labeled samples were eluted from the following bands of unstained cellulose acetate strips: (a) the α_2^{RAB} β_2^{A} band of Fig. 1b, (b) the rabbit hemoglobin band of Fig. 1a, (c) the rabbit hemoglobin band of Fig. 1d.

It was of interest to know if the electrophoretically disperse material shown in Fig. 1a contained soluble α or β chains as possible intermediates in hemoglobin assembly. Previous work with a cell-free system from reticulocytes had indicated that a similar component contained newly synthesized α chains (Shaeffer et al., 1967). The present soluble phase was assayed for α chains by incubation with isolated human hemoglobin β chains (see Experimental section). Fig. 1b shows the formation of a $^{3}H\ \alpha_2^{RAB}\ \beta_2^{A}$ hybrid hemoglobin with the concomitant disappearance of most of the disperse component. Chain separation chromatography of the eluted protein from this hybrid region showed that the radioactivity was exclusively in the α chain (Fig. 2a). Moreover, the quantity and β chain location of the radioactivity of the rabbit hemoglobin after the complementary chain assay (Fig. 1b) was the same as before (Fig. 1a). Additional evidence that incubation with human chains does not break down completed rabbit hemoglobin has been previously obtained in a similar assay with the cell-free system (Shaeffer et al., 1967). When the labeled soluble phase in the present study was incubated with human α chains instead, no corresponding $\alpha_2^{A}\ \beta_2^{RAB}$ hybrid was formed. These data suggest that reticulocytes contain a soluble non-hemoglobin component with newly synthesized α chains. London and co-workers (Vanderhoff et al., 1967) have recently found that reticulocytes from iron-deficient rabbits also contain soluble α chains.

In order to determine whether these α chains appeared to be hemoglobin intermediates, an aliquot of pulse-labeled cells was further incubated with non-radioactive amino acids (see Experimental section). Fig. 1c shows that after 90 min most of the disperse component had disappeared. Moreover, only a small amount of $^{3}H\ \alpha_2^{RAB}\ \beta_2^{A}$ hybrid was formed when the soluble phase was incubated with human β chains (Fig. 1d). On the other hand the rabbit hemoglobin radioactivity had more than doubled during the 90 min "chase" (note the change of ordinate scale between Fig. 1a and 1c). Fig. 2c shows that this rabbit hemoglobin band had a vastly increased number of labeled α chains compared to that originally pulse-labeled (Fig. 2b). Other experiments showed a

progressive conversion of soluble α chain radioactivity (as detected by hybrid formation) to rabbit hemoglobin radioactivity during the chase incubation. During this 90 min incubation the total soluble protein radioactivity increased by 17% presumably because the cell pools of ^{3}H leucine, ^{3}H leucyl-sRNA, and growing ribosomal chains were also being converted to hemoglobin.

Collectively these data suggest that soluble α chains are intermediates in hemoglobin assembly in the rabbit reticulocyte. Other workers have recently postulated such a role for α chains (Shapiro et al., 1966; Cline and Bock 1966). The reason for the electrophoretically disperse nature of the α chain component of this study is at present unknown. Perhaps the lack of enough protein to act as carrier prevents the α chains from forming a discrete band. On the other hand, they may exist in complexes having a various number of subunit monomers, or possibly in association with other soluble components.

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