IRON SUPPLEMENTATION IN VITRO AND THE STATE OF AGGREGATION AND FUNCTION OF RETICULOCYTE RIBOSOMES IN HEMOGLOBIN SYNTHESIS

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It has been established that the availability of iron for formation of heme (Kruh and Borsook, 1956; Borsook et al., 1957) or heme itself (Bruns and London, 1965) regulates the capacity of the rabbit reticulocyte not only for synthesis of hemoglobin but also for formation of the protein moiety, globin. Some characteristics of the control of globin synthesis by a non-protein component, iron, and its influence on the state of aggregation and activity of the ribosomes in the intact reticulocyte are considered in this communication.

METHODS

Rabbit reticulocyte formation was stimulated by a series of phenylhydrazine injections (Rabinovitz and Fisher, 1964). Cells were washed twice with cold magnesium-saline (Warner et al., 1963) and were incubated at 37° in this medium, buffered with tris(hydroxymethyl)aminomethane, 5×10^{-3} M, pH 7.6, and fortified with glucose 1 mg/ml. The following additions were made as indicated in individual experiments: ferrous ammonium sulfate (iron), to give a final concentration of 2×10^{-4} M, and an amino acid mixture (less leucine), as recommended by Borsook et al. (1957). The volume of cells was 0.25 ml in a final total volume of 2 ml.

Cells were incubated as described in the individual experiments, then one μmole of L-leucine-1-C^{14}, (6.8×10^5 c.p.m.) was added, and the incubation

continued for periods up to 20 minutes. Metabolism was terminated by adding cold magnesium-saline and shaking the flask in ice. For determination of the distribution of protein synthetic activity among the polyribosomal components, 0.01 μmole of L-leucine-U-C^{14}, (1.5×10^6 c.p.m.) was added after preincubation and the reaction terminated as described above after 2 minutes. The cells were isolated by centrifugation at 4°. The lysis and subsequent steps for separation of polyribosomes were performed in the cold as described by Warner et al. (1963) with minor modifications (Rabinovitz and Waxman, 1965).

Under the various incubation conditions, the incorporation rate of leucine-1-C^{14} into the soluble protein was constant during the assay period and thus served as an estimate for the rate of synthesis of complete protein molecules. The identity of the labeled soluble protein as globin was verified by the criterion of constant specific activity throughout the α- and β-chain chromatogram, as previously described (Rabinovitz and Fisher, 1964). The direct characterization of labeled soluble protein as hemoglobin is made difficult because the nascent hemoglobin can be separated from carrier hemoglobin by chromatography (Lingrel and Borsook, 1962; Rabinovitz and Fisher, 1964). All soluble protein synthesized under conditions of iron deprivation brought about by incubation with 2,2'-bipyridine was found to be globin (Rabinovitz and Waxman, 1965) and may be assumed to be present in the cell as hemoglobin.

RESULTS

Incubation of reticulocytes in a medium fortified with the amino acid mixture and glucose caused the rapid disaggregation of the polyribosomes (Fig. 1: A, B, E). Addition of ferrous iron after partial disaggregation resulted in reaggregation (Fig. 1: A, B, C, D). If the cells were incubated with iron throughout (Fig. 2), no disaggregation was found; all polyribosome profiles were identical to that shown in Fig. 1A. Hemin, at a concentration

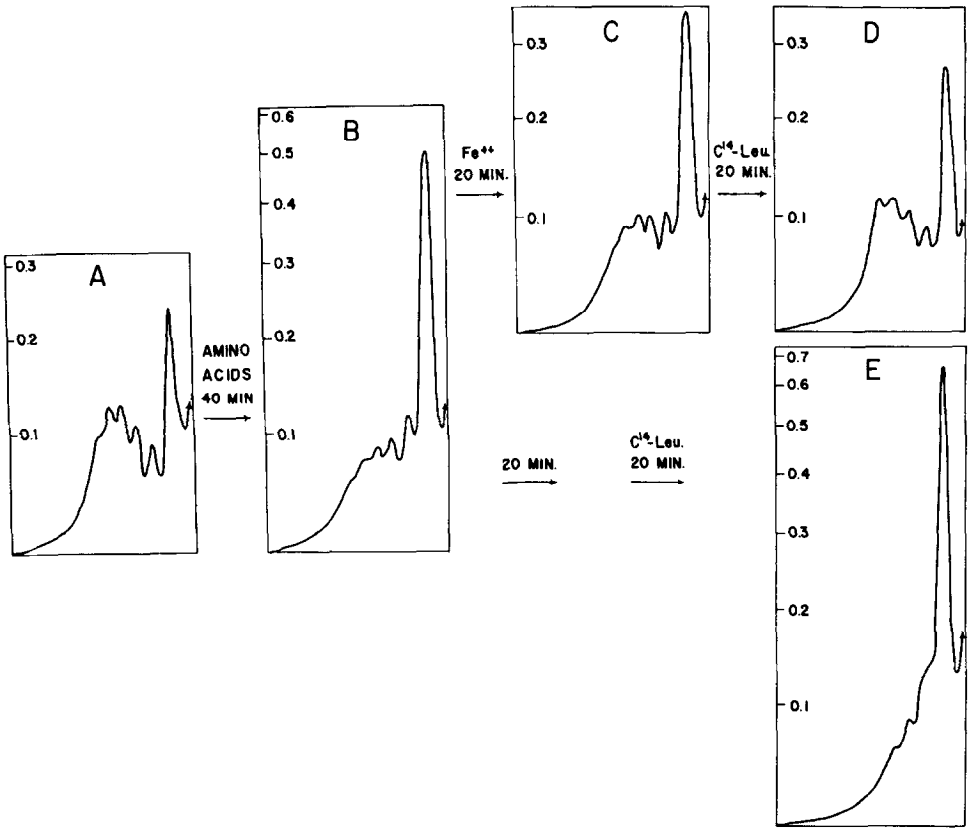

Fig. 1. Disaggregation of Reticulocyte Polyribosomes upon Incubation of Cells Without Iron, and Reaggregation upon its Addition

The ordinates represent absorbancy at 260 mμ and the abscissae the linear distance from the bottom of the 28 ml tube of 6 cm total length. Fig. 1A shows profile of unincubated control; Figs. B to E indicate the patterns found after incubation as indicated. Labeled leucine was added 20 minutes prior to termination to obtain rate of hemoglobin synthesis which is given in Table I.

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of 2×10^{-5} M produced identical maintenance of polyribosome integrity.

In some cell preparations, disaggregation of polyribosomes occurred when the cells were incubated in buffer without amino acids or iron. In others, no disaggregation was observed unless the amino acid mixture was present during the incubation; under these conditions, depletion of utilizable

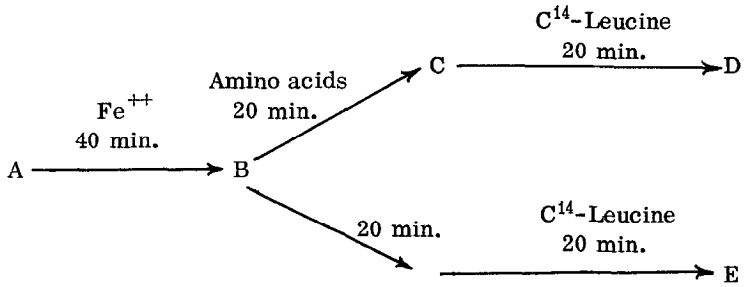

Fig. 2. Flow Diagram of Incubation in which Iron was Present Throughout

All absorbancy profiles at points indicated by letters were identical to Fig. 1A. Labeled leucine was added 20 minutes prior to termination to obtain rate of hemoglobin synthesis which is given in Table I.

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cellular iron was probably brought about by increased hemoglobin synthesis. In still other reticulocyte preparations, it was necessary to include a low concentration (5×10^{-5} M) of the ferrous chelating agent, 2,2'-bipyridine, to obtain polyribosome disaggregation (Rabinovitz and Waxman, 1965). These differences appear to be due to variations in the available endogenous iron of different reticulocyte preparations. Marked fluctuations in serum iron and iron-binding capacity after phenylhydrazine treatment have been reported (Schade and Stengle, 1959), and these factors have been found to affect the free iron content of reticulocytes (Jandl et al., 1959).

The delayed addition of either iron or amino acid mixture, following preincubation with the other, resulted in similar terminal profiles with well aggregated ribosomes (Figs. 1 and 2) as well as similarly enhanced hemoglobin synthesis (Table I). Preincubation with amino acids alone or with iron alone resulted in less active hemoglobin synthesis (Table I). Although preincubation of reticulocytes with amino acids alone was associated with a rate of hemoglobin synthesis 40 per cent higher than that of cells treated with iron

alone, the former was accompanied by a marked disaggregation of polyribosomes (Fig. 1E). Iron alone, although effecting a lower rate of hemoglobin

TABLE I

Hemoglobin Synthesis Under Conditions of Delayed Supplementation as Indicated in Figs. 1 and 2

Incubation Conditions	Ribosome-Polyribosome Profile	Incorporation
Amino acids, then iron	Fig. 1D	4.9
Amino acids only	Fig. 1E	1.7
Iron, then amino acids	Fig. 1A*	5.2
Iron only	Fig. 1A*	1.2

Incorporation is expressed as μmoles leucine incorporated per mg soluble protein $\times 10^4$ during the 20 minutes prior to termination of the incubation.

*When iron was initially present all profiles were identical to that of the unincubated control.

synthesis, produced complete stabilization of the polyribosomes.

These observations are brought into further contrast in Fig. 3, where incorporation of leucine into protein of the polyribosomes is also presented. As may be seen by comparison with the UV absorption profiles, when polyribosomes were partially disaggregated by incubation of cells with the amino acid mixture, the active ribosomes remained principally associated with the aggregates (Fig. 3B). Iron, in the presence or absence of amino acids, completely maintained ribosome aggregation, although incorporation of leucine into protein of the polyribosomes was somewhat less without the amino acid mixture (Fig. 3: C, D). Thus, there was a lack of correlation between the rate of hemoglobin synthesis and either the absorbancy profile

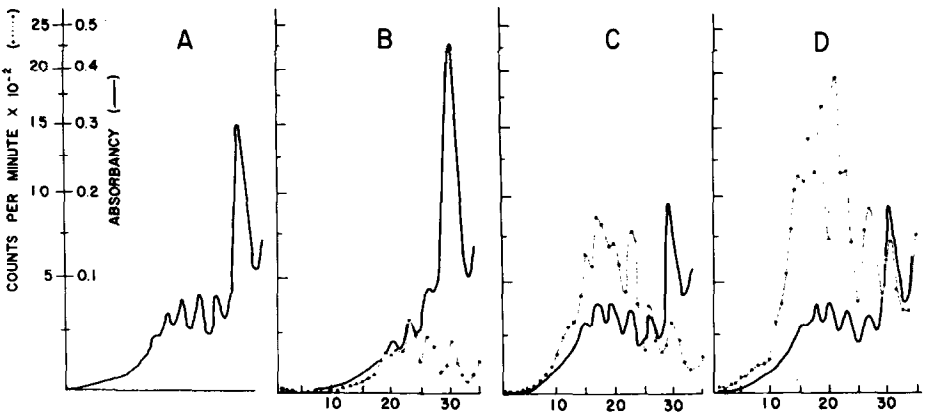

Fig. 3. Effect of Iron Deprivation and Supplementation on the Polyribosomes of Reticulocytes

A, —Unincubated control; B, —Incubation without amino acids or iron or with amino acid mixture only; C, —Incubation with iron only; D, —Incubation with amino acids and iron. The incubation time prior to labeling of the polyribosomes was one hour. Abscissae indicate distance from the bottom of a 6 cm tube; fraction number is also given where samples were isolated for determination of incorporated radioactivity.

TABLE II

Hemoglobin Synthesis Under Conditions of Supplementation as Indicated in Fig. 3

Incubation Conditions	Ribosome-Polyribosome Profile	Incorporation
Neither amino acids nor iron	Fig. 3B	0.61
Amino acids only	Fig. 3B	0.79
Iron only	Fig. 3C	0.54
Amino acids and iron	Fig. 3D	2.95

Duplicate one hour incubations of cells were performed under conditions for which profiles are shown in Fig. 3. L-Leucine-1-C^{14} was then added to give a final concentration of 5×10^{-4} M and the incubation continued for 10 minutes. Incorporation is expressed as μmoles leucine incorporated per mg soluble protein $\times 10^4$.

at 260 mμ or the leucine incorporation profile of the polyribosomes. The well-preserved and active polyribosomes of cells incubated with iron alone (Fig. 3C) could not support a high rate of hemoglobin synthesis in the absence of the amino acid mixture (Table II). In fact, as is shown in both Tables I and II, cells with polyribosomes disaggregated after incubation with the amino acid mixture alone show better hemoglobin synthesis than do cells with polyribosomes preserved by incubation with iron alone.

DISCUSSION

The behavior of iron and heme in maintaining polyribosome structure may be considered in light of the current hypothesis on the mode of action of polyribosomes — the tape theory of protein synthesis. The tape theory suggests that several monomeric ribosomes can attach themselves, one at a time, to the end of a messenger ribonucleic acid (m-RNA) tape (Hardesty, Hutton et al., (1963), and by migration along this tape decode its message and simultaneously carry forward a growing polypeptide chain (Warner et al., 1962; Wettstein et al., 1963). The completed polypeptide chain is released from each ribosome as soon as the ribosome completes its transit along the m-RNA and is itself released (Warner et al., 1962; Hardesty, Miller and Schweet, 1963). In agreement with this theory is the concept that terminal events, such as the association of the α- and β-polypeptide chains or the insertion of heme have no role in the peptide-forming process.

In an attempt to explain the role of iron or heme in terms of the above hypothesis, its introduction at only terminal stages of the protein synthetic process may be considered. For example, heme may associate with newly formed polypeptide chains and thus facilitate their removal from each ribosome as it completes its transit of the m-RNA. However, such an association would result in the stabilization of polyribosomes under conditions of iron

deficiency instead of the disaggregation observed. The opposite situation might be expected if heme participates in the removal of completed protein chains from ribosomes already released from their m-RNA. Under these conditions, iron deficiency would result in an increase of monomeric ribosomes with attached nascent protein. A delay in the removal of this nascent protein would prevent the ribosome from beginning a new synthetic cycle and thus promote polyribosome disaggregation. Since considerable protein synthesis still occurs in cells deprived of iron (Tables I and II) one might expect an exaggerated labeling of nascent protein on monomeric ribosomes which do complete the cycle. However, such an accumulation of newly formed protein on monomeric ribosomes in iron deprived cells was not found (Fig. 3B), nor was it observed when cells were made iron deficient with 2, 2'-bipyridine (Rabinovitz and Waxman, 1965). Thus it would appear that, within the framework of the tape theory, the combination of heme with globin precursor and the control of globin synthesis exercised by this process cannot be considered a terminal event but must be integrated with peptide chain formation.

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