POLYRIBOSOMAL CHANGES DURING INHIBITION OF RABBIT HEMOGLOBIN SYNTHESIS BY AN ISOLEUCINE ANTAGONIST

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Optimal hemoglobin synthesis in intact rabbit reticulocytes requires the presence of several amino acids in the incubation medium.[1] These may be termed "essential" amino acids. The rapid hydrolysis of particulate proteins during maturation of reticulocytes[2] provides an intracellular source of many other amino acids which need not be added to the medium. The omission of the complete amino acid component required for optimal hemoglobin synthesis does not affect the ribosome-polyribosome profile of these cells if an adequate source of iron for heme synthesis is available.[3] However, the selective omission of tryptophan from an otherwise complete medium brings about polyribosome disaggregation.[4] This disaggregation is due to the location of tryptophan only near the amino-terminal ends of rabbit hemoglobin, at position 14 in the α-chain, and in positions 15 and 37 of the β-chain.[5, 6] Since growth of the peptide chains proceeds from the amino-terminal end,[7, 8] during a deficiency of tryptophan the rate of translation of messenger RNA is retarded near this end at the sites of tryptophan residues. If the other essential amino acids are present, there will be a normal rate of translation beyond these sites, and the inhibition results in polyribosome disaggregation because of a failure in the system to maintain the steady-state number of ribosomes on messenger RNA.[4]

Isoleucine occupies positions 10, 17, and 55 from the amino-terminal end of the α-chain of rabbit hemoglobin[5, 9] and position 112 of the β-chain.[10] Since the α- and β-chains contain 141 and 146 amino acids, respectively, the isoleucine codon may be considered as located near the proximal end of messenger RNA of the α-chain and near the distal end of the messenger RNA of the β-chain. Under normal conditions, messenger RNA coding for peptide chains of hemoglobin can hold up to six ribosomes in a saturated polyribosome configuration.[11] Hence, under conditions of an isoleucine deficiency, the α-chain polyribosomes should become disaggregated to dimers, whereas the β-chain polyribosomes would consist principally as tetramers and pentamers.

Omission of isoleucine from a mixture of amino acids has no effect on hemoglobin synthesis or on the ribosome-polyribosome profile of rabbit reticulocytes. A sufficient supply of this amino acid is furnished to the amino acid pool of the cell by protein turnover.[2] It is possible, however, to limit the availability of isoleucine for protein synthesis with the use of the isoleucine antagonist O-methylthreonine. This isostere of isoleucine is activated by isoleucyl-RNA synthetase of mammalian cells, but is not transferred to tRNA.[12] In this communication we describe the effect of O-methylthreonine on the synthesis of rabbit hemoglobin by intact reticulocytes.

Materials and Methods.—Preparation of reticulocytes and incubation conditions have been described.[13] The amino acids reported as essential by Borsook *et al.* were present

at their recommended concentrations,[1] expressed as mM/liter: L-glutamine, 0.48; L-histidine, 0.58; L-leucine, 1.0; L-lysine, 0.45; L-phenylalanine, 0.40; L-serine, 0.43; L-tryptophan, 0.074; L-tyrosine, 0.21; and L-valine, 0.77. An additional supplement (group 2), found to stimulate hemoglobin synthesis, was also included: L-alanine, L-arginine, L-asparagine, glycine, L-proline, and L-threonine at a concentration of 0.2 mM, and L-cystine at 0.05 mM. Glucose, 1 mg/ml; ferrous ammonium sulfate, 0.2 mM; and rabbit transferrin (Pentex), 50 μg/ml were present in all cases. Hemoglobin synthesis was estimated by following the incorporation of L-leucine-1-C^{14} into soluble protein. Labeled leucine was added to give a final concentration of 0.5 mM and the nonradioactive leucine supplement was omitted. L-O-Methylthreonine was synthesized from L-threonine and purified by treatment with lithium periodate.[12] Procedures have been described for the isolation of α- and β-chains[8, 14] and for determination of the UV absorbance and the incorporated radioactivity of the ribosome-polyribosome component.[11, 15]

Results and Discussion.—Requirements for O-methylthreonine inhibition: Maximal inhibition of hemoglobin synthesis by O-methylthreonine can be achieved only when other amino acids are not limiting (Table 1). Under conditions of suboptimal incorporation (minus amino acid group 2), the inhibition by O-

TABLE 1. *O-methylthreonine inhibition of hemoglobin synthesis under different conditions of amino acid supplementation.*

	Leucine Incorporation with Amino Acid Mixture			
	Complete	Minus group 2	Minus histidine	With no amino acids
Control	1.10	0.634	0.164	0.180
O-methylthreonine	0.445	0.410	0.171	0.185
Per cent of control	41	65	100	100

Hemoglobin synthesis is expressed as μmoles leucine incorporated per gm soluble protein during the 20-min incubation period. One μmole leucine incorporated per gm protein corresponds to a specific activity of 420 cpm/mg protein. From 6 to 10 mg protein were plated. The O-methylthreonine concentration was 25 mM.

methylthreonine was markedly reduced. In the absence of histidine (the nutritionally most limiting amino acid in reticulocytes)[1] or when all amino acids were omitted, no further inhibition of hemoglobin synthesis by O-methylthreonine occurred. These results are in agreement with observations on the mode of action of the isoleucine antagonist in ascites tumor cells[16] and follow from the observation that it acts by inhibiting the synthesis of isoleucyl-tRNA in these cells and in rabbit reticulocytes.[12] Depletion of the isoleucyl-tRNA pool results in the equivalent of an isoleucine deficiency, but this can only become apparent when other amino acids are not limiting the rate of protein synthesis.

The effect of three concentrations of O-methylthreonine on inhibition of hemoglobin synthesis and the complete protection afforded by isoleucine is shown in Figure 1. An O-methylthreonine concentration of 25 mM was subsequently used in all experiments. Synthesis of both the α- and β-chains of hemoglobin, isolated from the total soluble protein, was about equally inhibited by the isoleucine antagonist (Fig. 2). Some protein of higher specific activity was present in the trough between the α- and β-chains obtained from inhibited cells. This has also been observed when hemoglobin synthesis was inhibited in iron-deficient cells,[15] but this protein represents only a small amount of the total protein synthesized under inhibited conditions. Inhibition of hemoglobin synthesis varied from 55

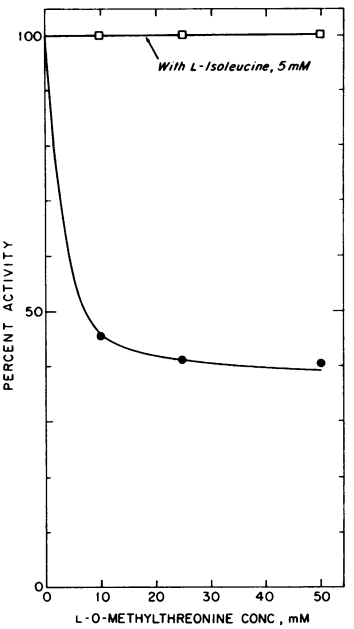

FIG. 1.—Inhibition of hemoglobin synthesis by O-methylthreonine and its protection by isoleucine. The incubation was performed as described under *Materials and Methods*. Leucine incorporation corresponding to 100% activity was 1.14 μmoles per gm hemoglobin during the 20-min incubation period.

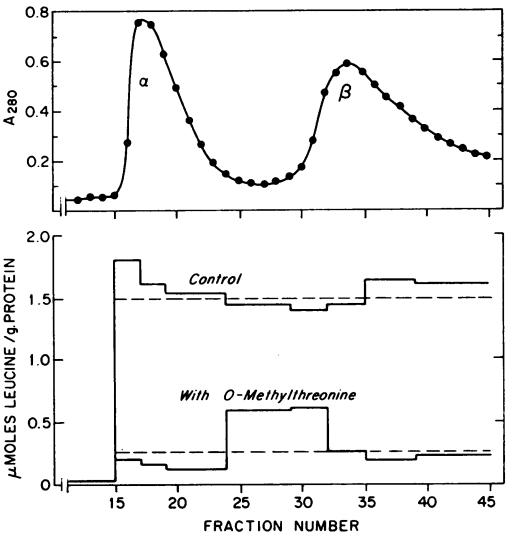

FIG. 2.—Inhibition of synthesis of both α- and β-chains of rabbit hemoglobin by O-methylthreonine. The cells, 0.75 ml packed volume, were incubated for 20 min in the absence or presence of O-methylthreonine, 25 mM, as indicated under *Materials and Methods*.

Top figure: separation of α- and β-chains on CM cellulose.

Bottom figure: ———, incorporation of leucine into α- and β-chain fractions; - - - -, incorporation of leucine into total globin.

to 80 per cent in different experiments and was relieved upon the addition of isoleucine (Fig. 3). Variations among different cell preparations are attributed to endogenous cellular conditions which determine the extent to which isoleucine can be made limiting.

Ribosome-polyribosome component during O-methylthreonine inhibition: The UV profile of the ribosome-polyribosome component under conditions of the O-methylthreonine-inhibition shown in Figure 3 is given in Figure 4. The data indicate the reversibility of both the inhibition of hemoglobin synthesis and the change in the ribosome-polyribosome profile. When histidine, leucine, or valine were omitted from the incubation medium, the UV profile remained normal in the presence of O-methylthreonine (unpublished observations), since isoleucine could not be made the limiting amino acid for hemoglobin synthesis under these conditions.

The unique pattern of polyribosomes obtained from O-methylthreonine-inhibited cells has four characteristics: (1) the level of 80*S* ribosomes (monomers) is unaltered in the inhibited system; (2) the dimers and trimers are elevated; (3) the normal components that are heavier than the trimers are reduced and;

(4) a new, very heavy polyribosome appears. Extrapolation of a logarithmic plot of polymer number versus distance traveled in the gradient[17-19] indicated that the new, heavy polyribosome contained approximately 12 monomer units. Since this number of monomers cannot be normally accommodated on messenger RNA coding for either α- or β-chains,[20, 21] this heavy polyribosome may have formed either by maximal packing of monomers on an extended messenger RNA strand[21] or by combination of lighter polyribosomes. It is highly unlikely to be

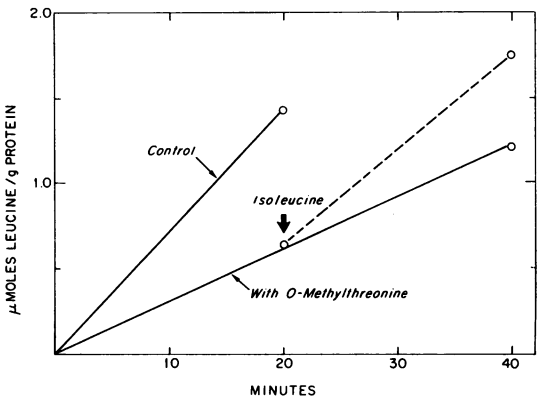

FIG. 3.—Relief of O-methylthreonine inhibition of hemoglobin synthesis by isoleucine. Control represents incorporation in the absence or presence of L-isoleucine, 0.2 mM. O-methylthreonine, when present, was at a final concentration of 25 mM, and L-isoleucine was added as indicated by the arrow to give a final concentration of 0.25 mM. The data illustrate the experimental procedures for obtaining the profiles shown in Fig. 4, and are not intended to indicate the rates of incorporation with precision.

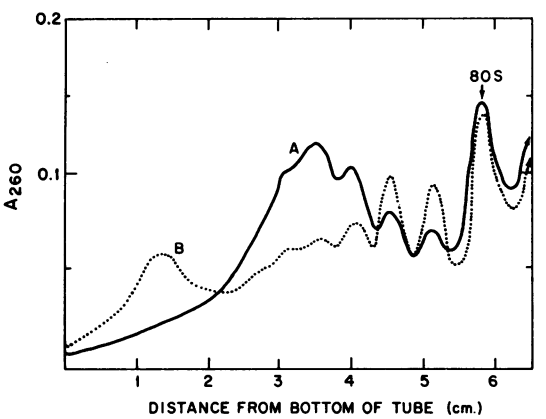

FIG. 4.—Ultraviolet absorption profile of the ribosome-polyribosome component of rabbit reticulocytes. The profiles correspond to cells incubated as shown in Fig. 3: *A*, controls or with O-methylthreonine for 0–20 min, and then isoleucine, 20–40 min; *B*, with O-methylthreonine alone for 20 or 40 min.

due to the emergence of polyribosomes engaged in the synthesis of a nonhemoglobin, isoleucine-free protein since iron[3] or heme[13] deficiency does not produce this component.

Identification of polyribosome components formed during O-methylthreonine inhibition: All polyribosomal components of cells with hemoglobin synthesis inhibited by O-methylthreonine incorporate amino acids into the growing peptide chains (Fig. 5). The marked differences between threonine and valine incorporation in the dimer and trimer components may be attributed to the relative deficiency of valine and abundance of threonine residues before the locus of the last isoleucine residue (no. 55) of the α-chain.[5, 9] Since translation of messenger RNA and movement of ribosomes would not be hindered beyond this isoleucine

codon, calculations based upon dimensions of reticulocyte ribosomes and messenger RNA given by Warner *et al.*[20] and Slayter *et al.*[21] would predict that polyribosomes bearing α-chains should become disaggregated to dimers. However, the inhibition by O-methylthreonine is not complete; therefore trimers are also prevalent. Thus, the increase in absorbance in the dimer-trimer region is attributed to the accumulation of polyribosomes bearing partially completed α-chains retarded in synthesis at the no. 55 isoleucine locus.

The single isoleucine residue of the β-chain is at position 112.[10] If ribosomal movement is retarded at the codon for this locus on β-chain messenger RNA, calculations similar to those indicated above would predict the formation of larger

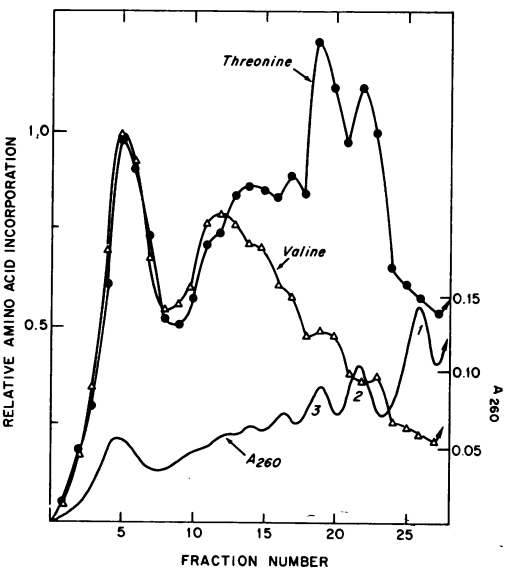

FIG. 5.—Incorporation of threonine and valine into peptide chains of the ribosome-polyribosome component of reticulocytes with hemoglobin synthesis inhibited by O-methylthreonine. The cells were incubated for 14 min in completely fortified medium with the following variations: cells to be labeled with valine were incubated with 10^{-4} *M* nonradioactive L-valine, which was found to be sufficient for optimal hemoglobin synthesis during a 20-min incubation period; cells to be labeled with threonine had this amino acid omitted from the incubation medium. At the end of this period, 0.01 μmole of L-valine-U-C^{14} (195 mc/mmole) or L-threonine-U-C^{14} (156 mc/mmole) were added. After 1 min incubation, L-O-methylthreonine was added to a final concentration of 25 mM and the incubation was continued for 5 min. Peaks 1, 2, and 3 correspond to the monomer (80*S*), dimer, and trimer, respectively. Incorporated radioactivity was normalized for fraction no. 5, the O-methylthreonine induced heavy polyribosome peak, and corresponded to 235 cpm for valine and 189 cpm for threonine.

polyribosomes (pentamers) bearing incomplete β-chains. Since the inhibition is not complete, hexamers would also be present, but there is no apparent reason to expect the formation of polyribosomes containing 12 monomers under these conditions.

Support for the characterization of the polyribosomal components of inhibited cells can be obtained by following the distribution of incorporated radioactive isoleucine. When isoleucine of high specific radioactivity is added at a concentration too low to prevent O-methylthreonine inhibition, the profile shown in Figure 6 is obtained. The higher levels of isoleucine incorporated into the peptide chains of the dimers and trimers support the conclusion that these bear incomplete α-chains. In this case, interpretation of the distribution of radioactivity differs from that of Figure 5, since when a C^{14}-isoleucyl-tRNA attaches to its

codon in a system where this aminoacyl-tRNA is limiting, the deficiency is not expressed in that particular molecular event. Thus, since only a single isoleucine residue is present in the β-chain, no isoleucine-C^{14} should label the nascent chains of β-chain polyribosomes which are characteristic of the inhibited system. In fact, as is shown in Figure 6, little isoleucine is incorporated into peptide chains

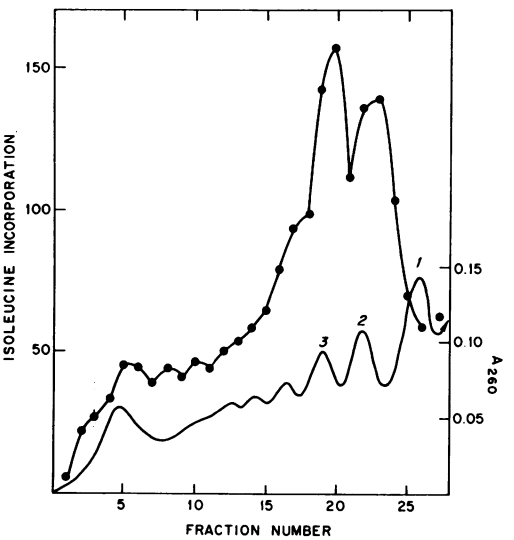

FIG. 6.—Incorporation of isoleucine into peptide chains of the ribosome-polyribosome component of reticulocytes with hemoglobin synthesis inhibited by O-methylthreonine. The cells were incubated for 14 min in completely fortified medium, 0.01 μmole L-isoleucine-U-C^{14} (250 mc/mmole) was then added, and the cells were treated with O-methylthreonine as described in Fig. 5. Isoleucine incorporation is given as counts per minute. The distribution of incorporated radioactivity of isoleucine, valine, and threonine into polyribosomes of uninhibited reticulocytes was essentially the same (unpublished observations) as that reported for leucine or a mixture of amino acids.[11]

associated with the heavy polyribosomes, and this is particularly evident upon comparison with results obtained with threonine and valine (Fig. 5). The small amount of isoleucine associated with the heavy polyribosome peak may be on nascent chains of ribosomes which had passed the isoleucine codon, but had not completed translation before a second inhibition event occurred.

The large polyribosomes obtained from cells with retarded synthesis of β-chains suggest that a selective block at the distal end of messenger RNA, such as a condition which may make the release of completed chains limiting, will also cause a major enlargement of the polyribosomes. Rifkind *et al.*[22] have found large polyribosomes in intact reticulocytes. Our observations indicate that these may be programmed for hemoglobin synthesis, but are blocked in some aspect of chain completion or release. Indeed, recent studies implicating a role for free α-chains in the release of β-chains from polyribosomes[23, 24] indicate that the block in β-chain synthesis by O-methylthreonine may be in part due to retarded progression of β-chain elongation at the 112 isoleucine locus and in part due to the failure of α-chain synthesis.

Summary.—The polyribosome profile of rabbit reticulocytes changes under conditions of isoleucine deprivation brought about by the antagonist O-methylthreonine. The loci of isoleucine in the hemoglobin chains and the corresponding loci of isoleucine codons in α- and β-chain messenger RNA permit interpretation of the data in terms of shortening of the α-chain polyribosomes and of enlargement of those synthesizing the β-chain.

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