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*A DEMONSTRATION OF CODING DEGENERACY FOR LEUCINE IN THE SYNTHESIS OF PROTEIN**

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sRNA acts as an adaptor in the transfer of amino acids into protein.[1–5] Since an organism may contain more than one variety of sRNA for a given amino acid, different coding specificities of the various adaptors would provide a mechanism for degeneracy in the code. In *E. coli* sRNA, several leucine-acceptors have been shown to respond differently to various synthetic polynucleotides.[5, 6] The experiments reported here were undertaken to determine whether the separate leucyl-sRNA's actually contribute leucine to different sites in natural polypeptide chains. Evidence that they do is presented in this paper.

Materials and Methods.—Separation of leucine acceptors: sRNA from *E. coli* strain B prepared by phenol extraction was obtained from General Biochemicals, Chagrin Falls, Ohio. Countercurrent distributions were performed with a 400-tube apparatus according to Holley *et al.* The solvent system was made by dissolving 550 gm K_2HPO_4 and 850 gm $NaH_2PO_4 \cdot H_2O$ in 4 liters of deionized water. To this, 300 ml formamide and 1535 ml isopropanol were added. Four hundred transfers were performed at 28°C. The 400 fractions obtained were combined into 80 sets of 5 tubes each. Assays of acceptor activity were done as previously described,[6] except that a partly purified *E. coli* leucine-activating enzyme preparation[8] was used instead of a crude *E. coli* extract. After incubating the fractions, the sRNA was precipitated with ice-cold 5% trichloroacetic acid (TCA). The precipitate was collected and washed with ice-cold 5% TCA on a membrane filter (Millipore). The filters were glued to planchets, dried, and counted.

Preparation of leucyl-sRNA: Attachment of labeled leucine to sRNA and isolation of C^{14}-leucine- and H^3-leucine-bound sRNA were performed as previously described[5, 6] except that a partly purified *E. coli* leucine-activating enzyme preparation[8] was used. Uniformly labeled C^{14}-leucine, specific activity 240 $\mu c/\mu M$ and 4,5 H^3-leucine, specific activity 5000 $\mu c/\mu M$ were products of the New England Nuclear Corp. Further details are given in the legends to the figures.

Preparation of reticulocyte ribosomes: Ribosomes from rabbit reticulocytes can be used to synthesize hemoglobin *in vitro*. They were prepared by the method described by Schweet *et al.*[9] ex-

cept that sucrose was omitted and Tris-HCl replaced the bicarbonate buffer. The temperature was 4°C unless otherwise stated. The following stock solutions were used:

Solution	Tris-HCl pH 7.6	$MgCl_2$	KCl	β-mercapto-ethanol
A	0.003 *M*	0.003 *M*	—	0.007 *M*
B	0.003 *M*	—	0.15 *M*	0.007 *M*
C	0.003 *M*	0.001 *M*	0.05 *M*	0.007 *M*

The packed reticulocytes were lysed by rapid suspension in 3 vol of solution A. After 5 min, 1 vol of solution B was added. Cell membranes were removed by centrifugation at 10,000 × *g* for 10 min. Ribosomes were obtained from the supernatant by centrifugation at 100,000 *g* for 120 min and suspended gently in solution C, at a concentration of about 20 mg RNA per ml, with the aid of a Potter-Elvehjem homogenizer.

Transfer of leucine into hemoglobin: The transfer mixture of von Ehrenstein and Lipmann[10] was modified to contain ATP and a low concentration of magnesium. The reaction was performed in 10 ml and contained (in μmoles unless otherwise noted): Tris-HCl, pH 7.6, 1000; KCl, 250; $MgCl_2$, 3; GSH, 80; GTP, 5; ATP, 10; PEP, 100; PEP kinase, 400 μg; C^{12}-leucine, 20; 19 C^{12}-amino acids (leucine omitted), 2 each; H^3-leucine attached to peak I sRNA, 1,600,000 cpm, 1200 μg sRNA; C^{14}-leucine attached to peak IIB sRNA, 142,000 cpm, 300 μg sRNA; reticulocyte ribosomes, 1500 $O.D._{260}$ units. After incubation for 30 min at 37°C, the mixture was chilled, and 400 mg of hemoglobin from rabbit reticulocytes added as carrier. The volume was adjusted to 13 ml with solution C, and ribosomes were removed by centrifugation at 100,000 × *g* for 2 hr.

Preparative phenolation of hemoglobin: To the supernatant obtained from the previous step, an equal volume of water-saturated phenol was added, and the mixture was agitated on a vortex mixer for 1 min at room temperature. After centrifugation at 10,000 *g* for 5 min, the mixture separated into a colorless upper aqueous phase containing the sRNA and a clear dark brown lower phenol phase containing the carrier- and *in vitro*-synthesized hemoglobin. The hemoglobin in the phenol phase, including a small amount of insoluble residue at the interface, was converted to globin by adding it dropwise at room temperature with vigorous stirring, to 10 vol of acetone containing 1 ml concentrated HCl per liter.[11] The flocculent tan-colored precipitate was collected by centrifugation at 2000 × *g* for 5 min, washed with acetone, and dissolved in water at a final concentration of 10 mg per ml yielding a clear, slightly brownish solution of globin. Formic acid and pyridine were added to final concentrations of 0.2 *M* and 0.02 *M*, respectively.

Chain separation: The globin was separated into its constituent α- and β-chains according to the method of Dintzis.[12] The sample containing approximately 400 mg globin was applied to a 15 × 2.5-cm carboxymethyl-cellulose column (8 gm dry weight, Serva Entwikelungslabor, Heidelberg, Germany, exchange capacity 0.71 meq/gm), and eluted with a linear gradient (500 ml 0.2 *M* formic acid plus 0.02 *M* pyridine in the mixing chamber; 500 ml 2.0 *M* formic acid plus 0.2 *M* pyridine in the reservoir).

Digestion of α-chain and β-chain with trypsin: The pooled α-chain fractions and pooled β-chain fractions were taken to near dryness on a rotary evaporator, and dissolved in 20 ml water. The pH was adjusted to 9.0 with 0.2 *N* NaOH. Two mg trypsin was added and the digestion mixture maintained at pH 9.0 for 3 hr, by addition of 0.1 *N* NaOH. The pH was then reduced to 6.4 with 1 *N* acetic acid. The digest was frozen and thawed, and the insoluble "core" removed by centrifugation at 10,000 *g* for 10 min.

Ion exchange chromatography of peptides: The supernatant containing the soluble peptides was fractionated according to the method of Jones.[13] It was evaporated to near dryness, dissolved in 2% formic acid, and applied to a 20 × 0.9-cm column of Dowex 50 × 8 (Type 15A resin, Beckman Instruments, Inc., Palo Alto, Calif.). The peptides were eluted with a concave gradient produced with the aid of a 7-chamber varigrad apparatus. Chambers 1, 2, 3, and 4 each contained 200 ml pH 3.1 buffer (1114 ml glacial acetic acid, 64.5 ml pyridine diluted to 4 l with distilled water), while chambers 5, 6, and 7 each contained 200 ml pH 5.0 buffer (573 ml glacial acetic acid, 645 ml pyridine diluted to 4 l with distilled water).

Aliquots were assayed with ninhydrin by the method of Moore and Stein.[14] Aliquots for liquid scintillation counting were dried down at 60°C in 20-ml glass counting vials and the samples dissolved in 0.2 ml 0.1 *N* HCl. Fifteen ml of scintillator solution (10 gm 2,5-diphenyloxazole (PPO); 0.5 gm 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), 500 ml ethanol, and 1500 ml toluene) was

added, and the H^3 and C^{14} content determined with a Packard liquid scintillation counter. The peak fractions were pooled, lyophilized, and further resolved by electrophoresis on Whatman 3MM filter paper at 50 v/cm for 90 min using pyridinium acetate buffer pH 6.4 (100 ml pyridine, 4 ml glacial acetic acid, 900 ml water). The purified peptides were located by staining guide strips with ninhydrin (0.2% ninhydrin in acetone).

The amino acid compositions of the purified peptides were determined following acid hydrolysis by column chromatography using a Technicon amino acid analyzer. The compositions of the α-chain peptides were compared with those reported by Diamond and Braunitzer[15] and the peptides numbered according to their convention.

Results.—Separation of leucine-acceptor sRNA's: Figure 1 shows the resolution of leucine-acceptor sRNA's into five components by countercurrent distribution. Peaks I*A*, I*B*, and I*C* represent further resolution of the peak I previously reported,[5, 6] while peaks II*A* and II*B* correspond to those found earlier, representing subdivisions of the one originally designated as peak II.[5, 6] Five components have also been found by Apgar and Holley.[16]

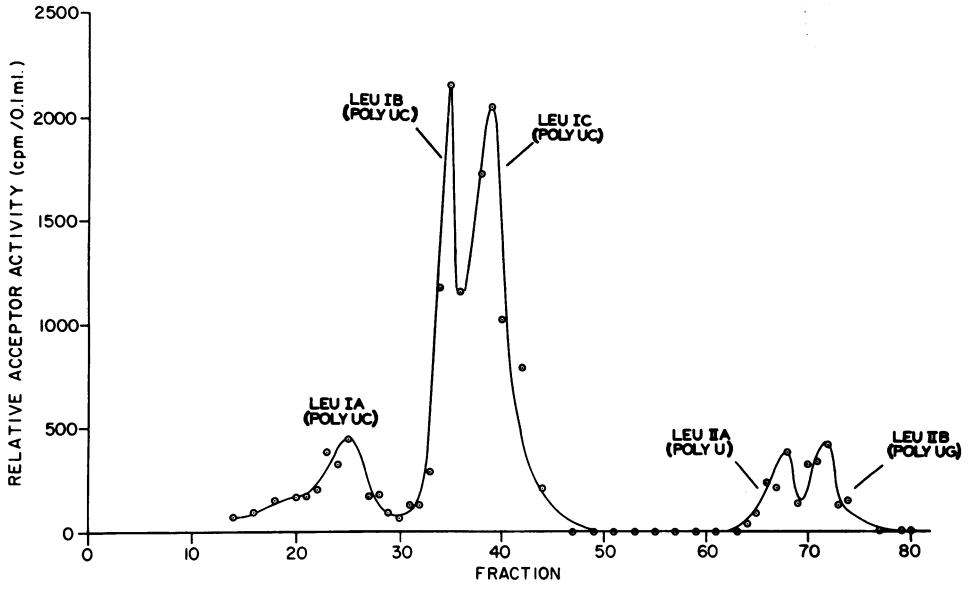

FIG. 1.—Resolution of 5 leucine acceptors by countercurrent distribution of *E. coli* sRNA. The polynucleotide most active in stimulating leucine transfer from each peak is indicated in parentheses.

In earlier experiments with synthetic polynucleotides[5, 6] peak I responded preferentially to poly UC, peak II*A* to poly U, and peak II*B* to poly UG. It has since been shown that the high magnesium concentrations employed can increase ambiguity in coding.[17] Therefore, the various leucine sRNA's were retested in the *E. coli* system at a low magnesium concentration (0.005 *M*). The differences between various peaks confirmed those reported previously.[5, 6] Peaks I*A*, I*B*, and I*C* all responded optimally to poly UC and were therefore pooled in preparing substrates for the hemoglobin transfer and referred to collectively as "peak I."

Distribution of leucine transferred into hemoglobin peptides: A double label experiment was performed in order to compare the specificity of transfer of leucine from peak I and peak II*B* sRNA's. H^3-leucine attached to peak I-sRNA, and C^{14}-

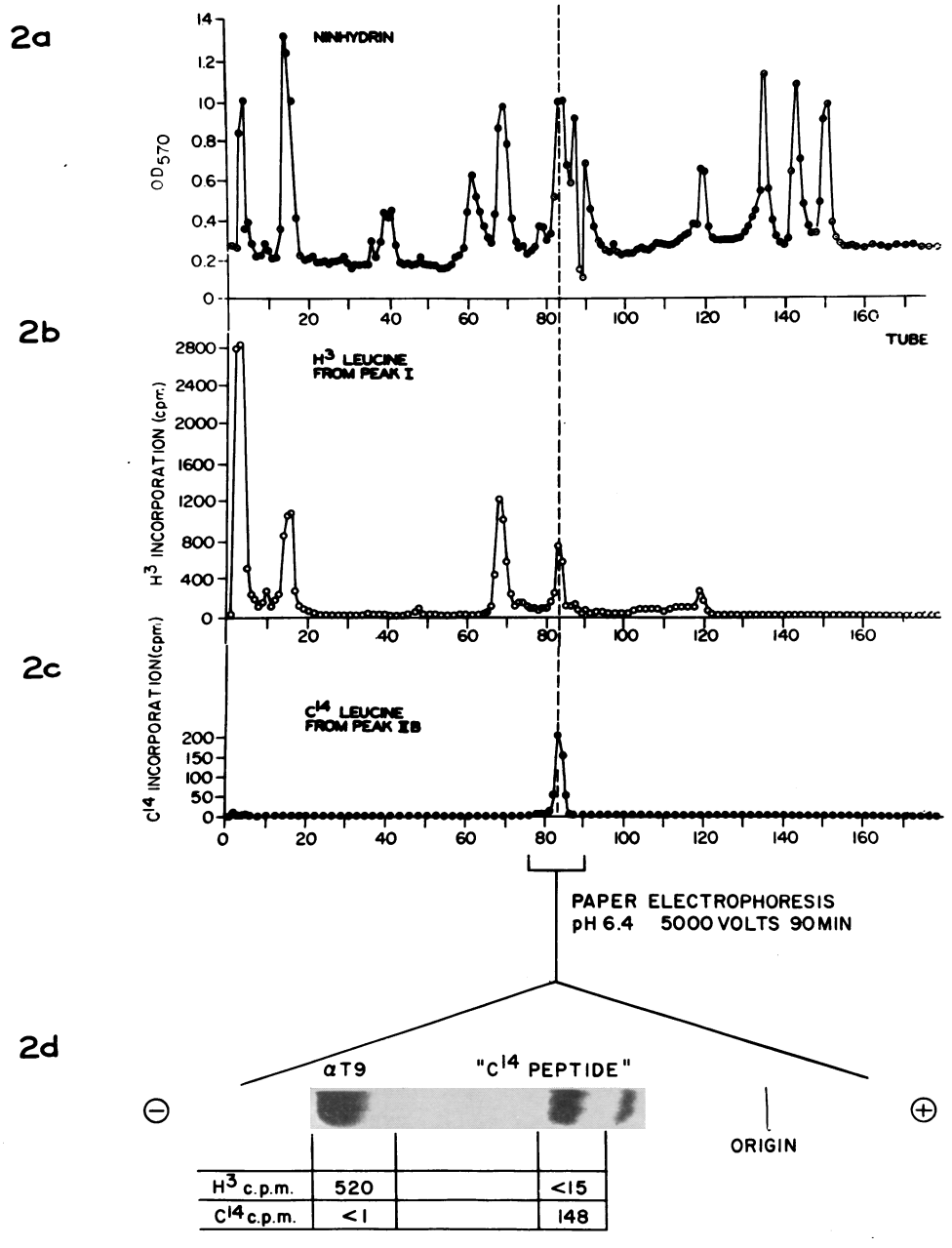

FIG. 2.—Separation of tryptic peptides of the α-chain of rabbit hemoglobin labeled by simultaneous transfer from H^3-leucine-charged peak I sRNA and C^{14}-leucine-charged peak II*B* sRNA. (*a*) Ninhydrin assay of fractions from Dowex 50 column. (*b*) H^3 counts in column fractions. (*c*) C^{14} counts in column fractions. (*d*) Tubes of special interest, 81–85 (see text), were pooled and further fractionated by paper electrophoresis. A guide strip was stained with ninhydrin to locate the peptides, and its photograph is shown. (Distance from the origin to peptide αT9 is 17 cm.) Corresponding parts of the remaining paper strip were eluted with 1 *N* acetic acid. After evaporation of the acetic acid, samples were dissolved in 0.2 ml 0.1 *N* HCl and assayed for H^3- and C^{14} content using liquid scintillation counting. Radioactivity contained in equivalent aliquots is tabulated.

leucine attached to peak II*B*-sRNA were mixed and transferred simultaneously into hemoglobin as described in *Materials and Methods*. Rabbit reticulocyte hemoglobin was added as a carrier, the soluble hemoglobin was purified by phenol extraction, and the α- and β-chains were separated, digested with trypsin, and the peptides fractionated by ion-exchange chromatography. Each fraction was assayed with ninhydrin (indicating the carrier peptides), and the radioactivity due to transferred H^3 and C^{14} was determined by scintillation counting.

The results for the α-chain are shown in Figure 2. At the top is the ninhydrin profile. The peaks were collected and further purified by electrophoresis. Following hydrolysis, their amino acid compositions were determined (see *Materials and Methods*). The peptides of interest here are those containing leucine, having their peaks at tubes 3, 15, 48, 70, 80, and 120. They could be correlated with the peptides reported by Diamond and Braunitzer,[15] with the exception of the peak at tube 3. The others correspond, respectively, to their peptides αT5 plus αT1 (unresolved), the ditryptide αT1 + 2, peptide αT10, peptide αT9, and peptide αT11.

Figure 2*b* shows the distribution of H^3-leucine transferred from peak I leucyl-sRNA. Note that six peaks (at tubes 3, 15, 48, 70, 80, and 120) received label. The heights of the peaks are not proportional to the number of leucine residues they contain, due to nonuniform labeling along hemoglobin chains synthesized in a cell-free system.[22]

In striking contrast, the C^{14}-leucine attached to peak II*B*-sRNA appears almost entirely in a single peak, as shown in Figure 2*c*. The material in this peak (tubes 81–85) was studied in further detail. Paper electrophoresis resolved two strong ninhydrin bands and two faint ones, as shown in Figure 2*d*. The radioactivity in the two strong bands is tabulated in the figure. The fastest band received only H^3 label. Its amino acid composition corresponded to peptide αT9, containing one leucine residue. Analysis showed that the radioactivity was all in the form of leucine. The peptide in the next band, designated "C^{14}-peptide," received its label from the C^{14}-leucine attached to sRNA peak II*B*. Analysis of the hydrolyzed peptide showed that the radioactivity was present solely as leucine.

TABLE 1

AMINO ACID COMPOSITION OF THE C^{14}-PEPTIDE COMPARED WITH PEPTIDE αT6

	C^{14}-peptide	αT6
Asp, $AspNH_2$	1	1
Glu, $GluNH_2$	2	2
Pro	1	1
Gly	1	1
Ileu	1	1
Tyr	1	1
His	2	2
Lys	1	1
Ala	0	0
Val	0	0
Met	0	0
Arg	0	0
Leu	1	0
Phe	2	3
Thr	1	2
Ser	2	1
Total	16	16

Amino acids showing differences are underlined. Try and Cys were not determined.

The amino acid composition of the C^{14} peptide, given in Table 1, does not correspond to any of the peptides previously described.[15–18] There is a close resemblance, however, between this peptide and αT6, shown for comparison. (In the column fractionation of Figure 2*a*, peptide αT6 emerged in tubes 86–90, immediately following the C^{14}-peptide.)

The amount of C^{14}-peptide is not accurately known; its ninhydrin color corresponds to about one fifth of that of αT6. Whether or not the C^{14}-peptide belongs to a minor hemoglobin component has not yet been

established. It should be recalled that this peptide is present in the carrier hemoglobin derived from intact reticulocytes, and fractionated together with hemoglobin through phenol extraction and with the α-chain during its separation from the β-chain. Ion-exchange chromatography of rabbit reticulocyte hemoglobin on carboxymethyl cellulose,[10] and on IRC-50,[19] does indeed reveal the presence of at least two components. We have found that electrophoresis on polyacrylamide gel also reveals two bands. However, further investigation is needed to see whether the C^{14}-peptide is associated with a separable hemoglobin type.

The two faint bands in Figure 2*d* each contained both arginine and lysine in about equal amounts and therefore do not represent pure tryptic peptides. They also contained small amounts of radioactivity (each contained 50 cpm H^3 and 10 cpm C^{14}). Characterization of these bands must await further purification.

The peptides of the β-chain were examined in a manner similar to that described in Figure 2. While several peaks contained H^3 transferred from leucyl-sRNA peak I, no C^{14} could be detected in any of the soluble β-chain peptides. They have not been investigated further.

Discussion.—If two sRNA's correspond to different codons, they should transfer amino acids into different positions in polypeptide chains. This was indeed found to be the case for the two leucine sRNA's examined.

Sueoka and Yamane[20] showed that, under certain conditions, leucine can be shifted from one sRNA to another. This transacylation is mediated by AMP and a leucine-activating enzyme that can attach leucine to both sRNA's. To minimize any such effect in our experiments, the reaction mixture contained a large amount of free C^{12}-leucine and ATP in order to keep the rabbit sRNA as fully charged as possible with C^{12}-leucine and the leucine-activating enzyme saturated with C^{12}-leucyl-AMP. Manifestly, the transacylation effect could not have played a significant role, or both H^3- and C^{14} label would have appeared in all the peptides.

These experiments employed a mixed system, using sRNA from *E. coli* to synthesize rabbit hemoglobin. Although it has not yet been specifically demonstrated that the rabbit has an sRNA with coding specificity corresponding to peak II*B* of *E. coli*, this does seem likely from the fact that leucine incorporation responds to poly UG in a rat liver system.[21] Heterogeneity has been observed for leucine-acceptor activity in rabbit liver sRNA fractionated by countercurrent distribution (von Ehrenstein, G., and D. Dais, unpublished).

The relation of the C^{14}-peptide to the main hemoglobin component remains to be clarified, but that is of secondary importance to the question under examination, since the peptide is, in any case, one among those synthesized in the cell-free system. Although the incorporated radioactivity is in the form of leucine, it has not yet been shown that it actually occupies a normal leucine position. The coincidence of the radioactivity and the leucine-containing peptide synthesized *in vivo* and visible with ninhydrin is, of course, consistent with this supposition. In any case, the clear-cut transfer of leucine from two separable sRNA's into different polypeptide positions leads to the apparently inescapable conclusion that leucine has at least two different kinds of coding specificity in the synthesis of natural polypeptides.

Summary.—Different leucine sRNA's in *E. coli* are separable by countercurrent distribution. When these sRNA's are used as donors of labeled leucine during the synthesis of rabbit hemoglobin, *in vitro*, they distribute leucine differently into

various peptides of the α-chain. One of the sRNA's introduces leucine only into a single position. The results indicate that there are at least two distinct codons for leucine in the amino acid code.

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