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Errors in Human Hemoglobin as a Function of Age[1]

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Introduction

Transcription and translation of the genetic message in cells are not infinitely precise and Orgel [9] has suggested that errors in protein synthesis may become more numerous with age and that the accumulation of error-containing proteins could contribute to the ageing process. Indeed, Lewis and Tarrant [5] have presented evidence for the accumulation of altered, inactive lactic dehydrogenase molecules in cultures of human fibroblasts prior to the death of these established cell lines. Moreover, Gershon and Gershon [4] have shown that fructose-1,6-diphosphate aldolase in livers of old mice is similarly altered; the specific activity of aldolase in the livers of old mice is only half that in young mice. They suggested that the inactive molecules may contain errors of mistranslation which arose at an increased frequency as the tissue culture cells and mice age. Loftfield [6] and Loftfield and Vanderjagt [7] have measured the frequency of substitution of one amino acid for another during the synthesis of egg albumin and rabbit hemoglobin. In the former study, valine or leucine was found to substitute for isoleucine at a frequency of about 3 per 10,000 residues; in the latter study where a better defined protein and better purification procedures were used, valine was found to substitute for isoleucine at two positions in rabbit hemoglobin at a frequency of 2–6 per 10,000 residues.

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Because human hemoglobin A, in comparison with most other proteins, has an unusual chemical property, i.e. it contains no coded isoleucine [3], we believed that chemical methods alone could be used to determine the substitution frequency of isoleucine for other amino acids in highly purified hemoglobin from individuals of various ages. An increase in the isoleucine substitution frequency with age could be evidence in support of the error theory of ageing. However, the small quantity of isoleucine found in hemoglobin A can be incorporated by more than one mechanism. These are: (1) infrequent errors in the transcription of DNA to mRNA may change nonisoleucine codons into isoleucine codons; (2) errors in transcription may produce altered forms of tRNA; these may have reduced fidelity as regards both the kinds of amino acids they accept and the species of mRNA codons they recognize; (3) errors in translation of the correct mRNA can occur through mispairing of tRNA anticodons and their proper mRNA codons; (4) amino acyl synthetase errors can cause the wrong amino acid-tRNA complexes to be formed, and (5) mistakes in the replication of DNA involving the exchange of appropriate base pairs [2] lead to the formation of mutant cells whose hemoglobin mRNAs contain isoleucine codons. Chemical determinations, as a composite measure of these errors, of the frequency at which isoleucine substitutes for other amino acids in hemoglobin of normal adults, ages 20–51, are reported in this paper.

Materials and Methods

Blood donors were composed of 3 laboratory personnel, 12 Marshall Island residents, and 13 Marshall Island residents exposed to γ-radiation fallout in 1954; the latter were positive controls. 10 ml of blood from each individual was collected in a heparinized syringe. Erythrocytes were separated from plasma by centrifugation for 10 min at 600 *g* and washed 3 times in 10 vol of saline. Samples from the Marshallese were resuspended in an equal volume of phosphate-buffered saline and shipped on ice by air and washed again upon arrival. The packed red cells were lysed by adding 4 vol of cold distilled water. Red cell stroma was removed by centrifugation for 15 min at 20,000 *g*. Hemoglobin in the supernatant was converted to the carbon monoxide form by bubbling with CO in a hood; one drop of octanol was added to prevent foaming. The hemoglobin solutions were kept cold during all subsequent processing (cold room at 4°C).

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Hemoglobin was separated from nonhemoglobin proteins first by molecular seiving over Sephadex G-200 (column size, 1.9 × 120 cm; buffer, 50 mM sodium phosphate, pH 5.8, saturated with CO; flow rate, 6 ml/h. Fractions containing the majority of the carbon monoxyhemoglobin (60–90 ml) were pooled and placed on a column (3.7 × 100 cm) of carboxymethyl cellulose (Whatman CM 23), which was washed according to manufacturer's instructions, and equilibrated with 50 mM sodium phosphate buffer, pH 5.8 (9 parts NaH_2PO_4 and 1 part Na_2HPO_4). Carbon monoxyhemoglobin was eluted using a nonlinear gradient of 1.7 l of 50 mM sodium phosphate buffer, pH 5.8, in a 2-liter reagent bottle, and 1.0 l of 50 mM Na_3PO_4, in a 1-liter reagent bottle (both solutions were bubbled briefly with CO); the flow rate was 1.5 ml/min. Carbon monoxyhemoglobin eluting between pH 6.7 and 6.95 was pooled and concentrated by pressure dialysis to a volume of less than 20 ml to remove much of the salt and then diluted to 40 ml with distilled water; then the pH was adjusted to 6.0 by adding 0.5 M H_3PO_4, and the carbon monoxyhemoglobin was converted to methemoglobin by making the solution 1 mM in $K_3Fe(CN)_6$. The second chromatography was performed on one of two columns of carboxymethyl cellulose (2.5 × 100 cm) using a gradient identical (one gradient supply for two columns) to the previous one but without CO. Methemoglobin eluting between pH 7.05 and 7.35 was concentrated, diluted, and pH adjusted as before. Methemoglobin was converted to cyanmethemoglobin by making the solution 1 mM in KCN and the cyanmethemoglobin was chromatographed on one of four columns (1.8 × 100 cm) of carboxymethyl cellulose using a gradient identical to the previous one but now also containing 10^{-4} M KCN. Cyanmethemoglobin eluting between pH 6.7 and 7.0 was concentrated by pressure dialysis and used to prepare globin [10], which in a few cases was further separated into α- and β-chains [1].

Globin or chains were hydrolyzed in 6 N HCl for 21 h at 110 °C. 2% of each hydrolysate was used to determine by amino acid analysis [11] the quantity of globin or chain in each sample. For reference markers, tracer amounts of L-leucine-^{14}C and L-isoleucine-4,5-^{3}H (New England Nuclear) were added to the remainder of the hydrolysate before it was chromatographed on a preparative ion exchange column (1.9 × 60 cm) of 8% cross-linked sulfonated styrene divinylbenzene copolymer (Beckman Type 150A) to separate and recover the isoleucine in the hydrolysate. The resin was equilibrated with 0.2 M sodium citrate buffer, pH 3.25 (Beckman), and the amino acids were eluted with 0.2 M sodium citrate buffer, pH 4.25 (Beckman); the flow rate was 68 ml/h. 3-min fractions of the eluate were collected and the radiotracers were used to locate fractions containing isoleucine but excluding

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leucine. Isoleucine-^{3}H radioactivity counts were also used to calculate the percentage of isoleucine eluting from the preparative column which was actually pooled for the quantitative analysis of isoleucine in the protein hydrolysate; i.e. the small quantity of isoleucine that did not completely resolve from leucine could be included in computing the quantity of isoleucine in the original sample. The pooled eluate must not contain much leucine because it would interfere with an accurate determination of the small quantity of isoleucine there. After adjusting the pH to 2.2 by adding 1 N HCl, the quantity of isoleucine in the pooled eluate was determined using a Beckman Model 120C amino acid analyzer [11]. The frequency at which isoleucine substitutes for other amino acids in human hemoglobin was calculated by dividing the nanomoles of isoleucine by the nanomoles of all other amino acids in the sample.

Results

About 3% of the total protein, mostly molecules of higher molecular weight, were separated by molecular seiving over Sephadex G–200. Based on an average isoleucine content of 5% for nonhemoglobin proteins, the carbon monoxyhemoglobin fraction still contained 3–4% nonhemoglobin protein after molecular seiving. This was removed by repeated chromatography of chemically derivatized hemoglobin over carboxymethyl cellulose.

The substitution frequencies of isoleucine for other amino acids in the highly purified hemoglobin from 15 controls of ages 20–60 years are shown in table I. Data on 13 Marshallese who were exposed to fallout radiation in 1954 are presented in table I to indicate that the method does detect individuals who have hemoglobin with a higher isoleucine content, presumably due to radiation-induced base substitutions in erythropoietic stem cells. One of the controls, sample 1547, showed a significantly higher value than the rest and it was not included in making the following calculations. The average isoleucine substitution frequency for the 3 laboratory personnel and the 12 Marshallese controls is 2.99×10^{-5}, and the computed line of best fit has a positive slope of 0.0296×10^{-5}/year.

The isoleucine substitution frequency in the separated α- and β-chains of a few samples was determined to show that the values equalled those obtained for the globin, although the substitution frequency in the β-chain was higher than in the α-chain (table I).

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Table I. Substitution frequency of isoleucine for other amino acids in human hemoglobin from 25 Marshall Island people and 3 ORNL laboratory personnel

Sample	Exposure	Age at exposure	Age at present years	Sex	Substitution frequency ($\times 10^{-5}$)	Average ± SEM ($\times 10^{-5}$)
3R	175	15 months	21	M	19.79	
10R	175	30 years	50	M	3.58	
18R	175	24 years	44	F	5.06	
24R	175	13 years	33	F	13.45	
33R	175	1 year	21	F	4.74	8.81 ± 1.96
35R	175	12 years	32	F	5.19	
42R	175	2 years	22	F	10.40	
71R	175	27 years	47	F	8.29	
6A	69	1 year	21	M	6.98	
8A	69	17 months	21	F	12.93	
44A	69	3 years	23	M	4.04	5.94 ± 1.92
45A	69	31 years	51	F	3.65	
81A	69	7 years	27	F	2.12	
813U	0		20	M	3.37	
815	0		24	M	2.17	
829U	0		35	F	3.47	
836	0		41	M	2.45	
839	0		46	F	1.89	
841U	0		41	F	3.56	
846	0.		51	F	2.41	3.20 ± 1.52
867U	0		46	F	2.12	
868U	0		51	F	4.35	
944U	0		49	M	3.93	
1547	0		60	F	7.15	
1549	0		21	M	1.57	
EB	0		34	M	2.84	
WL	0		45	M	4.37	3.52 ± 0.45
RAP	0		43	M	3.36	
836α					2.05	
836β					4.41	
867Uα					1.99	
867Uβ					3.40	
868Uα					1.76	
868Uβ					5.04	

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Discussion

The procedures used to purify human hemoglobin were chosen because nonhemoglobin proteins of higher and lower molecular weights than hemoglobin can be removed by molecular seiving and nonhemoglobin proteins of molecular weights similar to hemoglobin can be separated by repeated chromatography of derivatized hemoglobin on ion exchangers. Proteins that do not contain heme should chromatograph similarly on carboxymethyl cellulose each of the three times because the same ion exchanger, buffers and gradient are employed; however, the chromatographic properties of the derivatized hemoglobins are different. Thus, nonhemoglobin proteins that contaminate one form of hemoglobin should be removed during the next chromatography. Human fetal hemoglobin is a protein that does contain isoleucine and its chromatographic behavior on carboxymethyl cellulose is changed by derivatization much like that of hemoglobin A. However, hemoglobin F has a different isoelectric point and it has different chromatographic properties than adult hemoglobin; 95% of any hemoglobin F present is removed by the repeated chromatography over carboxymethyl cellulose. The potential contamination by hemoglobin F was determined by starting with a chemically determined mixture of 90% adult and 10% fetal hemoglobin. Chromatography over carboxymethyl cellulose removed 80, 50, and 50% of the fetal hemoglobin at each successive step using these procedures (unpublished). If human hemoglobin on the average contains 1% hemoglobin F, the hemoglobin F remaining after the third chromatography could contribute 7 parts of isoleucine per million amino acid residues. This is fourfold less than the isoleucine content found in highly purified human hemoglobin A (table I). Moreover, hemoglobin F cannot be the principal source of the isoleucine in the globin samples because isoleucine is found in both the α- and β-chain polypeptides (table I); the α-chain is, but the β-chain is not, cleanly separated from the γ-chain by the procedures used. Furthermore, in marmoset and sheep the ^{3}H-isoleucine and optical density profiles of the separated α- and β-chains are coincident [unpublished], which indicates that the isoleucine is in hemoglobin rather than in nonhemoglobin protein contaminants.

Chemical analyses show that highly purified human hemoglobin A has a very low content of isoleucine. A slight, but insignificant increase with age in the isoleucine substitution frequency is suggested on the basis of a least squares treatment of the data; the individual data points, except for sample 1547, fit well with a linear increase of 0.0296×10^{-5}/year between ages 20 and 51 years. Sample 1547 deviated significantly from linearity; this sample

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was obtained from a 60-year-old woman showing signs of senility. Little significance can be attached to this sample taken from the oldest person in this study; however, it does suggest that more data on older persons should be obtained to determine whether the isoleucine substitution frequency increases linearly or has an exponential component during advanced ageing [9].

As stated in the Introduction, isoleucine incorporation into human hemoglobin A may originate from genetic and/or nongenetic errors. One approach toward estimating the contribution of somatic mutations to the average isoleucine substitution frequency would be to assume that the mutation frequencies due to polymerase errors in germinal and somatic cells are similar. This may be a valid assumption for comparing spermatogonia and erythropoietic stem cells; both divide continually and differentiate into terminal cells. However, oogonia, which remain at prophase of the second meiotic division, may have a lower frequency of mutations owing to polymerase errors. The rate of germinal mutations in man has been estimated from the frequency of occurrence of inherited genetic diseases, which suggests an average mutations frequency of 1×10^{-5}/locus/generation. It has been argued that this estimate is too high, but a higher value based on molecular changes within proteins has been calculated recently [8]. Most inherited genetic diseases result from defective enzymatic mechanisms. Mutations within the active centers of enzymes (usually less than 5% of the molecule) cause inactivation or altered substrate specificity sufficiently different from normal to produce an altered phenotype, whereas mutations at amino acid residues outside the active center generally do not markedly change the specificity of an enzyme. Isoleucine is 1 of 20 amino acids that substitute for other amino acids but since only 5% of all mutations randomly occurs within an active site, one deduces that an isoleucine substitution for other amino acids occurs at approximately the same frequency as the appearance of expressed mutations, i.e. about 1×10^{-5}/locus/generation. Thus, this portion of the total isoleucine substitution frequency may be due to mutations while the remainder, 2×10^{-5}, may result from nongenetic error.

A better estimation of the contribution of somatic mutations and nongenetic errors toward the total isoleucine substitution frequency can be made in an experimental animal with the aid of isotopically labelled amino acid incorporation followed by sequential degradation of the polypeptide. Using such procedures, the absolute substitution frequencies of isoleucine for the various amino acids in the polypeptide can be determined. For amino acids whose codons can be changed from nonisoleucine codons into isoleucine codons by a single base substitution, e.g., GUU (Val) to AUU (Ile), the

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substitution of isoleucine may be caused both by infrequent nongenetic errors and by somatic mutations. However, isoleucine substitutions for amino acids whose codons require more than one base substitution to change into isoleucine codons can be considered to be due primarily to translational errors because the likelihood of two base substitutions within the same codon is the square of the frequency of a base substitution at one nucleotide, which would be very infrequent. Thus, isoleucine substitution for amino acids like glycine or alanine can be considered to result from translational errors only. Some amino acids are coded by triplets, some of which can and others which cannot mutate from nonisoleucine to isoleucine codons by single base substitutions, e.g. lysine, arginine, threonine and serine. Substitution of isoleucine for such amino acids would be by nongenetic errors at some positions, but by somatic mutations plus nongenetic errors at other positions. The distinction of these should be readily made by treatment of experimental animals with base substitution mutagens; marked increases in isoleucine substitution should occur only at positions where single base changes can mutate nonisoleucine codons into isoleucine codons. By comparing the substitution frequencies of isoleucine for lysine at positions which are and which are not affected by a base substitution mutagen, it should be possible to access the relative contribution of translational errors and somatic mutations toward the formation of erroneous proteins during protein synthesis in young and old animals.

In line with the above arguments, the data of LOFTFIELD and VANDERJAGT [7] are pertinent and interesting. If their data are handled in a manner similar to ours, i.e. to calculate the valine substitution for all the other amino acids in the tetra- and octapeptide (except for the C-terminal amino acids because valine substitutions for lysine would not yield the appropriate tryptic peptides), their average substitution frequency would be 7.5×10^{-5} per amino acid residue compared with our value of 2.99×10^{-5} per amino acid residue. These values are in good agreement and the twofold differences may actually be due to species differences. LOFTFIELD [6] has found that the majority of the valine incorporated in the tryptic peptides of the rabbit α-chain is located at positions where isoleucine is the coded amino acid (personal commun.). This would seem to support their view that most of these substitutions are caused by translational errors. However, amino acids that are likely to be involved in translational errors are the same ones which are coded for by triplets where single bases distinguish nonisoleucine and isoleucine codons. Of greater interest is the fact that the valine substitution in the tetrapeptide was lower than in the octapeptide. The octapeptide contains 3 glycine residues,

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1 glutamic residue and 1 isoleucine residue whose codons can mutate to valine codons by single base changes, whereas the tetrapeptide has only the isoleucine codon that can mutate to valine by a single base change. Assuming that the majority of valine at the position of coded isoleucine is incorporated through translational errors, that the valine substitution through translational errors is similar for isoleucine in both the tetra- and octapeptides, that the other amino acids have properties so different from isoleucine and valine that complexes between them and isoleucyl-tRNA would be very infrequent indeed, it would seem that the sum of the contributions of somatic mutations at the codons for the 3-glycine and 1-glutamic acid positions could be equal to the contribution of translational errors at the isoleucine position in the octapeptide. Correlations and speculations given above are no substitute for experimental facts! However, it does seem clear that translational variation can cause a small amount of a correctly coded mRNA to produce a chemically altered protein and that these alterations are chemically similar to those which can also arise by somatic mutations; to date, methods have not been judicially applied to distinguish between these alternatives in the analysis of errors in protein synthesis and ageing. Nevertheless, a combination of these errors can lead to a considerable amount of error-containing protein to be formed by cells. An average isoleucine substitution frequency per amino acid residue in human hemoglobin of 3×10^{-5}, where isoleucine is one of 20 amino acids each of which may show a similar frequency of amino acid substitution, suggests that a total of 8.5% of the polypeptide chains being synthesized contain one amino acid difference from correctly coded ones. For molecules composed of polymers of polypeptide chains, a large percentage of the molecules may contain one or more amino acid substitutions. If error-containing molecules are more prone to become enzymatically inactive but retain their antigenic properties, it is conceivable that the inactive enzyme molecules found in tissue culture [5] and liver [4] cells are molecules which contain errors.

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