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## The Frequency of Errors in Protein Biosynthesis

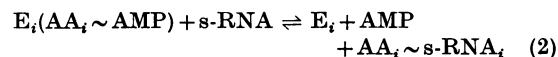
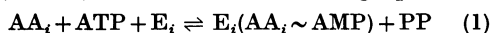
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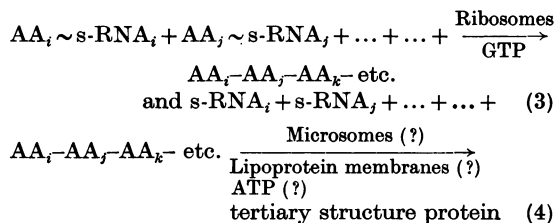
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It is widely recognized that variations in the amino acid sequence of particular proteins within and between species reflect corresponding variations in the genetic material of the cell in which the proteins are synthesized. Thus the 'error' in which valine replaces glutamic acid in haemoglobin synthesis (Ingram, 1958) may be regarded as a 'genetic error'. On the other hand, even where the genetic message is perfect, there may be errors in the transmission of the message or in the execution of the genetic instructions owing to the finite ability of macromolecular surfaces to distinguish between closely related molecules. Mistakes of this sort might be termed 'non-genetic errors in protein biosynthesis' and are the subject of the present paper.

It is generally believed that protein synthesis proceeds from free amino acids through amino acyl adenylates and amino acyl 'transfer' ribonucleic acid (s-RNA) as shown in the following equations:



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where  $AA_i$  represents a particular natural amino acid,  $E_i$  is the 'activating enzyme' that specifically converts  $AA_i$  into the mixed amino acyl adenylate ( $AA_i \sim AMP$ ), s-RNA<sub>i</sub> is the 'transfer' ribonucleic acid specific to  $AA_i$ , and  $AA_i-AA_j-AA_k$  represents the growing polypeptide chain containing various amino acids. Reaction (1) was first demonstrated by Hoagland (1955), and enzymes of the type  $E_i$  were shown to be quite specific for their particular amino acid by Sharon & Lipmann (1957). Reaction (2) was identified by Hoagland, Zamecnik & Stephenson (1957) and by Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958), who with others (Berg & Ofengand, 1958; Schweet, Bovard, Allen & Glassman, 1958) showed that natural amino acids did not compete with each other. From this latter observation it was inferred that there were 'transfer' ribonucleic acids unique

to each of the natural amino acids. Berg & Ofengand (1958) and Berg, Bergmann, Ofengand & Dieckmann (1961) showed that a single enzyme  $E_4$  catalysed both reactions (1) and (2) for a particular amino acid  $AA_4$ . Evidence for reaction (3) began to accumulate with observations by Keller & Zamecnik (1956) and by Hoagland *et al.* (1957, 1958), who showed that GTP was essential for the formation of a polypeptide. It is this step that appears to be sensitive to puromycin (Allen & Zamecnik, 1962; Yarmolinsky & de la Haba, 1959). Reaction (4) is the most poorly defined of these steps. It is reasonable to suppose that the formation of disulphide bonds and the folding and attachment of prosthetic groups and so forth should occur after the peptide chain is completed. The experiments of Campbell, Greengard & Kernot (1960) and of Decken & Campbell (1962) suggest that the intact microsome is required for the release of the complex three-dimensional structure whose antigenic, enzymic and physical properties characterize it as a native protein.

Something of the nature of each step can be learned by establishing whether or not there is discrimination between amino acids at that step. Thus if valine substitutes for isoleucine less often in step (3) than in step (2) we have presumptive evidence that step (3) involves a 'rereading' of the aliphatic side chain of the amino acid, a task that would probably require a specific enzyme. Alternatively, if the frequency of isoleucine-valine errors is the same after step (3) as after step (2), we might conclude that the amino acid has been supplied with an 'adaptor' (Crick, 1957) in step (2) which is read without further error or discrimination in the next step. Finally, if valine is found substituted for isoleucine more often in the polypeptide than at step (2) we deduce that the final reading mechanism is not precise.

Some information is available on the specificity with which isoleucine is preferred over the closely related but 'incorrect' valine in reaction (1) (Bergmann, Berg & Dieckmann, 1961; Loftfield & Eigner, 1961; R. B. Loftfield, unpublished work, 1963), the preference being about 50 : 1. For reaction (2) the corresponding ratio may be about 5000 : 1 (Loftfield, Hecht & Eigner, 1959, 1963), indicating that this step, though catalysed by the same enzyme, is far more discriminating than the first. There is as yet no explanation for this striking increase in discrimination. Although most biochemists now regard a particular protein molecule as having a unique sequence of amino acids, no precise data are available on the actual frequency of non-genetic errors in completed protein. Therefore, to compare the error frequency at step (2) with the error frequency at later steps, I have attempted to determine the frequency with which isoleucine, valine and leucine substitute for

each other or for other amino acids in the synthesis of chicken ovalbumin. A preliminary report of this work has been given (Loftfield, 1962).

*Experimental design.* If there is an occasional substitution of a valine for an isoleucine in the peptide chain, I assume *a priori* that this will have a small or negligible influence on many of the properties of the protein. The erroneous polypeptide can still coil and fold much like the correct chain and is not separable from the correct protein by the usual techniques of purification. The erroneous and correct proteins would have nearly identical susceptibilities with proteolytic enzymes such as trypsin or chymotrypsin and would yield on hydrolysis identical peptides except for one peptide in which a valine is substituted for an isoleucine. If the peptides can now be purified by methods, such as electrophoresis, that do not distinguish between the isoleucine peptide and the homologous valine peptide, a peptide may be isolated whose relative content of isoleucine and valine is a direct measure of the frequency with which valine naturally substitutes for isoleucine in protein biosynthesis.

These frequencies are too low to be determined by ordinary chemical methods. However, if the valine and isoleucine are radioactive, a determination is possible. In principle it would be best if the valine and isoleucine were incorporated into the protein *in vivo*. Consideration of economy and metabolic interconversions make this impracticable. As an alternative, the labelled proteins may be prepared by incubation of a surviving tissue, namely minced oviduct, with one or another of several labelled amino acids.

After purification by electrophoresis, the peptides were hydrolysed and the constituent amino acids isolated, to establish that the measured radioactivity was actually present in the particular amino acid that had been used.

## EXPERIMENTAL AND RESULTS

*<sup>14</sup>C-labelled amino acids.* L-[1-<sup>14</sup>C]Valine, L-[1-<sup>14</sup>C]leucine and L-[1-<sup>14</sup>C]isoleucine were prepared from Ba<sup>14</sup>CO<sub>3</sub> by way of K<sup>14</sup>CN (Loftfield, 1947) and the Bucherer hydantoin synthesis (Loftfield, 1950). The amino acids were resolved by the action of acylase I on their acetyl derivatives (Baker & Sober, 1953; R. B. Loftfield, unpublished work, 1963). Each was synthesized with 27 mc of <sup>14</sup>C/m-mole, which, under our counting conditions (Nuclear-Chicago Corp. C-115 low-background Geiger counters: infinitely thin samples on aluminium planchets), gave 16.5 × 10<sup>9</sup> counts/min./m-mole. The amino acids were purified from all other known amino acids by chromatography on a column (2.5 cm. × 220 cm.) of Dowex 50 (Na<sup>+</sup> form), with sodium citrate, pH 3.7, as eluent. Purity was ascertained by use of another column under different conditions. In no case was there a detectable amount of impurity (< 0.05%). Optical purity was determined with D-amino acid oxidase (R. B. Loftfield, unpublished work, 1963) and was greater than 99.9% in each case.

Table 1. Recovery of protein and radioactivity during the purification of  $^{14}\text{C}$ -labelled ovalbumin

Minced oviduct (about 1.5 g.) was incubated for 16 hr. at  $37^\circ$  in the presence of the indicated  $^{14}\text{C}$ -labelled amino acid and the distribution of the radioactivity then examined. Experimental details are given in the text. Counts/min. are expressed as millions.

	$^{14}\text{C}$ Isoleucine	$^{14}\text{C}$ Valine	$^{14}\text{C}$ Leucine
Radioactivity used in incubation (counts/min.)	46	144	100
Extracellular radioactivity after 16 hr. (counts/min.)	11	69	23
After homogenization, total soluble material (counts/min.)	26	84	33
Radioactivity in protein after treatment with Sephadex (counts/min.)	6.3	7.2	11
Ovalbumin after isolation with carboxymethyl-cellulose:			
(counts/min.)	3.6	3.7	6.0
(mg.)	132	160	139
( $\mu$ moles)	2.9	3.5	3.0
Estimated no. of residues/molecule (Tristram, 1949)	25	28	32
Estimated radioactivity of $^{14}\text{C}$ -labelled amino acid (counts/min./ $\mu$ mole of amino acid)	0.050	0.038	0.062
Conalbumin isolated:			
(counts/min.)	0.24	0.72	0.60
(mg.)	32	46	45
Ovalbumin after oxidation (mg.)	143	163	151

*Preparation of  $^{14}\text{C}$ -labelled ovalbumins.* A regularly laying White Leghorn hen from the stock of the School of Agriculture, Cambridge University, was killed 4 hr. after laying. The oviduct was cut open and the mucous layer was scraped from the muscular substratum. The mucous tissue was chopped finely in the medium used by Hendler (1956), which contains NaCl (33 mM), KCl (72 mM),  $\text{NaHCO}_3$  (40 mM) and glucose (12 mM) maintained at pH 7.5–7.8 with  $\text{CO}_2 + \text{O}_2$  (5 : 95). About 1.5 g. of the minced tissue was added to each of three flasks containing 10 ml. of the Hendler medium and 2.8  $\mu$ moles of L-[ $^{14}\text{C}$ ]isoleucine, 8.7  $\mu$ moles of L-[ $^{14}\text{C}$ ]valine or 6.1  $\mu$ moles of L-[ $^{14}\text{C}$ ]leucine respectively. The flasks were incubated for 16 hr. at  $37^\circ$  with gentle agitation and continuous gassing with  $\text{CO}_2 + \text{O}_2$  (5 : 95).

The contents of each flask were then frozen and thawed eight times to effect partial disruption of the tissue structure. Each preparation was homogenized in a Potter-Elvehjem homogenizer at  $0^\circ$  and centrifuged at 5000g for 10 min. The residue was extracted with 10 ml. of 0.15M-ammonium acetate, pH 7.0, and centrifuged. The supernatant and the extract were combined and assayed. The yield of radioactivity in the three experiments after various purification steps is shown in Table 1, which also shows that about half of the radioactivity in each case was still in soluble form in the tissue or medium after 16 hr. The balance was presumably bound to insoluble material or converted into volatile substances.

Each soluble extract of oviduct tissue was placed on a column (2.0 cm.  $\times$  60 cm.) of Sephadex G-25 that had been equilibrated with 0.15M-ammonium acetate, pH 7.0, and eluted with the same buffer. The eluate was collected in 10 ml. fractions. In each case there was substantial radioactivity associated with protein in fractions 5–9 followed by a peak associated with amino acids in fractions 10–18. The fractions containing [ $^{14}\text{C}$ ]isoleucine-labelled protein were combined, frozen and freeze-dried. The same was done with the other labelled proteins. Each preparation was then taken up in 5 ml. of 0.1N-acetic acid adjusted with

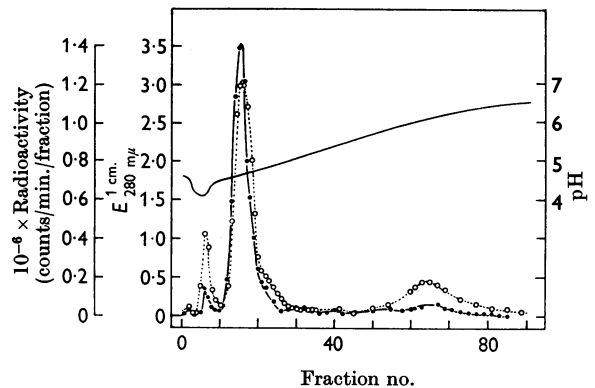


Fig. 1. Chromatography of [ $^{14}\text{C}$ ]isoleucine-labelled oviduct proteins on carboxymethylcellulose (9 cm.  $\times$  12 cm.) by using a pH gradient of ammonium acetate as described in the text. —, pH;  $\cdots\circ\cdots\circ\cdots$ ,  $E_{280}^{1\text{ cm}}$ ;  $\bullet\text{---}\bullet\text{---}\bullet$ , radioactivity (millions of counts/min./fraction). The major peaks are, from left to right, ovomucoid, ovalbumin and conalbumin.

aq. ammonia to pH 4.3 and chromatographed on a column (2 cm.  $\times$  9 cm.) of carboxymethylcellulose that had been equilibrated with pH 4.3 buffer. The column was developed with a gradient prepared by using an open mixing vessel containing 100 ml. of 0.1N-acetic acid adjusted to pH 4.3 with aq. ammonia and, at the same height, an open reservoir having twice the mixing vessel's diameter and containing 400 ml. of 0.1N-acetic acid adjusted to pH 7.0 with aq. ammonia. The gradient is almost linear in pH. Assay for radioactivity and extinction showed that the ovalbumin separated cleanly from the other egg proteins, although we never observed separation of the three forms of ovalbumin (Fig. 1; see also Rhodes, Azari & Feeney,

1958). The middle fractions from the peaks were pooled, dialysed against three changes of distilled water and freeze-dried. Starch-gel electrophoresis (Williams, 1962) showed the preparations of these three  $^{14}\text{C}$ -labelled albumins and conalbumins to be chemically and isotopically indistinguishable from known samples. In particular, the albumins showed the three-banded appearance that is typical of ovalbumin.

The three  $^{14}\text{C}$ -labelled ovalbumins were each taken up in 5 ml. of formic acid at  $0^\circ$  and agitated intermittently at  $0^\circ$  for 2 hr. Then 10 ml. of performic acid (prepared by adding 0.5 ml. of 30% hydrogen peroxide to 9.5 ml. of formic acid and holding at  $0^\circ$  for 2 hr.) was added and the mixture kept at  $0^\circ$  for 2 hr. (Ryle & Sanger, 1955). The mixtures were dried in high vacuum at low temperature, taken up in a small amount of water and again freeze-dried to remove the last traces of formic acid and performic acid.

**Chymotryptic digestion and separation of peptides.** Each of the oxidized ovalbumins was then taken up in 7 ml. of 1N-ammonia. After 1 hr. at  $37^\circ$  the solids were almost entirely dissolved, yielding a slightly brown viscous solution. 1M-Ammonium acetate, pH 7.0 (5 ml.), was added, which produced a flocculent precipitate. (The pH of the non-radioactive control experiment was 9.6 at this point.) To each preparation was added 200  $\mu\text{l}$ . of a solution containing 1.0 mg. of chymotrypsin and 0.05 mg. of soya-bean trypsin inhibitor (both from Worthington Chemical Corp., Freehold, N.J., U.S.A.). Digestion was carried out at  $37^\circ$  for 5 hr. Within 30 min. the flocculent precipitates were largely redissolved. In the non-radioactive control these conditions resulted in about 30 chain breaks/ovalbumin molecule as determined in a pH-stat. After the incubation 1 drop of 1% thymol blue was added and the solution was concentrated to near dryness on a rotary still. The residue was adjusted to pH 2 with 1.2 ml. of 6N-hydrochloric acid and kept frozen until it was used for column chromatography.

Because each of the digests contained more than 100 mg. of peptides, a preliminary crude fractionation was performed by chromatography on a column (2.1 cm.  $\times$  9.0 cm.) of Dowex 50 (2X;  $\text{Na}^+$  form) (J. T. Baker Co., Phillips

burg, N.J., U.S.A.). The peptide mixture was applied in a minimal volume at pH 2. Elution was carried out with a gradient buffer obtained by using an open mixing vessel and reservoir of identical size and shape, the one containing 500 ml. of 0.05M-citric acid plus 0.1M- $\text{Na}_2\text{SO}_4$  and the other 500 ml. of 0.1M- $\text{Na}_2\text{HPO}_4$  plus 0.02N- $\text{NaOH}$ . The eluate from each chromatogram was collected initially in 10 ml. fractions that were combined as indicated by the arrows in Fig. 2. These pooled fractions were designated according to the nature of the label and the pH of elution; thus I 2.0 indicated that peptide mixture derived by chymotrypsin treatment of [ $^{14}\text{C}$ ]isoleucine-labelled albumin that came off the column at pH 2, V 4.0 was the [ $^{14}\text{C}$ ]valine-labelled peptide mixture coming from the column at pH 4 and L 6.5 was a [ $^{14}\text{C}$ ]leucine-labelled peptide mixture eluted at pH 6.5.

Fractions I 2.0, V 2.0 and L 2.0 were deionized by passing them successively through a column (0.9 cm.  $\times$  25 cm.) of Dowex 50 ( $\text{H}^+$  form) and a column (2.2 cm.  $\times$  15 cm.) of Dowex 1 (formate form). All the other fractions were deionized by absorption on a column (0.9 cm.  $\times$  30 cm.) of Dowex 50 ( $\text{H}^+$  form), followed by washing with water and elution with 2N-ammonia containing thymol blue indicator (which facilitated the recognition of the ammonia front). All fractions were concentrated to dryness in a rotary still, taken up in 0.25 ml. of water and kept frozen. The radioactivity of these pooled fractions is shown in Table 2.

The electrophoretic separation of the peptides that is summarized in Table 3 was carried out by repeated electrophoresis. Thus fractions I 2.0, V 2.0 and L 2.0 were applied in three 7.5 cm.-long bands to the origin line of a sheet (40 cm.  $\times$  60 cm.) of Whatman no. 3MM paper. Small spots of each peptide mixture were also applied at the sides of the paper to determine whether there had been irregular movement of the peptides. The paper was then wetted with pH 3.5 buffer and subjected to ionophoresis essentially by the method of Ryle, Sanger, Smith & Kitai (1955). After ionophoresis, the paper was removed from the tank, dried in air and left overnight in contact with Kodak No-Screen X-ray film. Development of the X-ray film revealed the location of radioactive peptides. A strip of the paper, parallel to the origin, was cut out so that it included the radioactive peptide and other peptides of the same ionic mobility. The areas corresponding to the [ $^{14}\text{C}$ ]isoleucine-, [ $^{14}\text{C}$ ]valine- and [ $^{14}\text{C}$ ]leucine-labelled peptides were separately eluted with water into small test tubes and concentrated to 0.25 ml. by evaporation *in vacuo*. The paper was assayed for radioactivity before and after elution to establish that essentially all of the peptide had been eluted. The partially purified peptides were now subjected to the same treatment two or three times more, except that the electrophoreses were at different pH values, namely 3.5, 6.5, 8.9 and 2.1. Thus a [ $^{14}\text{C}$ ]isoleucine-containing peptide moved 10 cm. towards the anode at pH 3.5 in 2 hr. at 2000 v and was separated from other [ $^{14}\text{C}$ ]isoleucine-, [ $^{14}\text{C}$ ]valine- and [ $^{14}\text{C}$ ]leucine-labelled peptides. The strip of paper 10 cm. from the origin was cut, and the areas corresponding to [ $^{14}\text{C}$ ]isoleucine-, [ $^{14}\text{C}$ ]valine- and [ $^{14}\text{C}$ ]leucine-labelled albumin were separately eluted and applied to the origin line of another sheet of filter paper. Electrophoresis at 2000 v for 2 hr. at pH 6.5 moved the main radioactive band 17 cm. and separated it from a smaller amount of a slower peptide that also contained [ $^{14}\text{C}$ ]isoleucine. Finally, electrophoresis at pH 8.9 yielded an apparently homo-

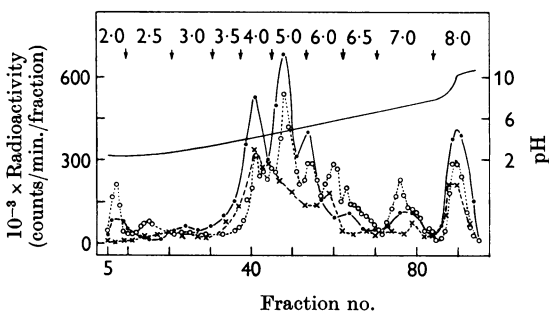


Fig. 2. Ion-exchange chromatography of the chymotryptic peptides from oxidized ovalbumin labelled with:  $\circ\circ\circ\circ\circ$ , [ $^{14}\text{C}$ ]isoleucine;  $\bullet\bullet\bullet\bullet$ , [ $^{14}\text{C}$ ]leucine;  $\times\times\times\times$ , [ $^{14}\text{C}$ ]valine. Experimental details are given in the text. The continuous line indicates the pH of the eluate. The arrows at the top separate the eluate fractions into ten pooled fractions designated by the indicated numbers according to the approximate pH of the eluate.

geneous peptide (A) that migrated 40 cm. in 2 hr. at 2000 v. Similarly, a peptide (D) was isolated that moved in 2 hr. - 3 cm. at pH 3.5, +1 cm. at pH 6.5 and +10 cm. at pH 8.9. This peptide had a substantial amount of radioactivity when derived from [<sup>14</sup>C]leucine-labelled ovalbumin and only a little radioactivity when derived from [<sup>14</sup>C]valine-labelled ovalbumin.

By this procedure we isolated micromole amounts of 16 peptides (in triplicate) from the chymotryptic digest of the three labelled ovalbumins. Samples of these were hydrolysed in 6N-hydro-

chloric acid and their amino acid compositions were determined by two-dimensional electrophoresis and chromatography. Other samples were treated with carboxypeptidase (Worthington) or trypsin (Worthington), subjected to electrophoresis and radioautographed to determine whether the peptide was sensitive to enzymic attack. These results are summarized in Table 3. About 20  $\mu$ m-moles of the [<sup>14</sup>C]isoleucine-labelled peptide A were incubated for 4 hr. at 37° with 1  $\mu$ g. of trypsin in 1% (w/v) ammonium carbonate, pH 8.2, evaporated to dry-

Table 2. Radioactivity in the fractions of chymotryptic peptides as eluted from the Dowex 50 column (Fig. 2)

Experimental details are given in the text.

Fraction	10 <sup>-3</sup> × Radioactivity (counts/min.)		
	[ <sup>14</sup> C]Isoleucine	[ <sup>14</sup> C]Valine	[ <sup>14</sup> C]Leucine
2.0	100	10	120
2.5	40	20	30
3.0	60	120	190
3.5	90	170	190
4.0	600	850	1250
5.0	1000	700	1200
6.0	700	450	1650
6.5	470	300	1050
7.0	470	300	420
8.0	400	350	700
Total	3930	3270	6800

Table 3. Properties of normal chymotryptic peptides isolated from labelled ovalbumins by repeated electrophoresis

Experimental details are given in the text.

Peptide	Approximate composition	Electrophoretic mobility (cm./hr. at 40 v/cm.)			Approx. counts/min./ $\mu$ mole in peptides derived from ovalbumin labelled with:			Mobility (cm./hr. at 40 v/cm.) of new peptides after treatment with:	
		pH 3.5	pH 6.5	pH 8.9	[ <sup>14</sup> C]Isoleucine	[ <sup>14</sup> C]Valine	[ <sup>14</sup> C]Leucine	Trypsin	Carboxypeptidase
A	Cy-SO <sub>2</sub> H, Pro, MetSO <sub>2</sub> , Ileu <sub>2</sub> , Ala	+5	+9	+20	58 000	55	80	—	+11 and 0 at pH 6.5
D	Ser, Leu <sub>2</sub> , Gly	-1	+1	+5	1 200	7 000	150 000	—	—
E	Leu, Val, Glu, MetSO <sub>2</sub> , Ala	-3	+5	+10	8 000	53 000	85 000	—	Carboxypeptidase releases free valine
H	Ala, Ser, Leu <sub>3</sub> , Asp, Lys	-5	+1	+12	17 000	15 000	200 000	-3 at pH 3.5	-3 at pH 3.5
I	Ileu <sub>2</sub> , Leu, Gly, Ala, Ser <sub>2</sub> , Glu, Lys	-3	+8	+18	125 000	10 400	106 000	+9 for [ <sup>14</sup> C]Ileu at pH 6.5; +1 for [ <sup>14</sup> C]Leu at pH 6.5	—
K	Glu, Ileu, Leu, Ala, Arg	-5	+8	+15	94 000	9 400	225 000	+5 for [ <sup>14</sup> C]Ileu at pH 6.5; +5 for [ <sup>14</sup> C]Leu at pH 6.5	—
L	Glu, Ser, Leu <sub>2</sub> , Lys	-11	0	+10	600	700	60 000	-15 at pH 3.5	—
N	Cy-SO <sub>2</sub> H, Phe, Pro, Val, Gly	-5	-2	+9	1 700	59 000	2 000	+3 for [ <sup>14</sup> C]Val at pH 3.5; -4 for [ <sup>14</sup> C]Phe at pH 3.5	—

ness and transferred to paper for electrophoresis. Another 20  $\mu\text{m}$ -moles of [ $^{14}\text{C}$ ]isoleucine-labelled peptide A were incubated with 10  $\mu\text{g}$ . of carboxypeptidase at 37° in 0.2 M-N-ethylmorpholine-acetate buffer, pH 8.4, evaporated to dryness and placed on the same sheet which contained also an untreated sample of [ $^{14}\text{C}$ ]isoleucine-labelled peptide A. After 2 hr. of electrophoresis at 2000 v in pH 6.5 buffer, the trypsin-treated sample produced a single radioactive spot with the same electrophoretic mobility as the untreated control. The carboxypeptidase-treated sample yielded a radioactive component of greater mobility than the control and a neutral radioactive spot that was very strongly ninhydrin-positive.

Each group of three peptides was then treated with the enzyme that most markedly changed the electrophoretic mobility of the main radioactive component. (Although peptide D was insensitive to either enzyme, it was treated with carboxypeptidase in the hope that contaminating peptides might be affected.) The products of this second enzymic treatment were again purified by successive electrophoresis.

When the three peptides A were treated with carboxypeptidase, they gave three amino acids (isoleucine, alanine and methionine sulphone) and three new peptides that moved +10 cm. at pH 3.5 and +22 cm. at pH 6.5 in 2 hr. at 2000 v. These peptides were hydrolysed in 250  $\mu\text{l}$ . of 6N-hydrochloric acid for 20 hr. at 110° and the hydrolysates were placed on paper. The appropriate carrier (0.2  $\mu\text{mole}$ ) of [ $^{12}\text{C}$ ]valine or [ $^{12}\text{C}$ ]leucine was added. Standards of 0.1  $\mu\text{mole}$  each of valine, isoleucine and leucine were also placed on the base-line. After 16 hr. of development with the upper phase of a

mixture of 2-methylbutan-2-ol, water and the pH 6.5 buffer used in electrophoresis (2 : 1 : 1, by vol.), the part of the paper containing the standards was developed with ninhydrin to locate the isoleucine, leucine and valine. The appropriate sections were cut out, eluted with water, dried on aluminium planchets and assayed for radioactivity.

Each sample was assayed with the low-background end-window counter for a minimum total of 2000 counts. The amino acids were transferred from the planchets with water and treated with ninhydrin to determine the total amount of amino acid. The planchets were assayed again in the same holders to establish that removal of the amino acid was quantitative. The residual radioactivity, including natural background of the apparatus, did not exceed 3.5 counts/min., and was usually about 2.0 counts/min. (also determined for 2000 counts). The observed activities varied from 7000 to 3.0 counts/min.

Typical results are shown in Table 4 for this peptide (A). Table 4 shows that  $^{14}\text{C}$  activity was present as [ $^{14}\text{C}$ ]isoleucine to the extent of 2450 counts/min./0.051  $\mu\text{mole}$  of peptide in the N-terminal moiety of peptide A derived from [ $^{14}\text{C}$ ]isoleucine-labelled ovalbumin. On the other hand,  $^{14}\text{C}$  activity was present as [ $^{14}\text{C}$ ]valine to the extent of 2.6 counts/min./0.075  $\mu\text{mole}$  of peptide (determined by isoleucine content) in the same peptide derived from [ $^{14}\text{C}$ ]valine-labelled ovalbumin, and there were 1.4 counts/min. of [ $^{14}\text{C}$ ]leucine activity/0.088  $\mu\text{mole}$  in the peptide from the [ $^{14}\text{C}$ ]leucine-labelled ovalbumin. Table 5 summarizes the corresponding results from the other peptides that were isolated and analysed. After the valine, isoleucine and leucine areas were cut out, the remainder of the

Table 4. *Distribution of radioactivity among the amino acids in the hydrolysate of the N-terminal moieties of the three peptides A derived from ovalbumin labelled with [ $^{14}\text{C}$ ]isoleucine, [ $^{14}\text{C}$ ]valine or [ $^{14}\text{C}$ ]leucine*

After isolation the peptides A were treated with carboxypeptidase. The resultant tripeptides were hydrolysed in 6N-HCl and chromatographed on paper. The amino acid content and radioactivity of the leucine, isoleucine, valine and 'blank' areas of the chromatogram were determined as described in the text.

	Peptides derived from ovalbumin labelled with:		
	[ $^{14}\text{C}$ ]Isoleucine	[ $^{14}\text{C}$ ]Valine	[ $^{14}\text{C}$ ]Leucine
Leucine band			
Net counts/min.	68	1.0	1.4
$\mu\text{mole}$ of leucine	0.0	0.0	0.20*
Isoleucine band			
Net counts/min.	2450	1.7	6.6
$\mu\text{mole}$ of isoleucine	0.051	0.075	0.088
'Blank' area between the isoleucine and valine markers			
Net counts/min.	26	1.6	0.5
$\mu\text{mole}$ of amino acid	0.0	0.0	0.0
Valine band			
Net counts/min.	18	2.6	1.4
$\mu\text{mole}$ of valine	0.01	0.19*	0.0

\* Carrier.

Table 5.  $^{14}\text{C}$  Activity in several peptides derived from [ $^{14}\text{C}$ ]isoleucine-, [ $^{14}\text{C}$ ]valine- and [ $^{14}\text{C}$ ]leucine-labelled ovalbumin found to be present as the corresponding amino acid on hydrolysis

Experimental details are given in the text and Table 4.

Peptide	Isoleucine		Valine		Leucine	
	(Counts/min./ $\mu\text{mole}$ of peptide)	(Moles/mole of peptide)	(Counts/min./ $\mu\text{mole}$ of peptide)	(Moles/mole of peptide)	(Counts/min./ $\mu\text{mole}$ of peptide)	(Moles/mole of peptide)
A (C-terminal)	47 500	0.96	24	0.006	7	0.0001
A (N-terminal)	48 000	0.98	35	0.001	16	0.0003
D	25	0.0005	1 120	0.029	129 000	2.08
H (C-terminal)	620	0.012	1 660	0.044	196 000	3.16
I <sub>1</sub>	25	0.0005	165	0.004	58 000	0.93
I <sub>2</sub>	49 000	0.98	1 080	0.028	870	0.014
K	45 000	0.90	75	0.002	65 000	1.05
L	3.6	0.00007	6	0.0002	60 000	0.97
N	18	0.0004	41 000	1.08	6	0.0001

## Composition

A	(Cy-SO <sub>3</sub> H, Ileu, Pro) (Ala, Ileu, MetSO <sub>2</sub> )
D	Leu <sub>2.0</sub> , Ala <sub>0.12</sub> , Gly <sub>0.25</sub> , Ser <sub>0.69</sub> , Thr <sub>0.20</sub> , Val <sub>0.10</sub>
H	Leu <sub>1.0</sub> , Val <sub>0.1</sub> , Ileu <sub>0.08</sub> , Ala <sub>0.11</sub> , Gly <sub>0.15</sub> , Glu <sub>0.03</sub> , Ser <sub>0.18</sub> , Thr <sub>0.03</sub> , Asp <sub>0.03</sub> , CyS <sub>0.02</sub>
I <sub>1</sub>	Leu <sub>1.0</sub> , Tyr <sub>1.0</sub> , Phe <sub>1.0</sub> plus traces of Asp, Thr, Ser, Glu, Ala, Val, Ileu
I <sub>2</sub>	Ser <sub>2.0</sub> , Glu, Gly, Ala, Ileu plus trace of valine
K	Glu, Pro, Tyr, Ileu, Leu, Arg
L	Glu, Ser, Leu, Lys (plus trace of Asp?)
N	CyS, Ser, Pro, Val

paper chromatogram was treated with ninhydrin to show that the three peptide hydrolysates were essentially identical. In addition, one of the hydrolysates was analysed by use of a Beckman Spinco Amino Acid Analyser.

## DISCUSSION

The successful elucidation of the structures of several proteins shows that one amino acid does not often substitute for another. However, this does not mean that no such errors occur. Thus, immediately after isolating the protein in question, the investigator chromatographs or recrystallizes his material repeatedly. This may remove not only impurities but proteins in which amino acid errors have occurred. Once purified, the protein is hydrolysed chemically or enzymically. The substitution of one amino acid for another may lead to substantial changes in reactivity; consequently a peptide containing an erroneous amino acid will either not be formed as fast as, or may be further hydrolysed than, the corresponding correct peptide. The peptides are then isolated and purified, usually by chromatography; again a small amount of an erroneous peptide may be lost. Finally, the isolated purified peptide is hydrolysed and its composition determined under circumstances where traces of amino acids are unlikely to be recorded or where they will be ignored if they are recorded.

Expecting that the incidence of errors would be low, I relied therefore on highly radioactive amino

acids to show their presence when colorimetric techniques would have failed. The amino acids were incorporated into chicken ovalbumin under conditions not far different from those *in vivo*. The ovalbumin was isolated by a method that probably would not distinguish between homologous ovalbumins (i.e. differing by no more than the substitution of a valine for an isoleucine residue). The enzymic hydrolysis was catalysed by chymotrypsin because there is no evidence that the enzyme would distinguish between a valine peptide and the corresponding isoleucine peptide, although it might behave differently towards a leucine peptide. The very short column of Dowex 50 used for partially fractionating the peptides probably would not have separated closely related peptides, and the repeated electrophoresis would not be expected to distinguish between peptides of identical charge differing by not more than 14 molecular weight units (see, for example, Naughton, Sanger, Hartley & Shaw, 1960). Thus, if valine had substituted for part of the isoleucine at a particular locus in ovalbumin, e.g. in peptide A, the hexapeptides containing isoleucine and valine might be produced by chymotryptic hydrolysis in proportion to their original amounts. The valine content can then be determined by dividing the radioactivity due to [ $^{14}\text{C}$ ]valine by the specific radioactivity of valine, which is estimated from the total valine radioactivity of the ovalbumin and the number of valine, residues or from the specific radioactivity of a valine-containing peptide such as peptide N. De-

spite the initial purity of the  $^{14}\text{C}$ -labelled amino acids, metabolic conversion into other amino acids could have occurred, so it was necessary to establish that the radioactivity measured was actually in the form of isoleucine, valine or leucine. The paper-chromatographic system used for isolating these amino acids from the hydrochloric acid hydrolysate separates these three amino acids cleanly from each other and from all other amino acids that could be present in the digest. Hence one can be confident that the radioactivities reported in Table 5 are probably present as the indicated amino acid. Carrier amino acids were used during the chromatography and radioassay to establish whether the yield was nearly quantitative and to insure against loss by sublimation of amino acids that otherwise would have been present in micromicrogram amounts.

The question of whether the electrophoretic technique yields pure peptides is partly answered by the results. Five of the peptides ( $A_C$ ,  $A_N$ , K, L, N) were isolated in analytically pure condition, free from any discernible trace of other amino acid or peptide impurity. The analyses showed each component amino acid to be present at 1 mole/mole of peptide within 10%, except for serine, which was a little lower. These are the most acidic or basic peptides that were closely investigated. The hydrolysates of the other four peptides in Table 5 all showed fractional amounts of one or more amino acids, from which it is concluded that purification had not been complete. Less weight should be given to the results from these latter peptides. There were in addition at least 20 other peptides whose isolation was begun by electrophoresis. These did not run well on paper electrophoresis and could not be adequately purified in the time available.

Among the five peptides that appear to be purest, the  $^{14}\text{C}$ -labelled amino acids are present either at 1 mole/mole of peptide or at values ranging between 2000 and 70  $\mu\text{moles/mole}$  of peptide with a median figure of 0.3 m-mole/mole. Though the experiments involve several assumptions on the possibility of inadvertent separation of the genetically correct peptide from its incorrect homologue, it seems unlikely that the assumptions would prove false in every case where I have succeeded in isolating an analytically pure peptide. Thus these experimentally observed ratios are probably not lower than the true ratio of errors in the original albumin. We have no assurance that all traces of unrelated contaminating peptides have been removed from the peptides, nor that the trace amino acid replaces only one of the several amino acids of the peptide. Thus in peptide A valine may have substituted equally for cysteine, methionine, isoleucine and alanine, so that the error ratio valine : isoleucine is nearer to 0.0003 than to 0.001. Hence

it is most appropriate to regard the observed ratios as maximum estimates of the error of protein biosynthesis. These estimates may be reduced as the peptides are more rigorously purified.

The other peptides of Table 5 are not analytically pure and are included only for completeness. It could be argued that these results are evidence for errors ranging from 0.05 to 4.4%. In some cases a somatic mutation may have occurred and some of the oviduct cells may be making a mutant ovalbumin. It is, however, most probable that the purification of the peptides is inadequate. For instance, peptide I may be largely free of all unrelated isoleucine peptides while it remains contaminated with a small amount of a valine peptide. In several cases the contamination as measured by radioactivity is less than found in the amino acid analysis for the hydrolysate of the most radioactive peptide (i.e. [ $^{14}\text{C}$ ]leucine-labelled peptide H). Presumably this indicates a slight variation in the extent of purification of the three differently labelled peptides.

The uncertainty in these estimates could be eliminated by establishing the validity of my assumptions and by taking greater pains to purify the peptides. This kind of study could also be extended to other proteins and other amino acids. None the less, I consider that there is evidence that closely related amino acids do not substitute for each other more often than once in 3000 times, perhaps much less frequently. This differs substantially from the estimate of 1:20 based on physicochemical calculations (Pauling, 1958).

The adaptor hypothesis of s-RNA function requires that, once an amino acid is attached to its specific s-RNA, the further reactions of the amino acid will be determined by the base-pairing properties of the s-RNA and not by the structure of the amino acid. To the extent that polymerization of amino acids on synthetic uridylic acid-guanilyc acid polymers resembles protein synthesis, this idea has experimental support from the work of Chapeville *et al.* (1962). The present work, which shows that the frequency of errors in overall ovalbumin synthesis may be of the same order as the frequency of errors in the attachment of amino acids to s-RNA, is also consistent with this adaptor hypothesis; but, since the determination of errors in each of these two steps yields only a maximum error level, one cannot say definitely whether one or the other is higher. Hence it is not certain that steps (3) and (4) involve no discrimination based on the amino acid side chain.

If indeed the reactions subsequent to the formation of the amino acyl s-RNA are insensitive to the side-chain character of the amino acid, it follows that the frequency of error cannot be lower than the frequency with which the wrong adaptor



s-RNA is bound to the protein-coding template. It is generally believed that this template or 'messenger' contains sequences of nucleic acid bases that specifically 'code' for a particular amino acid, most probably, three bases to one amino acid (Crick, Barnett, Brenner & Watts-Tobin, 1961). It is presumed that the code is 'read' by a particular portion of the s-RNA molecule which possesses the complementary bases in correct sequence. Because of the limited number of base combinations and the possible degeneracy of the code, some of the code sequences for different amino acids will be identical except for a single base. Thus both Wittmann (1961) and Speyer, Lengyel, Basilo & Ochoa (1962) propose that the code for isoleucine is UUA, UAU or AUU, and that the code for valine is identical except for the substitution of a guanine for an adenine. Therefore, the adaptor s-RNA of isoleucine, which by inference might possess the code-reading sequence AAU, will fit the UUG valine message except for the poor fit between a uracil and a guanine. As shown in Fig. 3, one hydrogen bond can surely form between these two bases and quite possibly two or three bonds can form without distorting the steric configurations of the complementary ribotides (Lawley & Brookes, 1962). Neither of the indicated forms of uracil predominates

under physiological conditions, the ionic form being about 0.15% (Lawley & Brookes, 1962) and the enolic form somewhat more. To this extent at least, uracil can behave like cytosine in hydrogen-bonding to guanine. Thus the force that opposes fitting the wrong s-RNA to a given position on the template is the lowered binding energy resulting from the formation of one or possibly two fewer hydrogen bonds. [Similar considerations apply to the enzyme-catalysed replication of deoxyadenylic acid-thymidylic acid polymer (Trautner, Swartz & Kornberg, 1962): guanine was observed to pair with thymine less often than 1:28 000 and with bromouracil less often than 1:2000.] In water one hydrogen bond may have an energy about 1.5 kcal. (Schellman, 1955).

Can the ability or inability to form one hydrogen bond result in discrimination of the order reported in this research? By assuming (as the triplet-code adaptor theory does) that all other factors are equal, the difference between binding an isoleucyl-s-RNA or a valyl-s-RNA to a valine site on the template must determine the frequency of error according to the equation:

$$\Delta(\Delta F) = \Delta F_{\text{Ileu}} - \Delta F_{\text{Val}} = -RT \ln \frac{K_{\text{Ileu}}}{K_{\text{Val}}}$$

where  $K_{\text{Val}}$  and  $K_{\text{Ileu}}$  are the binding constants of the corresponding s-RNA molecules with the valine template. From the present results  $K_{\text{Val}}/K_{\text{Ileu}}$  appears to be about 3000, perhaps larger. Hence  $\Delta(\Delta F)$  equals approx. 5.0 kcal., substantially more than would be expected from the formation of one or two hydrogen bonds in water.

There are many other pairs of triplet codes where the distinction is presumed to lie entirely in the replacement of a single cytosine by a uracil or of an adenine by a guanine (e.g. all the nitrous acid tobacco-mosaic-virus mutants described by Wittmann, 1961). In every such case the triplet-code adaptor hypothesis would require that selection between the amino acids depends on the difference in hydrogen-bonding energy between adenine and guanine or uracil and guanine, and adenine and uracil or guanine and uracil etc. In other cases, such as in the potential competition between isoleucine and leucine, the codes would differ much more, thus UUA for isoleucine and UAU or AUU for leucine. In such cases, failure to form several hydrogen bonds and steric repulsion might combine to exclude the wrong s-RNA.

The energy of a hydrogen bond between bases in water is not accurately known. None the less, this argument raises doubts whether hydrogen-bond formation can account for the specificity of protein synthesis—or equally for the specificity of nucleic acid replication. Several possibilities present themselves. There is as yet no experimental

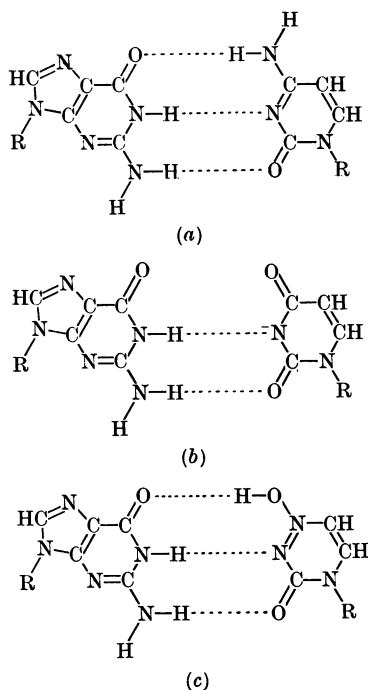


Fig. 3. Possible hydrogen bonding (a) between guanine and cytosine, (b) between guanine and ionized uracil (Lawley & Brookes, 1962) and (c) between guanine and enolized uracil.

proof that cytosine-guanine or adenine-uracil interactions are as specific as our calculations would require. Extension of work by Randerath & Weimann (1963), Gilham (1962) and Adler & Rich (1962) may permit a determination of  $\Delta(\Delta F)$ . If this quantity is greater than 5 kcal., it may be that bonding between adenine and uracil (or thymine), and between cytosine and guanine, involves forces other than hydrogen bonds, i.e. that highly specific intermolecular orbitals are formed. Also more than three bases may be used to 'code' for a single amino acid: thus, if nine bases are used, the distinction between the valine code and the isoleucine code will be the difference between three adenines and three guanines. Finally, the template may be 'read' by one of several enzymes, each of which in turn is able to select specifically the appropriate amino acyl s-RNA. Alternatively, one enzyme may 'read' a given base sequence and be altered in its conformation so as to select only a particular s-RNA (i.e. an allosteric enzyme as postulated by J. Monod, personal communication). In these latter cases there is no necessary complementary relationship between the template code and the base sequence of the corresponding s-RNA.

The polynucleotide-catalysed polymerization of amino acids that has been used to assign nucleotide code words for the recognition of amino acids has yielded the surprising conclusion that poly(uridylic acid) codes, not only for phenylalanine, but also to a limited extent for leucine (Jones & Nirenberg, 1962). From this one might expect occasional leucine-phenylalanine errors. The present research was carried out with [ $^{14}\text{C}$ ]phenylalanine and [ $^{14}\text{C}$ ]tyrosine, as well as with the other three amino acids. Although the purification of the [ $^{14}\text{C}$ ]phenylalanine- and [ $^{14}\text{C}$ ]tyrosine-labelled peptides was not carried as far as those labelled with [ $^{14}\text{C}$ ]valine, [ $^{14}\text{C}$ ]isoleucine and [ $^{14}\text{C}$ ]leucine, and although there is a greater chance of inadvertent loss of erroneous peptides, no more than 2% of isoleucine or leucine was found in the phenylalanine moiety of peptide N and no more than 3% of phenylalanine was found in the leucine or isoleucine regions of peptides A, D, E, H, I, K or L; in most cases the amounts were less than 1%. It seems likely that phenylalanine-leucine errors are as rare as valine-isoleucine errors.

#### SUMMARY

1. Surviving hen oviduct was incubated with [ $^{14}\text{C}$ ]leucine, [ $^{14}\text{C}$ ]isoleucine or [ $^{14}\text{C}$ ]valine.

2. From these preparations three differently  $^{14}\text{C}$ -labelled ovalbumins were isolated, purified, oxidized and enzymically hydrolysed.

3. The resulting peptides were purified by ionophoresis techniques that should not have separated homologous peptides differing only by the sub-

stitution of a valine or leucine for an isoleucine residue.

4. By use of the radioactivity it was shown that these amino acids do not substitute for each other in ovalbumin biosynthesis more often than once in 3000 times.

5. This degree of discrimination cannot be explained by the ability or inability of a particular s-RNA to form one or two particular hydrogen bonds with the bases of the template code. Other more substantial and specific bonds are necessary, or an enzyme is required as an intermediate in the 'reading' process.

6. Tentative results suggest that confusion between leucine and phenylalanine is also very rare.

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## Nicotinamide Nucleotide Coenzymes and Glucose Metabolism in the Livers of Foetal and Newborn Lambs

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Many physiological adjustments take place in the newly born animal. Some of these are associated with cardiovascular changes (Dawes, 1961; Mott, 1961), and others can be more closely related to metabolism. During the later part of foetal development, the glycogen content of the liver rises to high levels and falls rapidly immediately after birth in many animals (Shelley, 1960). Similar changes occur in the lipid content of the liver of the guinea pig in the perinatal period (Raiha, 1961). Dawkins (1959) has shown that respiratory enzymes in the liver of the newborn rat increased in activity in the first few days after birth. In the prenatal guinea pig, activities of glucuronyltransferase, tryptophan pyrrolase and glucose 6-phosphatase are low, and rise toward adult values soon after birth, whereas that of glucose 6-phosphate dehydrogenase shows a gradual decrease and that of 6-phosphogluconate dehydrogenase shows little change during development (Nemeth & Dickerman, 1960). These changes in relative enzymic activities and in carbohydrate and lipid contents

reflect the changes in functional activities of the liver after birth (see Villee, 1961).

Caiger, Morton, Filsell & Jarrett (1962) have shown that during development of the rat and the sheep the concentration of total nicotinamide nucleotide coenzymes in the liver increased and reached a maximum value as growth ceased. The changes in concentrations of coenzymes and the relative stage of development of the animal were assessed by using a number of parameters including liver wet wt., liver dry wt. and number of nuclei/liver.

In the studies of these coenzymes (expressed as amount/g. fresh wt.) in the livers of foetal, newborn and adult guinea pigs (Nemeth & Dickerman, 1960; Raiha, 1961) and of rabbits (Roux, Gordon, Dinnerstein & Romney, 1962), similar trends in relation to age were observed.

The present paper describes relationships of nicotinamide nucleotide coenzymes to growth and metabolism, and includes measurements on livers of foetal and newborn lambs as well as on older