Nonuniform Labeling of Egg White Lysozyme

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The incubation of minced hen's oviduct with C^{14}-labeled L-arginine and with H^3-labeled D,L-leucine 4:5-T led to the formation of radioactive lysozyme which was isolated and purified by chromatography on carboxymethylcellulose columns. The distribution of radioactivity in the leucine residues along the polypeptide chain of the reduced, carboxymethylated protein was determined by isolation of leucine-containing peptides produced by trypsin digestion, followed by hydrolysis and quantitative estimation of the specific radioactivities of individual leucine residues. Samples of radioactive lysozyme isolated from oviduct minces that had been incubated for 3 and 5 minutes, showed unequal labeling of leucine residues, and the relative specific radioactivities were found to increase from the NH_2-terminal to the COOH-terminal end of the chain. Longer incubations of 10 and 40 minutes led to uniform labeling. These data are consistent with a model of amino acid assembly in which the polypeptide chain grows unidirectionally, initiated at the NH_2-terminal end and terminated at the COOH-terminal end.

The concept that radioactive amino acids, when added to a protein synthesizing system, might be unequally distributed within a newly synthesized polypeptide chain was first documented with the demonstration of nonuniform labeling of newly synthesized ovalbumin in minced hen's oviduct (Anfinsen and Steinberg, 1951; Steinberg and Anfinsen, 1952; Flavin and Anfinsen, 1954). Various models to explain this phenomenon were suggested (Dalgliesh, 1953; Steinberg *et al.*, 1956), but decisive evidence in support of any particular model did not become available until recently. Thus, studies by Bishop *et al.* (1960), have demonstrated that, in a cell-free hemoglobin-synthesizing system, the NH_2-terminal valine residues show lower than average specific activities shortly after a radioactive pulse. In this study it was also shown that the NH_2-terminal valine residues had higher specific activities when ribosomes that had been preincubated with C^{14}-valine were allowed to complete the hemoglobin chain in the presence of nonradioactive valine. These combined experiments were taken in support of a model for sequential growth of newly synthesized polypeptide chains initiated at the NH_2-terminal residue. Extensive data on the rabbit reticulocyte system, which are in complete accord with the results of Bishop *et al.*, have been reported by Naughton and Dintzis (1962). The model is best tested by examining the specific activities of amino acids at different positions within a polypeptide chain whose entire amino acid sequence is known. This communication reports such a study for newly synthesized egg white lysozyme. A preliminary report of these experiments, which were performed in conjunction with the determination of the amino acid sequence of this protein, has appeared.[1]

MATERIALS AND METHODS

Isotopes and Materials.—L-Arginine HCl-Guanido-C^{14}, Lot 106797, with a specific activity of 5 mc/mmole was obtained from the California Corporation for Biochemical Research. Tritiated DL-Leucine 4:5-T, Tra. 75, batch 1, containing 5000 mc/mmole was obtained from the Nuclear Chicago Corporation. The oviducts used in these experiments were obtained from laying, white leghorn hens. The carboxymethylcellulose was obtained from H. Reeve-Angel Company, Bridewell, New Jersey.

Oviduct Mince and Incubation.—Oviducts were removed from laying hens immediately after sacrifice, chilled, and minced in the buffer described below. All incubations were in buffer of the following composition; 0.072 M KCl, 0.032 M NaCl, 0.04 M $NaHCO_3$, 0.2% glucose. This mixture was equilibrated with 5% CO_2–95% oxygen. The buffer also contained 0.03% CoA. The CoA was added on the basis of a report that it enhanced protein synthesis in oviduct (Hendler, 1957). Approximately 1.5 ml of buffer was used per g of oviduct tissue during the incubations, and the flasks were continuously flushed with 5% CO_2–95% O_2. Each incubation will be separately described.

Lysozyme Isolation.—At the completion of the experiment the oviduct was chilled in ice-acetone and immediately homogenized in a Potter-Elvehjem homogenizer. The chilled homogenate was further treated in a Waring Blendor for two periods of 1 minute each. The homogenate was then centrifuged in a Spinco Model L preparative ultracentrifuge at 78,000 × *g* for 1 hour. Nonradioactive carrier lysozyme was added (approximately 150 mg) to the supernatant fraction which was poured over a carboxymethylcellulose column (2.4 × 25 cm) previously equilibrated with 0.05 M NH_4HCO_3. This exchanger was chosen on the basis of a report of the favorable chromatographic behavior of egg white proteins on carboxymethylcellulose (Rhodes *et al.*, 1958). In the present experiments an ammonium carbonate system was chosen because the salt is volatile. The column was washed with approximately 8 volumes of 0.05 M NH_4HCO_3 and a two-chamber Varigrad buffer reservoir was then used for lysozyme elution. The first chamber contained 1000 ml of 0.05 M ammonium bicarbonate and the second an equal volume of 0.5 M $(NH_4)_2CO_3$. All chromatography was carried out at 4°. Figure 1 depicts the results for such a chromatographic run for the purification of C^{14}-arginine-labeled lysozyme. The values for lysozyme activity were calculated by dividing the arbitrary figure 100 by the time in minutes required for a fall in optical density of *Micrococcus lysodeikticus* suspension from 0.9 to 0.7 OD at 570 mμ (Boasson, 1938; Smolelis and Hartsell, 1949). Aliquots of 50 μl of effluent fractions were counted in planchets on a

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[1] A preliminary report of this work has appeared. Abstracts of the Biophysical Society Meeting, Washington, D.C., February, 1962.

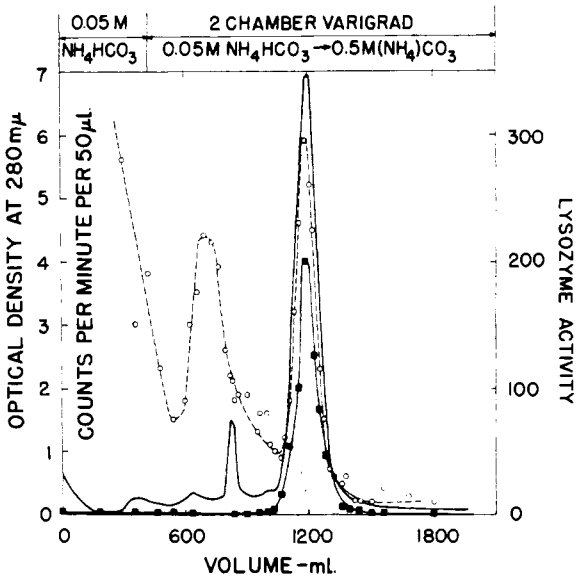

FIG. 1.—Elution of radioactive and carrier lysozyme from a column of carboxymethylcellulose. The solid line depicts optical density at 280 mμ, the dashed line and open circles represent radioactivity, and enzymic activity is represented by the solid squares and stippled area (see text). The radioactivity values are corrected for a background of 1.5 cpm.

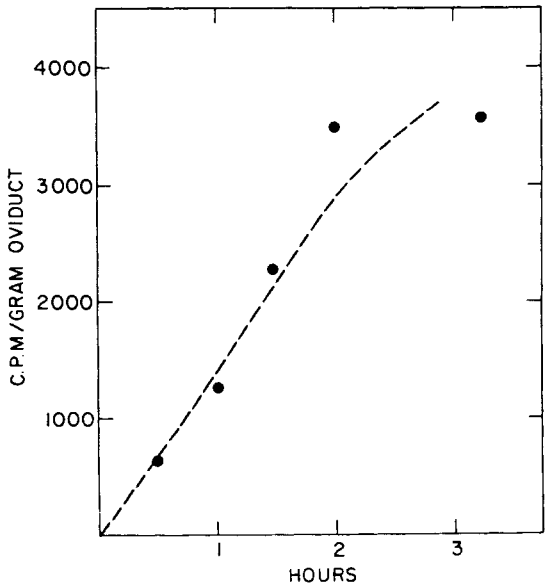

FIG. 2.—Incorporation of C^{14}-arginine into lysozyme following varying periods of incubation of minced oviduct.

Nuclear Chicago low background counter which had an average background of 1.5 cpm. Tubes containing lysozyme were pooled and lyophilized.

Peptide Isolation.—The lyophilized lysozyme was reduced with 2-mercaptoethanol in 10 M urea and subsequently alkylated with iodoacetic acid (Anfinsen and Haber, 1961; Canfield and Anfinsen, 1963). The carboxymethylated product was digested with 2% (w/w) trypsin at 37° for 1.5 hours as in earlier experiments (Canfield and Anfinsen, 1963). Following digestion and lyophilization, the tryptic peptides in experiment 4 were separated on a 2.0 × 150-cm column of Dowex 50 × 4. (BioRad Laboratories, AG50WX4, Richmond, California), using a pyridine acetate gradient system (Canfield, 1963a). In experiments 2 and 3, the peptides were separated on a column of phosphocellulose (Canfield and Anfinsen, 1963). Since some of the peaks contained several peptides, further purification was achieved by strip electrophoresis at *p*H 3.6 and the purified peptides were eluted from the paper.

Determination of Specific Radioactivities.—The isolated peptides, which contained either C^{14}-arginine or tritiated leucine, were hydrolyzed in constant boiling HCl in evacuated and sealed tubes for 24 hours at 110°. Following evaporation of the HCl, the samples were dissolved in 0.001 N HCl. Carefully measured aliquots were taken for (1) amino acid analysis on a Spinco Model 120 amino acid analyzer, and (2) determination of radioactivity in Bray's solution (Bray, 1960) using a Packard Tricarb scintillation counter. After determination of a number of radioactive disintegrations sufficient to make the standard deviation of counting less than 2%, equal aliquots of an internal standard were added to each sample of a given experiment, so that a correction for quenching in each sample could be applied. Several standard amino acid mixtures were run on the amino acid analyzer using the same batch of ninhydrin that was used for the analysis of the peptides, and the experimental analyses were corrected for the usual slight decay in ninhydrin color value. The amino acid analysis also served to confirm the identification and purity of the peptides. Each peptide reported here was found to be over 90% pure.

The ratio of observed disintegrations to micromoles of amino acid, corrected for the relative size of the aliquots taken, yielded the estimates of specific radioactivities reported here for the leucine or arginine residues in each peptide.

Experiment 1. Determination of Incorporation of C^{14}-Arginine into Lysozyme.—Five aliquots of an oviduct mince of approximately 6 g each were weighed in tared flasks, suspended in buffer, equilibrated with 5% CO_2–95% O_2, and 10 μc of C^{14}-arginine was added to each flask. The flasks were incubated at 37° for varying periods of time following which the tissue was homogenized and the lysozyme recovered as outlined above. In each case a solution containing 15 mg of nonradioactive carrier lysozyme was added to the supernatant and, following isolation of the lysozyme using carboxymethylcellulose columns (1.0 × 15 cm), the number of counts per minute per gram of oviduct incorporated into recoverable lysozyme was determined.

Experiment 2.—One hundred fifty μc of C^{14}-arginine was added to 70 g of minced oviduct and the suspension was incubated with shaking at 37° for 40 minutes. The lysozyme was isolated and the tryptic peptides were separated on phosphocellulose as outlined above.

Experiment 3.—Twenty-five mc of H^3-leucine was added to 24 g of chilled, minced hen's oviduct, and the flask was immediately shaken in a 37° water bath. After a total incubation time of 5 minutes, the flask was quickly chilled in ice-acetone. Just prior to homogenization the chilled mince was centrifuged in the cold for 1 minute and the supernatant was added to a second batch of 51 g of chilled, minced oviduct. This second batch of oviduct tissue was shaken for 5 minutes, chilled, homogenized, and pooled with the homogenate from the first batch. The double incubation was carried out in this fashion so that a larger quantity of oviduct might be exposed to high levels of isotope under similar conditions. The remainder of the experiment was carried out as outlined above. The tryptic peptides were separated on phosphocellulose and further purified by paper electrophoresis.

Experiment 4.—Twenty-five mc of H^3-leucine was

TABLE I

SPECIFIC RADIOACTIVITIES OF INDIVIDUAL LEUCINE RESIDUES IN THE POLYPEPTIDE CHAIN OF NEWLY SYNTHESIZED EGG WHITE LYSOZYME

Peptide	Amino Acid (μmoles)	cpm/μmole	Peptide	Amino Acid (μmoles)	cpm/μmole	Rel.[a] Sp. Act.
Experiment 2 (40 minutes)			Experiment 4 (3 minutes)			
Protein	1.08	38	T-(3 + 4)	0.71	36	1.00
T-(1 + 2)	1.55	40	T-5	0.49	34	0.96
T-5	0.68	33				
T-9	1.72	34	T-8	0.08	42	1.17
T-10	0.65	39	T-11	0.74	51	1.43
T-14	1.13	36	T-(17 + 18)	1.58	76	2.13
T-(17 + 18)	1.23	39				
Experiment 4 (10 minutes)			Experiment 3 (5 minutes)			
Protein	0.30	508	Protein	1.79	336	
T-(3 + 4)	0.67	441	T-3	0.74	116	1.00
T-5	1.05	405	T-5	2.8	133	1.15
T-6	0.79	443	T-6	0.34	134	1.15
T-8	0.22	498	T-11	0.51	234	2.00
T-11	0.89	468	T-(17 + 18)	2.1	343	2.94
T-(17 + 18)	0.66	440				

[a]The specific radioactivities of H^3-leucine residues relative to the specific radioactivity of the leucine residue in peptide-T-3 are plotted as a function of their relative positions in lysozyme. Peptide T-(3 + 4) differs from peptide T-3, (Cys-Glu-Leu-Ala-Ala-Ala-Met-Lys), by the presence of an arginine residue following the COOH-terminal lysine residue of T-3.

added to 79 g of minced hen's oviduct that had been equilibrated with 95% O_2–5% CO_2 at 37° (in contrast to experiment 3 in which the oviduct tissue was chilled at the time of isotope addition). The incubation was stopped after 3 minutes, and the flask was chilled in ice and acetone and centrifuged for 1 minute as before. While the oviduct tissue was being homogenized, the supernatant (enriched with 5 mc of H^3 leucine) was added to 101 g of oviduct at 37° and incubated with shaking and CO_2–O_2 equilibration for 10 minutes. The lysozyme was isolated as above. In this instance the tryptic peptides were separated on a Dowex 50 × 4 column (Canfield, 1963a).

RESULTS

Figure 1 illustrates the purification of the lysozyme labeled with C^{14}-arginine that was produced in experiment 2 (see Methods). Almost all the incorporated amino acids, as well as most of the proteins of the oviduct tissue, washed through the column or appeared in the first column volume. However, approximately 6 volumes of starting buffer are necessary to eliminate significant levels of radioactivity from the effluent. Shortly after the two-chamber Varigrad was started, a distinct peak of radioactivity emerged which was unassociated with a corresponding peak of optical density at 280 mμ. The identity of this peak is unknown, but the work of Rhodes *et al.* (1958) has demonstrated that several proteins synthesized by oviduct tissue emerge from columns of carboxymethylcellulose near the lysozyme region. This early peak is followed by the carrier lysozyme which is characterized by corresponding patterns of optical density, enzymic activity, and radioactivity, the latter representing the newly synthesized lysozyme.

Figure 2 presents the results of experiment 1 which demonstrate that increasing amounts of radioactivity can be recovered from the lysozyme of oviduct tissue for at least 2 hours after the addition of the labeled arginine.

The specific activities of the labeled amino acids in each isolated peptide are listed in Table 1. These tryptic peptides are identified by a number which represents their sequential position in the polypeptide chain of egg white lysozyme (Canfield, 1963b). The table summarizing the results of experiment 2 lists specific radioactivities of C^{14}-arginine values while those for experiments 3 and 4 list values for H^3-leucine. As noted earlier (Canfield and Anfinsen, 1963), phosphocellulose chromatography does not provide good resolution and yields of all the tryptic peptides of reduced, carboxymethylated lysozyme, and, in this instance, only six of the eleven arginine containing peptides were recovered in sufficient yield for analysis. These six values, however, are well distributed throughout the polypeptide chain. The samples were counted for a sufficient length of time to reduce the standard deviation of counting to less than 2% for each sample except for peptide T-8 in the 3-minute H^3 incubation. This latter peptide was low in quantity and radioactivity, and the calculated standard deviation of counting was ±14%. It would be misleading, however, to imply that the standard deviation of counting represented the accuracy of the value reported for specific activity as there are other factors that could introduce error. The reproducibility of amino acid analysis by the technique used here has been shown to be ±3% (Spackman *et al.*, 1958). Incomplete acid hydrolysis of a peptide may result in low amino acid yields, but under the conditions used here the peptides studied appeared to have been completely hydrolyzed as judged from the excellent stoichiometry of the analytical data. Materials that contaminate the pyridine acetate or are eluted from papers, as well as the colored material which results from acid hydrolysis of a tryptophan containing peptide, may all contribute variable degrees of quenching in scintillation counting. This error was minimized by the addition of internal standards to each sample to determine an appropriate correction factor for quenching, and all the values in Table I represent specific activities corrected in this way.

Table I also lists the specific activities of the labeled amino acids for samples of whole protein prior to digestion with trypsin. The values for the H^3-leucine experiments were determined for samples of lysozyme prior to reduction and alkylation, and they are unexpectedly high. This may represent contamination

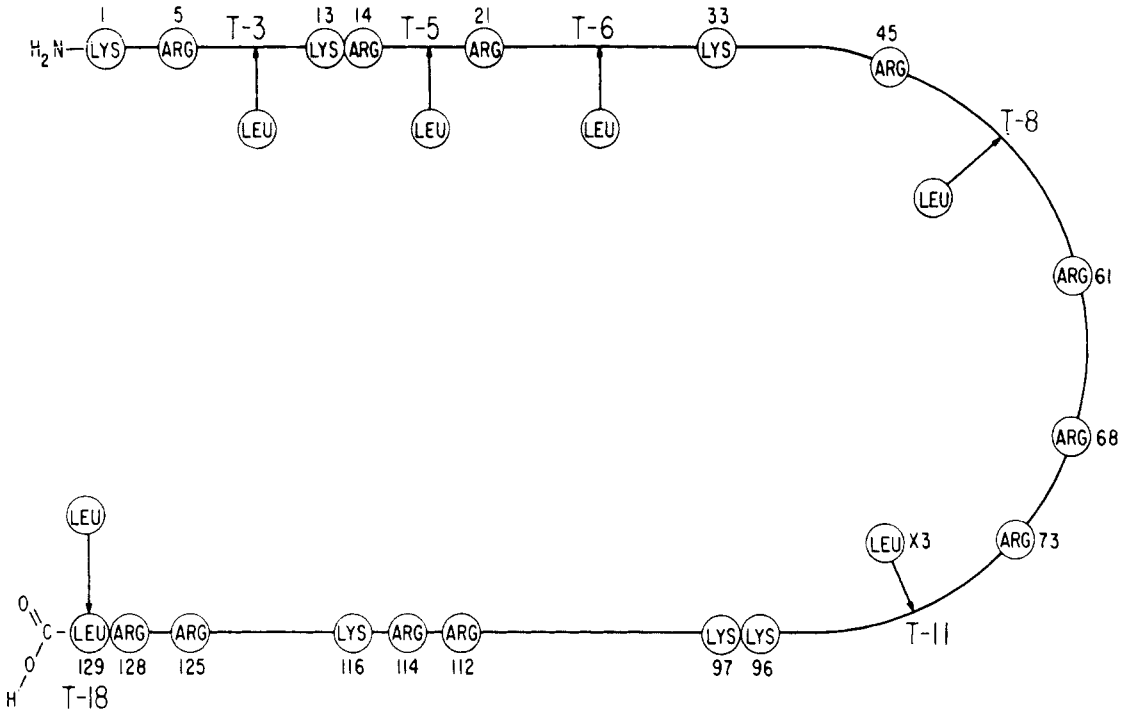

FIG. 3.—The distribution of leucine residues in the sequence of tryptic peptides which constitute the single polypeptide chain of egg white lysozyme. The position of each lysine and arginine residue is numbered.

with a minute amount of the unincorporated H^3-leucine which trails off the column. It could also be due to contamination with another protein. The protein sample from the C^{14}-arginine experiment was taken following reduction and alkylation of the lysozyme, and the specific activity compares favorably with that of the individual peptides. The rest of the values are for peptides that were isolated by column chromatography and a second purification by electrophoresis. The specific activities of these peptides are almost certainly free of error due to radioactive contamination.

Figure 3 depicts the order of tryptic peptides in lysozyme (Canfield, 1963b). The peptides containing leucine are identified, and the arginine residues are, by definition, the COOH-terminal residues of the tryptic peptides in which they reside. T-11 contains three leucine residues, and since all measurements are expressed as specific, rather than total, radioactivity, the three have been treated as though they were one leucine residing in the middle of the peptide.

Figure 4 shows plots of the specific activities listed in Table I, relative to the specific radioactivity of the leucine residue in peptide T-3, as a function of the relative position of each of the amino acids as they exist in lysozyme (Figure 3).

DISCUSSION

The potential usefulness of nonuniform labeling data for understanding the process of amino acid assembly in protein synthesis has been discussed, and several possible model systems have been suggested (Dalgliesh, 1953; Steinberg *et al.*, 1956; Naughton and Dintzis, 1962; Yoshida and Tobita, 1960; Dintzis, 1961). The emerging knowledge of the amino acid sequence of egg white lysozyme provided a further opportunity to test some of these models particularly since studies on the incorporation of amino acids into oviduct protein have demonstrated that this tissue can be useful for studies of protein synthesis (Steinberg *et al.*, 1956; Hendler, 1961).

The particular model tested in these experiments is shown in Figure 5. It depicts four theoretical templates for the synthesis of lysozyme, and these are meant to be representative of the many templates that are carrying out this synthesis at any one time. The positions "L" represent regions of the template coded for leucine in the polypeptide chain. There are two essential assumptions in this model. The first is that the amino acid assembly process is initiated at one end of the polypeptide chain with continuous growth to the other end (in Figure 5, the stippled blocks above the templates represent growing polypeptide chains initiated at the left). The second assumption is that the synthesis is not synchronous; that is, at any given time one template may be initiating chain growth while another is completing it and a third may have only half completed its chain. The model depicts four representative polypeptide chains at various stages of completion at a time, t_0, when radioactive leucine is introduced. Template 1 has not yet incorporated a leucine residue and H^3-leucine will thus have access to all the "L" positions (positions to be filled by H^3-leucine are represented by black boxes). In contrast, template 4, at time t_0, has almost completed its chain, so that only one position is left to be filled from the H^3-leucine pool. By the same reasoning, templates 2 and 3, when they are completed, will have H^3-leucine more heavily incorporated at the right ends of the chains. If, at the end of approximately one cycle of protein synthesis, the newly synthesized polypeptide chains are isolated and each individual leucine position is examined for its specific activity, the distribution of radioactivity shown at the bottom of Figure 5 would be found. The specific activity would be relatively low in positions near the end that initiated growth and would continuously increase toward the end that terminates growth. The same phenomenon might be envisaged to persist

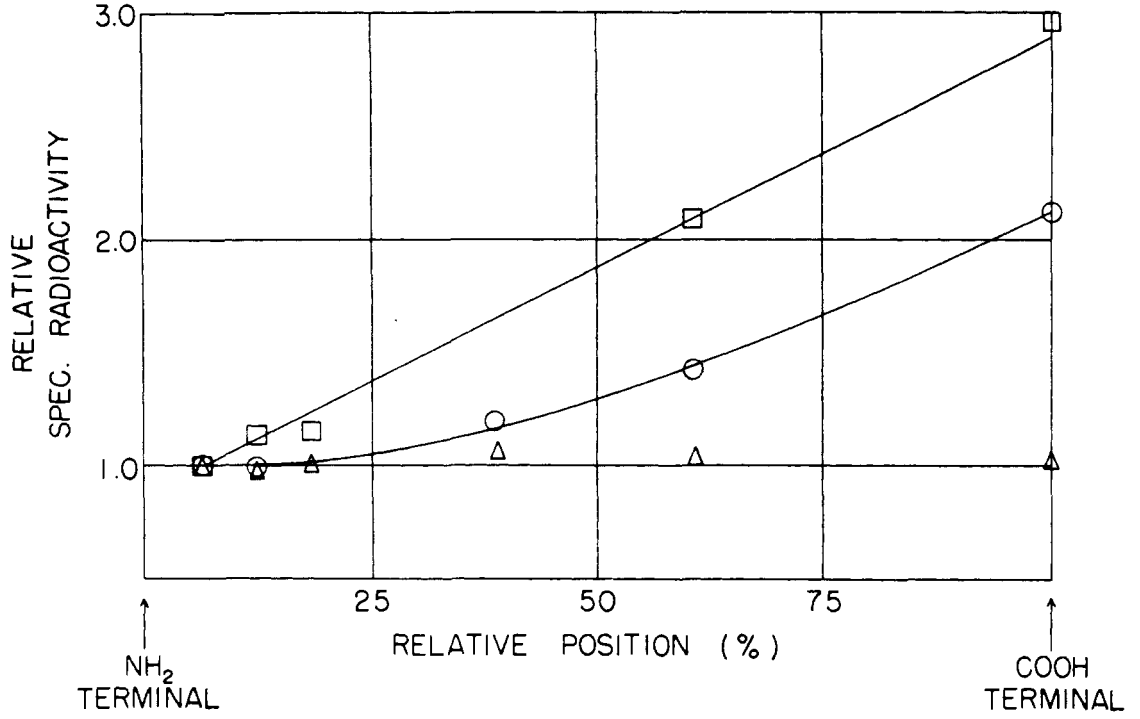

FIG. 4.—The specific radioactivities of H^3-leucine residues relative to the specific radioactivity of the leucine residue in peptide T-3 (see Table I), plotted as a function of their relative positions in lysozyme: circles, 3-minute incubation (experiment 4); squares, 5-minute incubation (experiment 3); triangles, 10-minute incubation (experiment 4).

through several cycles of protein synthesis if the specific activity of the pool were rising at a very rapid rate.

The data plotted in Figure 4 illustrate that this situation exists during lysozyme synthesis. In both the 3-minute and the 5-minute incubations, the relative specific activities are lowest for those leucines near the amino end and rise to a highest value at the carboxy terminal leucine residue. At later times, when several cycles of protein synthesis have been completed, significant amounts of radioactivity should appear in all positions of the polypeptide chain. It is consistent with the model that the 10-minute and 40-minute incubations lead to relatively uniformly labeled lysozyme.

It was not convenient to design these experiments to compare absolute radioactivities. Furthermore, experience has shown that oviducts may vary considerably from one to another and it is reasonable to as-

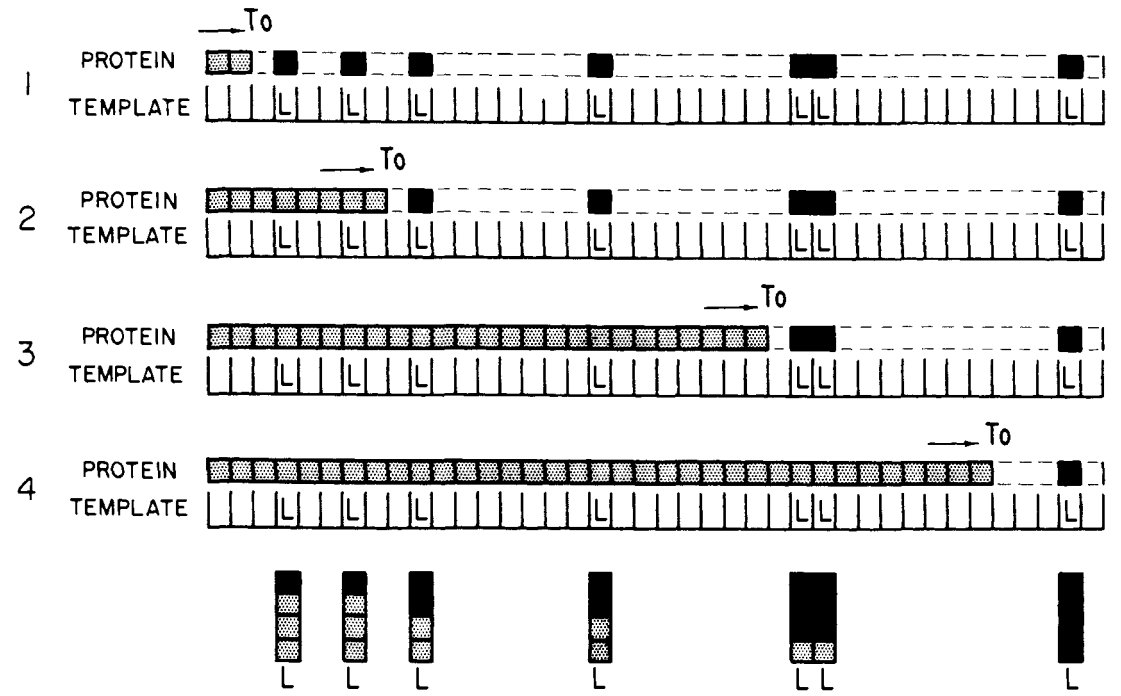

FIG. 5.—A scheme depicting four representative templates in the process of lysozyme synthesis. Stippled boxes represent amino acids incorporated prior to the radioactive pulse. Black boxes represent leucine positions to be incorporated after the addition of radioactive leucine.

sume that variations in oviduct protein synthesis may occur during different phases of the egg-laying cycle. Varying amounts of carrier lysozyme were added in each experiment, depending upon the type of peptide isolation procedure used, and, as noted earlier, each incubation represented different ratios of isotope to oviduct tissue. For these reasons the absolute levels of radioactivity cannot be meaningfully compared, but the relative values for any given experiment can. It is of interest, however, that the relative specific radioactivity of the COOH-terminal leucine residue in the lysozyme isolated from the 3-minute incubation relative to the specific radioactivity of the leucine residue in peptide T-3, is slightly less than that found in the 5-minute incubation (Figure 4). Aside from differences in the hens (the experiments were performed 6 months apart) there is one experimental condition which may account for this. The 5-minute incubation began with chilled oviduct while the 3-minute incubation began with oviduct previously warmed to 37°. It is possible, therefore, that the pool of leucine in the chilled oviduct was enriched with H^3-leucine before the increase in temperature initiated protein synthesis. If the specific activity of the COOH-terminal leucine residue is compared with the average specific activity of the three leucine residues near the NH_2-terminal end of the polypeptide chain, one observes a 2.9-fold difference in the 5-minute chilled incubation and a 2.1-fold difference in the 3-minute incubation. These data support the model for sequential growth of a new polypeptide chain illustrated in Figure 5.

Radioactivity was found in positions near the amino terminal end of completed chains of lysozyme as early as 3 minutes after addition of the labeled leucine. It is probable that very little synthesis occurred in the chilled tissue between the end of the incubation and the homogenization of the tissue, and it may be tentatively concluded that a complete chain of lysozyme can be synthesized in less than 3 minutes at 37°. There are 129 amino acids in the polypeptide chain (Canfield, 1963a) and it is therefore probable that incorporation occurred at a rate of at least one residue per second.

Although these experiments support the hypothesis of linear growth of a polypeptide chain at the time of synthesis, they give no information concerning the formation of secondary or tertiary structure. Lysozyme contains four cystine residues, and it has been shown that fully reduced lysozyme can reacquire its native conformation (Isemura *et al.*, 1961; White, 1962). All that can be inferred here is that the newly synthesized lysozyme chains assume the native structure some time prior to elution from the carboxymethylcellulose columns with the carrier lysozyme.

Studies, on several systems, leading to nonuniform labeling now lend support to the model for amino acid assembly summarized in Figure 5. Incorporation of C^{14}-leucine into α-amylase, associated with studies of the amino and carboxyl terminal sequences, revealed higher specific activities in the carboxyl fragment (Yoshida and Tobita, 1960). Studies by Bishop *et al.*, (1960) on rabbit reticulocyte hemoglobin were mentioned above. Carboxypeptidase digestion of the pool of newly synthesized proteins in *E. coli* revealed that labeled amino acids were more concentrated near the carboxyl terminal ends of the chains (Goldstein and Brown, 1961). Recently, Naughton and Dintzis (1962), using various sequence data for human and rabbit hemoglobins (Braunitzer *et al.*, 1962; Braunitzer and Matsuda, 1961; Diamond and Braunitzer, 1962; Guidotti *et al.*, 1962), have elegantly determined a similar isotope distribution pattern for peptides of both the α and β chains of rabbit reticulocyte hemoglobin.

Sequential nonuniform labeling has been shown to be a very transient phenomenon in the *E. coli*, the rabbit reticulocyte, and the hen's oviduct systems. Thus, a short time after addition of a labeled amino acid, it can be shown that uniform specific activity exists throughout the protein chain. It is of interest that nonuniform labeling has been observed after much longer times of incubation for the oviduct (Steinberg and Anfinsen, 1952) and the reticulocyte systems (Kruh *et al.*, 1960) and it is possible, therefore, that more than one mechanism may exist to bring about conditions of unequal labeling in recently synthesized protein.

Acknowledgment

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