ERRATA

In the paper entitled "Assembly of the Peptide Chains of Hemoglobin," by Howard M. Dintzis, which appeared in volume 47, number 3, pages 247–261, the author wishes to make the following correction:

On page 251, five lines below Figure 2, the phrase "and 2 *M* formic acid—0.02 *M* pyridine" should read "and 2 *M* formic acid—0.2 *M* pyridine."

In the paper entitled "The Thermostatic Control of Human Metabolic Heat Production," by T. H. Benzinger, A. W. Pratt, and Charlotte Kitzinger, which appeared in the May issue of volume 47, where the caption for Figure 3, p. 735, reads "*internal*-sensory receptor site, *D*," the "*D*" should read "*A*." Throughout the remainder of the caption, for "*D*" read "*A*."

reduced and fully oxidized pyridine nucleotide. We thus envision the following scheme:

$$DPNH + FMN \rightleftharpoons DPNH^+ \cdot FMN^- \rightleftharpoons DPNH^+ + \dot{F}MN^-$$

$$2DPNH^+ \rightleftharpoons DPN^+ + DPNH + H^+$$

with possibly

$$\dot{F}MN^- + H^+ \rightleftharpoons \dot{F}MNH.$$

The net result would be that DPNH reduces FMN to a semiquinone without forming a free radical itself.

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[1] Fisher, H. F., E. E. Conn, B. Vennesland, and F. H. Westheimer, *J. Biol. Chem.*, **202**, 687 (1953).

[2] Hoewus, F. A., F. H. Westheimer, and B. Vennesland, *J. Am. Chem. Soc.*, **75**, 5018 (1953).

[3] Singer, T. P., and E. B. Kearney, *J. Biol. Chem.*, **183**, 409 (1950).

[4] Commoner, B., and B. B. Lippincott, these PROCEEDINGS, **44**, 1110 (1958).

[5] Isenberg, I., and A. Szent-Györgyi, these PROCEEDINGS **45**, 1229 (1959).

[6] Isenberg, I., and A. Szent-Györgyi, these PROCEEDINGS, **46**, 1307 (1960).

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*ASSEMBLY OF THE PEPTIDE CHAINS OF HEMOGLOBIN**

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The mechanism by which proteins are synthesized has been a matter of intense speculation in recent years.[1, 2] Some published speculative models propose simultaneous bond formation between all neighboring activated amino acids on a preloaded template (a sort of stamping machine operation). Others suggest various forms of sequential addition of amino acids to a steadily growing polypeptide chain. In addition there have been hypothesized all degrees of exchange between amino acids already incorporated into growing peptide chains on the template and various classes of precursor "activated" amino acids in solution.[3]

A common concept of how peptide chains may grow is based on their linear chemical nature and assumes serial addition of amino acids, starting at one end of the chain and progressing steadily to the other end. A less orderly picture involves peptide sections growing randomly here and there on the template and finally coalescing into a single chain. Since we know very little about the geometric nature of the templates upon which protein synthesis occurs,[4] we cannot a priori rule out all manner of complex growth mechanisms. For example, if the substructure of the template is folded or coiled in a regular manner it is possible that short, evenly spaced bits of peptide chain are made first on those parts of the template most accessible to the external solution and that the intervening bits are added later at a slower rate. Also since nothing is known about the type of bonds

holding the activated amino acids to the template just prior to peptide bond formation, we cannot assume that chain growth is necessarily unidirectional. It is possible that chain growth is initiated at both the amino end and the carboxyl end and progresses towards the middle, or conversely, begins in the middle and proceeds toward both ends.

It is apparent that there exists no shortage of hypothetical models of protein chain growth. The difficulty lies in finding an analytical technique capable of yielding enough information to eliminate conclusively most wrong models and, if possible, to narrow the choice to a single correct one.

Data concerning the actual mechanism of protein assembly should in principle be obtainable by studying both the newly formed protein molecules and the ribosome templates on which they are supposedly formed. However, no method exists for fractionating from a cellular extract all ribosomes engaged in the production of a single type of protein molecule. If a type of cell could be found which is engaged solely in the synthesis of a single kind of protein molecule, then presumably all ribosomes in such a cell would contain incomplete bits of that kind of protein molecule and no others.

Fortunately, a close approximation to this highly desirable situation exists in the case of immature mammalian blood cells producing hemoglobin. These cells, reticulocytes, account for 80 to 90 per cent of the red cells present in the blood of rabbits made anemic by daily injection of phenylhydrazine. The cells may be isolated from the blood and placed in an incubation medium where they will continue producing hemoglobin for many hours.[5, 6] During such an incubation over 90 per cent of the soluble protein produced appears as hemoglobin. It is therefore reasonable to expect most of the growing peptide chains present in the ribosome fraction of such cells to represent incomplete hemoglobin molecules.

If we have available a technique for splitting the peptide chains of both completed and incompleted hemoglobin molecules at a definite number of specific sites, we should be in a position to test which one, if any, of the above hypothetical mechanisms of protein assembly is correct. This is so because each model of protein assembly leads to a definite prediction as to the time and space distribution of newly added amino acids in short sections of peptide chain, both in finished hemoglobin and in the ribosomal particles. Amino acids labeled with radioactive isotopes provide a means of detecting newly added amino acids. In living reticulocyte cells there exist a very large number of finished hemoglobin molecules (10–20% of the cell by weight) and in addition a large number of ribosomal particles, supposedly containing unfinished hemoglobin molecules in different stages of completion. If, at a given moment, we add a radioactively labeled amino acid to the incubation medium containing reticulocytes, then we expect polypeptide produced thereafter to be labeled with radioactive amino acid.

The data to be presented in this paper strongly support a model of protein synthesis involving growth by some kind of sequential addition of amino acids. In Figure 1 are shown some of the predicted consequences of this type of model. For the purposes of illustration we have chosen a model involving chain initiation at one end of the polypeptide followed by sequential addition of one amino acid after another until the other end is reached. We shall assume that some digestion technique can be used to split each polypeptide chain at a definite number of

specific sites, yielding the set of peptides *a*, *b*, *c*, . . . *g*, and that furthermore, the set can be separated and the amount of newly added amino acid present in each member *a*, . . . *g* determined quantitatively.

In the finished hemoglobin at short times, we would then expect a steep gradient of radioactive label through the peptides, with only a few peptides labeled at very short times. At longer times the gradient of radioactivity along the peptide chain should become shallower as more and more completely labeled molecules are produced. At all times, the peptide *g*, closest to the finish line, should have the most radioactive label, and the peptide *a*, closest to the starting line, should have the least radioactive label.

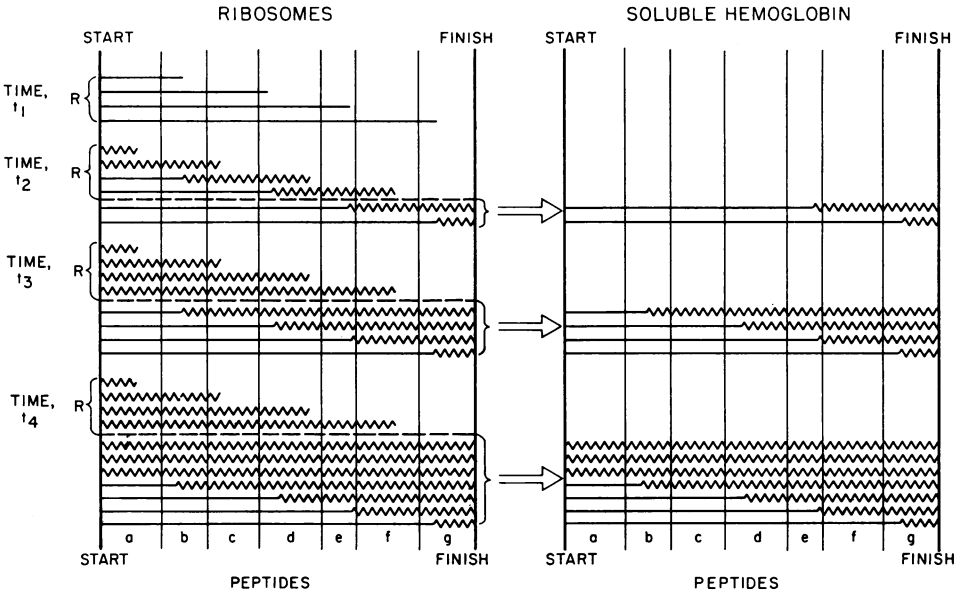

FIG. 1.—Model of sequential chain growth. The straight lines represent unlabeled polypeptide chain. The zigzag lines represent radioactively labeled polypeptide chain formed after the addition of radioactive amino acid at time t_1. The groups of peptides labelled *R* are those unfinished bits attached to the ribosomes at each time; the rest, having reached the finish line, are assumed to be present in the soluble hemoglobin. In the ribosome at time t_2, the top two completely zigzag lines represent peptide chains formed completely from amino acids during the time interval between t_1 and t_2. The middle two lines represent chains which have grown during the time interval but have not reached the finish line and are therefore still attached to the ribosomes. The bottom two chains represent those which have crossed the finish line, left the ribosomes, and are to be found mixed with other molecules of soluble hemoglobin.

On the other hand, in the ribosomes at very short times we would expect an almost uniform distribution of total label among the various peptides since each growing chain will have added only a small radioactive section (Fig. 1). After times long enough to flush out the nonradioactive bits of growing chain, there should be a gradient of total radioactivity from the initial peptide *a*, with the most, to the final peptide *g*, with the least. For this model this is so because there exist in a population of ribosomes at any moment, more sections of peptide *a* than *b*, more *b* than *c*, and so on. Thus, the expected gradient of label in the ribosomes is opposite to that in the finished hemoglobin, both in space direction and in time development.

General Experimental Considerations.—The technique used for forming and separating a reproducible set of peptides was a modification of the method involving a combination of paper electrophoresis and chromatography, at right angles, used by Ingram for human hemoglobin,[7] and termed "fingerprinting" by him. The enzyme trypsin, which splits polypeptides with high specificity wherever the amino acids lysine and arginine occur, furnishes the means of splitting at a definite number of sites. For various reasons, many details of Ingram's procedure for tryptic digestion, paper electrophoresis, and chromatography were modified in the present study.

The problem of obtaining quantitative data on the amount of radioactivity in each peptide was solved by the use of two different isotopic labels. Short incubations were done with H^3-leucine, and very long incubations with C^{14}-leucine. The very long incubations were assumed to give hemoglobin of uniform specific activity in each leucine position. The H^3- and C^{14}-labeled preparations were mixed and carried through the stages of digestion, electrophoresis and chromatography together. The ratio of H^3 to C^{14} was taken as a measure of the amount of label in each peptide obtained from the short time incubations. This method gave an internal standardization automatically correcting both for the differential losses and for the different number of leucine residues in the peptides.

In order to slow the rate of hemoglobin synthesis to the point where samples could be handled with convenience, incubations were tried at various temperatures below body temperature. It was found that the rate of incorporation of C^{14}-leucine into hemoglobin fell slowly with temperature until a point about 10° was reached, whereupon incorporation abruptly stopped. Incorporation of labeled amino acid was found to proceed smoothly at 15° at approximately 1/4 of the rate at 37° (Table 1) and this temperature was routinely used for all short-time experiments.

TABLE 1

INCORPORATION OF C^{14} LEUCINE INTO RABBIT HEMOGLOBIN AT VARIOUS TEMPERATURES OF INCUBATION

Temperature of incubation	Experiment 1		Experiment 2	
	Cpm/mg	% of 37° value	Cpm/mg	% of 37° value
0	0	0.00	...	...
5	14	0.22	...	...
10	280	4.3	...	...
15	2,230	34	8,150	17
20	...	...	17,700	38
25	...	...	52,600	110
30	...	...	45,000	95
37	6,500	100	47,000	100

Hemoglobin was dialyzed for 5 days against water, precipitated with trichloroacetic acid, dissolved in dilute NaOH, reprecipitated with trichloroacetic acid, washed with acetone and ether, and then plated in thin layers containing approximately 20 mg. Counting was done using a Nuclear Chicago end window gas flow counter, the results corrected to zero thickness.

It has been previously shown that the structural protein of ribosomes is not appreciably labeled at short times of incubation.[8] It was therefore assumed that the labeled peptides resulting from a digest of ribosomes with ribonuclease and trypsin represent growing hemoglobin chains and not ribosomal structural proteins. On tryptic digestion the ribosome structural protein did yield a large number of ninhydrin staining peptides which were distinct from those of hemoglobin but, as expected, they did not contain radioactive label.

Incubation of cells: Rabbit reticulocytes prepared from phenylhydrazine-treated animals were washed and incubated according to the procedures of Borsook *et al.*[6] The cells were incubated at 37° for 15 min to allow them to renew metabolites, then at 15° for 5 min. To 1.8 ml cells in a total volume of 4 ml incubation mixture was added 0.24 mg 4, 5 H^3-DL-leucine (5 mc, New England Nuclear Corporation, 3.6 mc per μmole) and the incubation continued at 15°. At various intervals aliquots of approximately 1 ml were removed with a pipet and quickly placed in precooled vials surrounded by solid carbon dioxide.

Uniformly labeled C^{14} leucine hemoglobin was prepared in a similar manner from approximately 1 mg of L-leucine, uniform C^{14}, (50μc Nuclear Chicago), which was incubated with 10 ml of sterile whole blood at 37° for 5 or 24 hr. During 24-hr incubations a significant amount of cell lysis occurred, partly offsetting the approximately 50 per cent higher specific activity obtainable. Typi-

cal incubations with C^{14} L-leucine of specific activity 6–8 c/millimole gave hemoglobin of approximate activity 1×10^5 dpm/mg.

Preparation of hemoglobin and ribosomes for tryptic digestion: Samples containing approximately 0.45 ml cells were thawed and the broken cells diluted to a volume of 7 ml with cold solution containing 0.14 *M* KCl, 0.001 *M* $MgCl_2$ and 0.01 *M* Tris-Cl pH 7.3. Solution of this composition had been previously shown to stabilize rabbit reticulocyte ribosomes[8] and was used in all operations where ribosomes were present. The solutions were then centrifuged at 20,000 *g* for 10 min to remove cell walls and debris, and then at 130,000 *g* for $1^1/_2$ hr to remove ribosomes.

Hemoglobin: The ribosome-free supernatant was dialyzed for 5–7 days in the cold against daily changes of 5×10^{-4} *M* KH_2PO_4, 5×10^{-4} *M* K_2HPO_4 saturated with toluene to prevent bacterial growth. The slight precipitate which formed was centrifuged off and the supernatant hemoglobin frozen until used.

Short time labeled H^3-leucine hemoglobin (4–60 min at 15°) solution was mixed with long time labeled C^{14}-leucine hemoglobin (5–24 hr at 37°) solution in a ratio such that both the H^3 and C^{14} could be counted with good accuracy. This ratio was usually near 10 dpm H^3 per dpm C^{14}. The combined hemoglobin solution was then used to prepare globin by acid acetone precipitation.[4]

Ribosomes: The ribosome pellets were dissolved in 7 ml stabilizing buffer at 0°C, centrifuged for 5 min at 20,000 *g* to remove denatured protein and then reprecipitated by centrifuging at 130,000 *g* for $1^1/_2$ hr. The ribosomes were redissolved and recentrifuged three times to remove

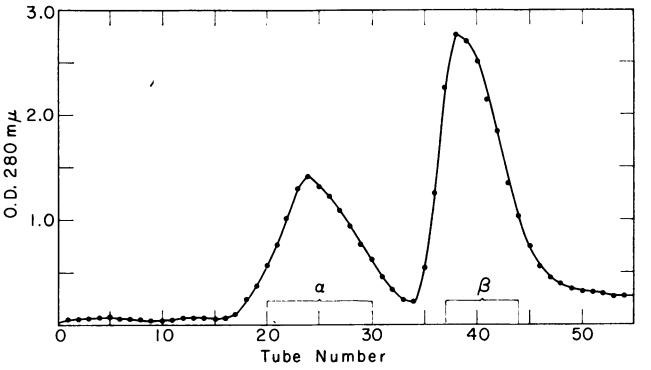

FIG. 2.—Separation of α- and β-chains of rabbit hemoglobin on carboxymethylcellulose column.

free leucine and hemoglobin. The final ribosome pellet was a very light yellowish color and completely transparent.

Separation of peptide chains of hemoglobin: The α- and β-chains of rabbit globin were separated on carboxymethyl cellulose using a linear concentration gradient of buffer between 0.2 *M* formic acid—0.02 *M* pyridine and 2 *M* formic acid—0.02 *M* pyridine (Fig. 2). Two samples of carboxymethyl cellulose were found to give good results: a preparation of 0.47 meq/g (Brown Co., Berlin, N. H.) and a preparation of 0.06 meq/g (Serva, Heidelberg, Germany). Several preparations of higher capacity from various companies did not give as good results. Solutions of separated chains were dried under vacuum in the presence of sulfuric acid and soda lime.

Tryptic digestion of hemoglobin samples: Autotitrator: Dried samples were dissolved in water to a concentration of 10–20 mg/ml. The pH was adjusted to 9.5 with 0.10 *N* NaOH from an initial value between 3 and 4. Dense precipitation occurred near neutral pH but the solution became clear again at pH 9.5. 0.01 ml of 1% trypsin (Worthington 2x crystallized, salt-free in 10^{-3} *M* HCl) was added for each ml of solution and the digestion was allowed to proceed at 37° until definite evidence of a plateau in base uptake was obtained (approximately $1^1/_2$ hr).

Buffer: 10 mg of dried sample was dissolved in 0.5 ml water, 0.015 ml 0.5 *M* NH_4OH was added, followed by 0.01 ml 1% trypsin and 0.025 ml buffer made of 1 *M* NH_4OAc + NH_4OH to pH 9.75. Digestion proceeded for 4 hours at room temperature.

In all cases digestion was stopped by the addition of several drops of glacial acetic acid. The samples were then dried under high vacuum in the presence of sulfuric acid and soda lime and then dissolved at a concentration of 100 mg/ml in 0.4% acetic acid-0.1% pyridine, giving a preparation which was often clear, but sometimes had slight to medium turbidity.

Tryptic digestion of ribosome samples: The ribosome pellet from 0.45 ml cells, approximately 3 mg dry weight, was dissolved in 1 ml water. 10–20 mg uniformly labeled C^{14} leucine globin was dissolved in 1 ml water. The two solutions were mixed and adjusted to pH 8.5 in an autotitrator at 37°. 0.02 ml 1% ribonuclease (Worthington crystalline) was added, followed, after 15 min, by 0.02 ml 1% trypsin. The digestion was followed in the autotitrator for 15 min, then the pH was raised to 9.5 and the digestion followed for approximately $1^1/_2$ hr until a plateau was reached. The samples were acidified and dried as in the case of hemoglobin digestion.

Paper electrophoresis: Electrophoresis was carried out on a water-cooled metal plate insulated with a thin sheet of polyethylene. Strips of Whatman No. 3MM paper 12 in. wide and 37 in. long were wet with buffer of pH 4.5 (2.5% pyridine, 2.5% acetic acid, 5% n-butanol, all concentrations v/v) and blotted. Eight-inch wicks made of 4 thicknesses of the same paper were overlapped at each end, and 0.02 ml of solution containing 2 mg of sample was applied at the origin. The paper was then covered with polyethylene sheeting pressed flat by weights applied over a sponge rubber pad. Electrophoresis was carried out at 2,000 volts and approximately 100 ma, for 16 hr, after which the paper was dried.

Chromatography: The dried papers were trimmed to a length of 33 in. and stapled into cylinders 12 in. high. Chromatography was then conducted at room temperature in glass jars 12 in. wide and 24 in. high, using a mixture of 42.5 vols n-butanol, 27.5 vols pyridine, 30 vols water. Occasionally it was necessary to increase the chromatographic resolution by sewing a 4-in. strip of paper to the top of the sample sheet before stapling into a circle.

Isolation and counting of peptides: The dried chromatograms were dipped in 0.25 per cent ninhydrin in acetone, dried, and heated at 90° for 5 min. The resulting blue paper spots were cut out, placed in 20 ml counting vials and 5 ml of water was added to each. The vials were then heated in an oven at 90° for 30 min to extract the peptides from the paper, after which time the paper was removed from the vial with a tweezer and the solution evaporated to dryness overnight in an oven at 90°. 0.20 ml of 0.01 HCl was added to each vial, followed by 20 ml of scintillator solution made up of three parts toluene, one part absolute ethanol, and containing 1% phenylbiphenylyloxadiazole-1,3,4(PBD) and 0.05% p-bis [2-(5-phenyloxazolyl)]-benzene (POPOP). The resulting solutions were measured for C^{14} and H^3 activity simultaneously using a TriCarb scintillation counter equipped with split channel operation so that the lower voltage channel counted both C^{14} and H^3 while the upper voltage channel counted mainly C^{14}. The recovery of radioactivity from eluted peptides of hemoglobin amounted to approximately 50 per cent of the amount applied at the origin spot for paper electrophoresis.

The TriCarb scintillation counter was run with 1040 volts on the photomultiplier tubes. The lower pulse height discriminator was set to register pulses between 10 and 50 volts, giving an efficiency of 6.5% for H^3 and 20% for C^{14} with a background of 40 cpm. The upper pulse height discriminator was set to register pulses of 100 volts or higher, giving an efficiency of 0.14% for H^3 and 37% for C^{14} with a background of 60 cpm. Mixtures of isotopes ranging from 2 dpm H^3/dpm C^{14} to 40 dpm H^3/dpm C^{14} were used.

Figure 3 shows a rather typical peptide separation. To improve photographic reproduction, the ninhydrin staining was done with twice the usual concentration of ninhydrin. The result shows more clearly than usual the presence of "ghost" spots, which are defined as weak spots sometimes present but usually absent or barely detectable. The spots which are always or almost always present have been numbered arbitrarily from left to right.

Peptide 31 is the leucine-containing peptide farthest from the origin as determined by radioactivity count on peptides made from uniformly labeled hemoglobin. There are approximately four ninhydrin staining spots farther from the origin than peptide 31, but since they were not labeled by leucine, they were routinely removed from the paper by electrophoresis, to increase the separation of the remaining peptides. The total number of peptides found with reasonable reproducibility is thus about 35, appreciably above the number 26 reported in human hemoglobin by Ingram.[7] It should be noted that a number of peptides, e.g., 2, 7, 16, 19, 23, stain quite weakly

and may represent products of incomplete tryptic digestion, or partial digestion by other enzymes such as chymotrypsin which may be present as trace impurities in the trypsin.

The separation and identification of peptides was not uniformly good. In some runs spots were either missing or badly smeared into other spots. Consequently it was necessary to eliminate

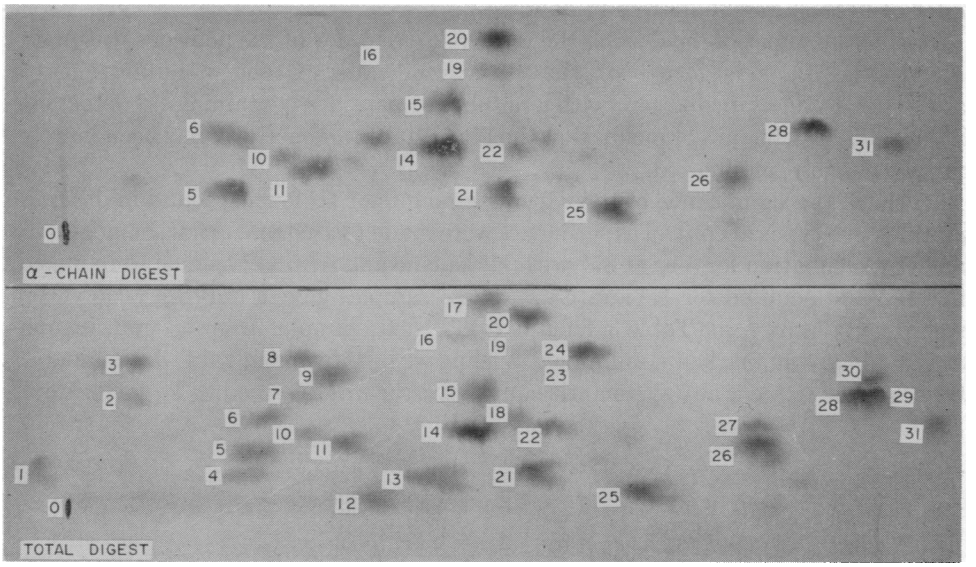

FIG. 3.—Peptide maps of tryptic digest of total rabbit globin (bottom) and column separated α-chain (top). The point of application of digest to the paper is marked by O. The positive electrode is to the left.

from elution and counting in each run those peptides which could not be identified with certainty or which badly overlapped with neighbors known to contain leucine.

A total of 18 peptides, 9 from the α-chain and 9 from the β-chain, were found reproducibly to contain leucine in significant quantity. The average relative yield of C^{14} in these peptides, obtained from digests of uniformly labeled single chains, is shown in Table 2. In addition, smaller

TABLE 2

RELATIVE YIELD OF C^{14} LEUCINE FROM TRYPTIC PEPTIDES

α-Chain Peptides		β-Chain Peptides	
Peptide number	Average relative yield	Peptide number	Average relative yield
10	0.4	1	1.2
11	0.7	3	0.8
14	4.5	9	2.6
16	0.3	12	0.5
20	1.0	13	1.0
21	0.4	17	1.2
22	0.2	18	0.5
25	0.6	24	1.0
31	0.6	27	0.6

amounts of label were found in peptides 2 (β), 6 (α), 19 (α), and 28 (β), but since their yields were quite small and variable, no attempt was made to do quantitative measurements on them (except for a few studies on peptide 28, reported below). This study, which is dependent on the use of radioactive leucine, is therefore based on slightly over half of the total number of recognizable peptides produced from hemoglobin by trypsin digestion. Extension to the remaining peptides awaits the availability of other amino acids, preferably lysine and arginine, of very high H^3 specific activity.

Results.—After 7 min of incubation at 15° in the presence of H^3 leucine, a marked difference in the relative amount of tritium contained could be found in the peptides of both the α- and β-chains. The peptides could be arranged in a more or less definite order of increasing tritium content (Fig. 4), such that only the relative order of nearest neighbors was in doubt.

At different times of incubation the same relative order of the peptides was maintained (Fig. 5). The shape of the curves indicates extreme nonuniformity of labeling at 4 min of incubation, with a number of peptides containing no detectable H^3 leucine. By 60 min of incubation the gradient of radioactivity has been largely, but not entirely, eliminated.

To check the significance of the varying amounts of H^3 leucine found in different peptides two types of control experiments were made (Table 3). First, hemoglobins made by incubation for 5 hr at 37° with H^3 leucine and with C^{14} leucine were mixed, digested and counted for H^3 and C^{14}. These samples gave a uniform ratio within experimental error; see Table 3, column (*a*). Next, samples from a 7-min incubation at 15° giving marked nonuniform labeling with H^3 leucine (Table 3, column (*b*)) were checked to see if any systematic counting error was involved. To each sample

TABLE 3

CONTROTS ON COUNTING ACCURACY

Peptide number	(*a*) Long-time incubation	(*b*) Short-time incubation	(*c*) Increment ratio
α-Chain			
21	1.01	0.08	1.02
10	0.94	0.08	0.98
20	1.06	...	...
25	1.04	0.36	1.03
11	1.04	0.38	1.05
14	1.07	0.69	1.05
31	1.02	...	...
22	1.02	0.84	1.00
16	0.88	1.06	1.02
β-Chain			
13	...	0.05	0.98
24	0.93	0.10	1.02
1	1.01	0.16	1.03
17	0.94	0.23	1.02
3	0.94	0.34	0.88
9	0.99	0.54	1.02
18	0.97	0.59	0.89
12	1.05	0.70	1.00
27	0.86	...	...

(*a*) 5-hr incubation H^3 leucine, 5-hr incubation C^{14} leucine, relative amount of tritium.
(*b*) 7-min incubation H^3 leucine, 30-hr incubation C^{14} leucine, relative amount of tritium.
(*c*) Ratio of increases in H^3 to C^{14} after adding H^3 leucine and C^{14} leucine to each counting vial of (*b*).

vial a constant amount of H^3 standard and C^{14} standard were added, and the radioactivity redetermined. The measured increments in H^3 and C^{14} activity were constant within experimental error and the normalized ratio of increments $\Delta H^3/\Delta C^{14}$ was also constant (Table 3, column (c)).

The results obtained by digesting ribosomes were less reproducible for a number of reasons. First, the α- and β-chains could not be separated, since by definition we were looking for incomplete chain fragments in the ribosomes, and thus did not dare lose fragments in an attempt at fractionation. Secondly, the over-all background of radioactivity between ninhydrin staining spots was much higher in the ribosomes. This is perhaps to be expected from the model in Figure 1, where there

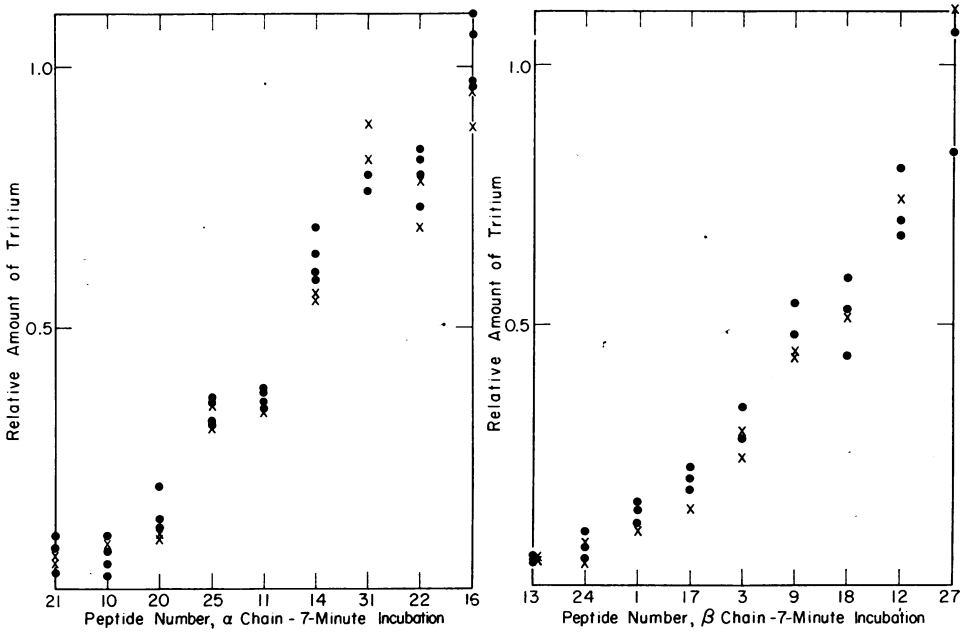

FIG. 4.—Distribution of H^3-leucine among tryptic peptides of soluble rabbit hemoglobin. Peptides produced by tryptic digestion in an autotitrater are indicated by ●. Peptides produced from a separate incubation by tryptic digestion in buffer are indicated by X.

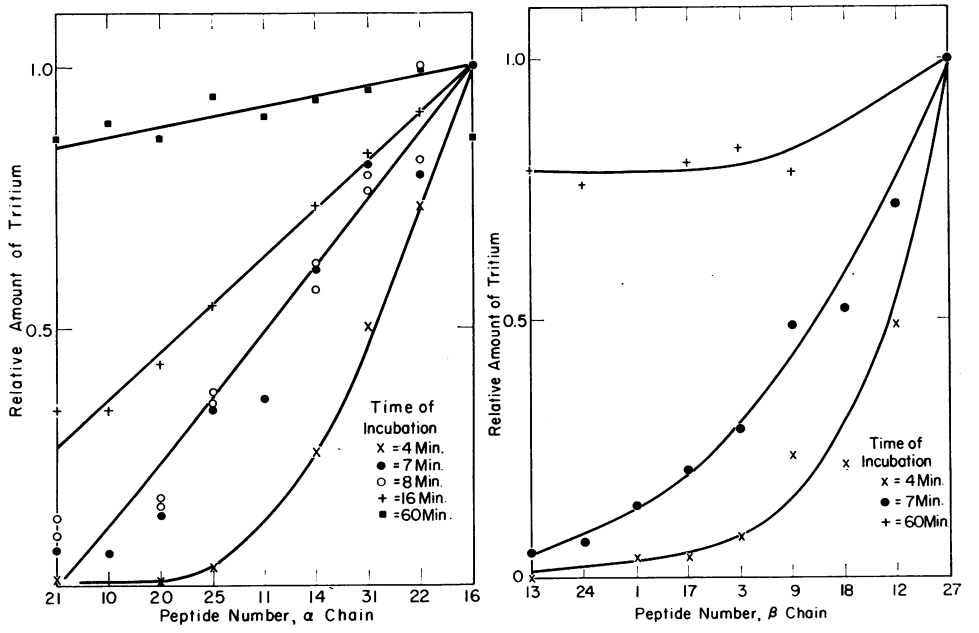

FIG. 5.—Distribution of H^3-leucine among tryptic peptides of soluble rabbit hemoglobin after various times of incubation at 15°C. The points indicated for 7 minutes (●) are the result of averaging all points shown in Figure 4.

are shown end bits of growing chain which do not span vertical lines. Such bits would not correspond to tryptic peptides from hemoglobin and would not be expected to separate with the known peptides; hence they would contribute to the background of radioactivity.

Figure 6 shows the data obtained from ribosomes of cells which had been incubated for short periods (4 to 7 min) at 15°. It is hard to see any significant trend to the data, with the possible exception that the terminal peptides (16 and 27) seem lower than the rest. It thus appears that at these short times of incubation the hemoglobin peptides in ribosomes are labeled almost uniformly.

Figure 7 shows results from ribosomes of cells which had been incubated 60 min at 15°. In this case there is a clear trend visible in the peptides of the α-chain with a less definite result in the case of the β-chain. The gradient of radioactivity is opposite to that in Figure 4.

After 7 min of incubation with H^3 leucine at 15° the hemoglobin peptides isolated from soluble hemoglobin (Fig. 4) had an average specific activity of 1.2×10^5 dpm H^3 per mg. The average specific activity of the hemoglobin peptides prepared from the ribosomes isolated from the same cells may be calculated if one can make an estimate of the weight fraction of ribosomal particles which is present as growing hemoglobin chains. If we make the extreme assumption that the purified ribosomes are pure hemoglobin, then the specific activity of the average peptide in the ribosome (Fig. 6) is 7×10^6 dpm H^3 per mg, or 60 times that of the average peptide in soluble hemoglobin. If we take as more likely the previously reported[8] estimate that growing peptide chains amount to approximately 0.1 per cent of the ribosomal mass, then the ratio of peptide specific activity in ribosomes to that in soluble hemoglobin becomes 60,000. This latter assumption also leads to the conclusion that the specific activity of the H^3-leucine in the ribosomal hemoglobin peptides is approximately 1.5 times that of the H^3-leucine used for the incubation, a result obviously too high but within the combined errors of experiment and assumptions. These results indicate conclusively that the tryptic peptide fragments of hemoglobin isolated from ribosomal particles are precursors of finished hemoglobin molecules and do not represent contamination of the ribosomal particles by completed molecules from the soluble pool.

The results given in Figures 4, 5, 6, and 7 are in agreement with the model shown in Figure 1 in all particulars. The predicted gradient of radioactivity in the peptides of soluble hemoglobin, becoming less pronounced with time, and the inverse gradient in the peptides of the ribosomes, becoming more pronounced with time, are both found. The development of a gradient of radioactivity in the ribosomal hemoglobin peptides at long times is perhaps the most direct proof to date that ribosomes contain incomplete growing peptides. A gradient might be expected at short times in the ribosomes due to contamination from nonuniformly labeled molecules produced elsewhere, but it is hard to see how a gradient could develop with increasing time except by means of the mechanism shown in Figure 1.

It must be stressed that the data given thus far do not constitute proof of the correctness of the particular model in Figure 1, although they are in complete agreement with it. This is the case because all the data presented above are limited to time measurements. To test the model completely, the sequence of amino acid residues along the peptide chain must also be known. Specifically it must be

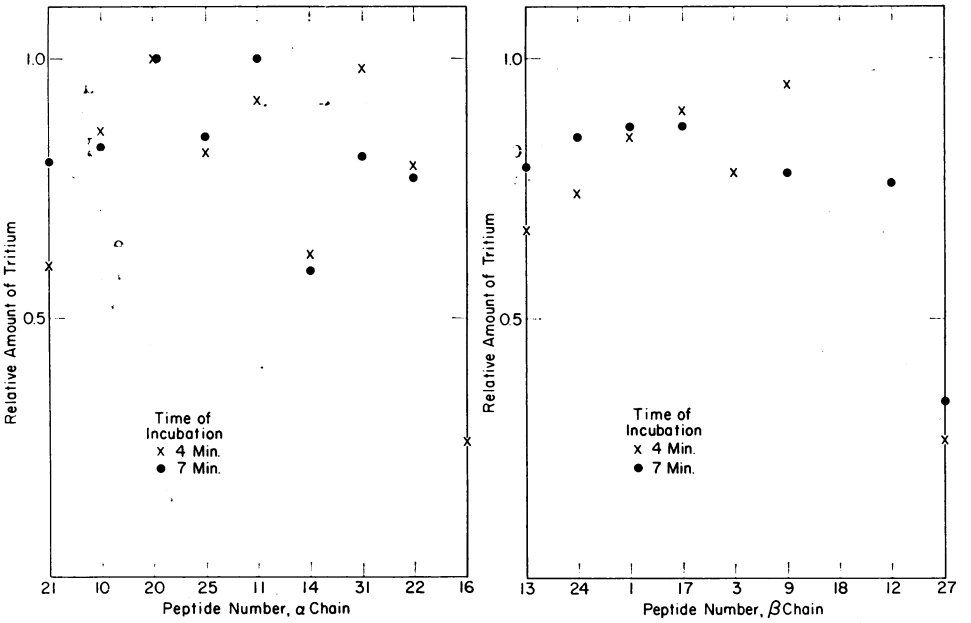

FIG. 6.—Distribution of H^3-leucine among tryptic peptides of ribosomes after short incubations.

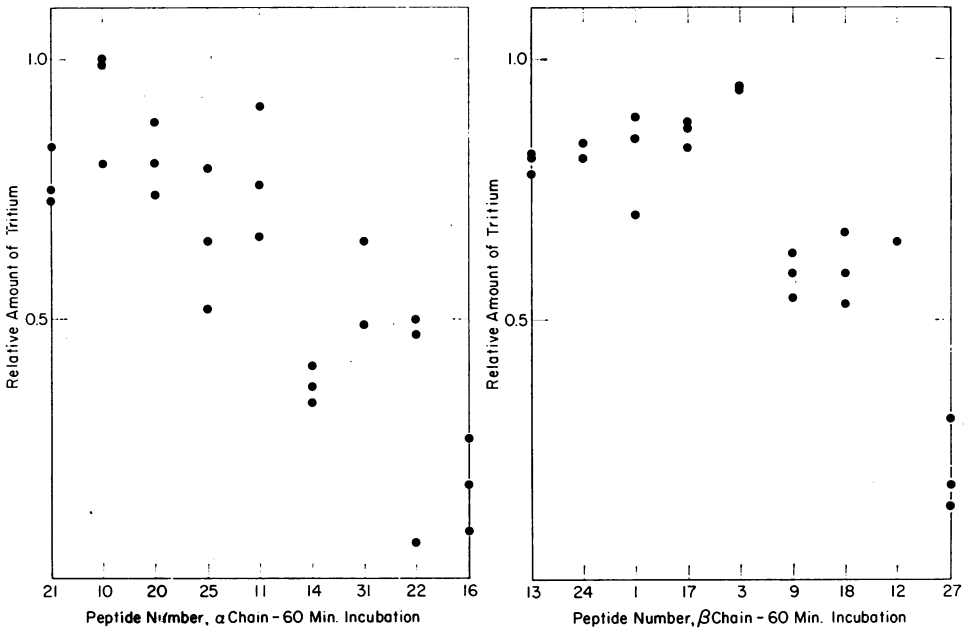

FIG. 7.—Distribution of H^3-leucine among tryptic peptides of ribosomes after long incubations.

shown that peptides at the end of the time sequence, e.g., 21 and 31, are nearest to the end of the polypeptide chain, and furthermore that peptides which are neighbors in the time sequence of labeling with H^3 leucine, e.g., 14 and 31, are also neighbors on the polypeptide chain.

An attempt has been made to identify the tryptic peptide nearest to the free carboxyl end of each hemoglobin chain. Guidotti has reported that by using a mixture of carboxypeptidases A and B he was able to remove sequentially approximately a dozen amino acids from the carboxyl end of the α- and β-chains of human globin.[10] In the case of human globin a leucine residue was one of those removed from each chain. It was therefore reasonable to assume that a similar operation on rabbit globin could remove a leucine residue from the peptide nearest to the carboxyl end.

Uniformly labeled C^{14} leucine globin was incubated according to the conditions of Guidotti with carboxypeptidase A (Worthington, DFP treated) and carboxypeptidase B (kindly donated by Dr. Martha Ludwig). After digestion it was heated at 100° for 15 min to denature the enzyme, and then mixed with undigested uniformly labeled H^3 leucine globin which had received the same treatment, except that no carboxypeptidase had been added to it. The mixture of C^{14} leucine globin and H^3 leucine globin was then digested with trypsin and the peptides were separated and counted as described above. If the carboxypeptidase had no effect on any recognizable leucine-containing peptide, then we would expect to obtain a constant ratio of H^3 to C^{14} in each resulting tryptic peptide (e.g., see Table 3, column (*a*)). If, however, C^{14}-leucine were removed from a peptide by the action of carboxypeptidase, we would expect a decrease in C^{14} leucine content in that peptide with a corresponding increase in the ratio of H^3 to C^{14}. The experiment was also done in reverse, with the H^3 leucine globin being digested with carboxypeptidase. As a final control both C^{14} leucine globin and H^3 leucine globin were carried through all operations except that no carboxypeptidase was added to either. The results are indicated in Table 4.

TABLE 4

RELATIVE TRITIUM CONTENT OF TRYPTIC PEPTIDES FOLLOWING CARBOXYPEPTIDASE ACTION

Peptide number, α-chain	(*a*) C^{14} Globin Digested with Carboxypeptidases			(*b*) H^3 Globin Digested with Carboxypeptidases		(*c*) No Digestion with Carboxypeptidases	
	Digest 1		Digest 2				
10	1.0	1.0	1.2	1.0	0.8	1.3	1.1
11	1.0	1.0	1.2	...	1.0	1.1	1.3
14	1.0	1.0	1.1	1.1	1.0	1.0	1.1
16	11.0	20.0	2.7	0.07	0.0	0.3	0.4
20	1.0	1.0	0.7	1.0	1.0	1.0	1.0
21	1.0	1.0	1.0	1.0	1.1	1.0	1.0
22	0.9	0.9	0.9	1.2	1.6	1.0	1.1
25	1.0	1.0	1.2	1.1	1.1	1.0	1.1
31	1.0	1.1	1.0	1.2	1.0	1.2	1.0
Peptide number, β-chain							
1	1.0	1.0	1.0	0.9	1.0	0.9	1.0
3	0.9	1.0	1.0	0.9	1.0	1.0	1.0
9	0.9	0.9	0.9	0.9	1.0	0.9	0.9
12	1.4	0.8	1.2	1.1	1.1	1.0	0.9
13	1.2	0.8	1.0	0.9	0.9	0.9	1.2
17	1.0	1.0	1.0	1.0	1.0	1.0	1.0
18	0.8	0.9	1.0	0.9	0.9	2.0	0.9
24	1.0	1.0	1.1	0.9	0.9	1.0	1.0
27	1.6	1.4	0.9	0.8	0.8	0.8	0.9
28	30.0	8.0	13.0	0.07	0.01	0.8	0.9

The only α-chain peptide which shows significant deviation from constant ratio

in the expected direction is peptide 16. Unfortunately, the control experiment shows some ratio deviation in peptide 16 (column (*c*)) but not enough to upset the conclusion that peptide 16 is near the carboxyl end of the α-chain. The ratio variation of peptide 16 in the control experiment (Table 4, column (*c*)) may be due to the fact that the proteins were heated at 100° for 15 min to inactivate carboxypeptidase, whereas in previous experiments (Table 3, column (*a*)) this was not done.

In the β-chain none of the major yield peptides showed a significant ratio change, but peptide 28, which was previously sometimes present in minor yield, was present in good yield and clearly showed the behavior expected of a peptide near the carboxyl end of the β-chain. On re-examination of the data from 4- and 7-min H^3-leucine incubations, four clear cases were found where peptide 28 had been present but in low yield. The average tritium content of peptide 28 in these four runs was found to be 1.06 ± 0.27 times the tritium content of peptide 27. Although the average yield of peptide 28 was only 0.18 ± 0.04 times the yield of peptide 27, it is tempting to conclude that peptide 28 is closely related to peptide 27 in the time sequence of labeling with H^3 leucine.

It would thus appear that in both the α- and β-chains those leucine-containing peptides which are the first to be labeled with H^3-leucine in the soluble hemoglobin are nearest to the free carboxyl end of the chain. According to the model shown in Figure 1, this implies that chain growth terminates at or near the free carboxyl end of the molecule.

Discussion.—The NH_2-terminal amino acid of both the α- and β-chains of rabbit hemoglobin is valine.[11] Attempts have been reported to find the rate of short time radioactive labeling of the NH_2-terminal valine relative to the average of all other valines in the hemoglobin molecule. Using whole rabbit reticulocytes, Loftfield[2] reported results indicating that the NH_2-terminal valine is labeled last. On the other hand, Bishop *et al.*,[12] using a cell-free system from rabbit reticulocytes, reported results indicating that the NH_2-terminal valine is labeled first. Reports on other protein-synthesizing systems are equally conflicting. Thus the work of Yoshida and Tobita[13] on bacterial amylase indicates that synthesis proceeds from the amino-terminal toward the carboxyl-terminal end. Complications are present in the interpretation of their work because of the very long times of incubation involved and the presence of various protein precursor pools. Shimura *et al.*[14] using the fibroin synthesizing gland of the silk worm obtained results indicating that the NH_2-terminal glycine is added last.

Muir *et al.*[15] reported finding uniform labeling in hemoglobin labeled *in vivo*. This is to be expected from the results reported in this paper. Thus Figure 5 shows that labeling is uniform within 20 per cent after 60 min of incubation at 15°, corresponding to 15 min of incubation at 37°. Kruh *et al.*[16] have reported nonuniform labeling in hemoglobin after very long *in vivo* experiments. This result is not consistent with the data reported in this paper and possibly represents phenomena different from the original synthesis.

A different approach was used by Loftfield and Eigner who reported kinetics of amino acid incorporation into ferritin[17] and hemoglobin[2] after short times of incubation. Their data indicate that for the first few minutes of labeling the specific activity of newly formed protein increases as the square of the time, becoming linear only after several minutes. From these data they concluded that a scheme

essentially the same as that of Figure 1 is indicated. However, this result cannot distinguish between a random and a sequential process of attaching amino acids to the template.

It is perhaps worth noting that if the model shown in Figure 1 is finally proved to be correct, then the experimental technique described in this paper could be useful for structure determination. Thus, it should be possible to determine the spatial sequence of tryptic peptides in proteins of unknown structure by determining the time order of labeling.

It has previously been reported[8] that to account for the production of new hemoglobin in living rabbit reticulocytes, each ribosomal particle must, on the average, make one polypeptide chain of hemoglobin in 1.5 min. That result was obtained by dividing the total rate of hemoglobin synthesis by the total number of ribosomal particles. From Figure 5 it may be seen that the last peptide on each chain to be labeled receives its label at some time between 4 and 7 min of incubation at 15°. Since the rate of labeling was found to be approximately $^1/_4$ as great at 15° as at 37° (Table 1), this implies that the total time of assembly of each polypeptide chain at 37° is approximately 1.5 min. The agreement between the average rate of synthesis, 1.5 min, and the individual rate of chain synthesis, also 1.5 min, strongly implies that most of the ribosomal particles present in rabbit reticulocytes are, in fact, producing hemoglobin. Since there are approximately 150 amino acid residues in each chain, the average rate of growth is close to two amino acids added per second.

In all of the above discussion a number of possible complications have been ignored because of insufficient data to evaluate their effects. Thus we have ignored the effects of both delay time and dilution of specific activity suffered by labeled leucine during its passage into the cells and subsequent reactions prior to actual peptide bond formation. The fact that we have not needed to invoke these processes to explain the results suggests that the effects are small. Likewise we have ignored the possible existence of hemoglobin in transitory forms between completed polypeptide chains and final soluble hemoglobins. We might imagine, for example, that α-chains and β-chains are produced on separate ribosomal particles and that furthermore single α- and β-chains are insoluble and stay on the ribosomes, while α_2 and β_2 dimers are soluble. This leads to the notion of a small pool of completed chain attached to the ribosome, which would change slightly the results expected in Figure 1, and would lead to a less steep predicted slope in Figure 7.

The figures for this paper have been drawn with uniform spacing between adjacent peptides. This, of course, does not imply that the labelled amino acids are uniformly spaced along the actual polypeptide chain. When the actual sequence of the peptide chains is determined we shall be in a position to plot the relative amount of labeling in each amino acid against its position in the chain. Only when that is done will it be worthwhile to consider the detailed shape of the curves for evidence concerning uniformity of growth rate along the polypeptide chain.

In summary it may be concluded that the growth of the peptide chains of hemoglobin is not a random process but a steady sequential addition of amino acids to growing chains at the rate of approximately two amino acids per second. The number of initiation points per chain is, at most, very small and most likely only one.

The chain growth terminates near or at the free carboxyl end. Taken together, these conclusions indicate that chain growth proceeds steadily from the free amino end toward the free carboxyl end in rabbit hemoglobin.

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[1] Steinberg, D., M. Vaughan, and G. B. Anfinsen, *Science*, **124**, 389 (1956).
[2] Loftfield, R., *Proc. 4th Intern. Congr. Biochem.*, **8**, 222 (1960).
[3] Borsook, H., *Proc. 3rd Intern. Congr. Biochem.*, **92** (1956).
[4] Dibble, W. E., and H. M. Dintzis, *Biochim. et Biophys. Acta*, **37**, 152 (1960).
[5] Kruh, J., and H. Borsook, *J. Biol. Chem.*, **220**, 905 (1956).
[6] Borsook, H., E. H. Fischer, and G. Keighley, *J. Biol. Chem.*, **229**, 1059 (1957).
[7] Ingram, V. M., *Biochim. et Biophys. Acta*, **28**, 539 (1958).
[8] Dintzis, H., H. Borsook, and J. Vinograd, in *Microsomal Particles and Protein Synthesis*, ed. R. B. Roberts (New York: Pergamon Press, 1958), p. 95.
[9] Wilson, S., and D. B. Smith, *Can. J. Biochem. and Physiol.*, **37**, 405 (1959).
[10] Guidotti, G., *Biochim. et Biophys. Acta.* **42**, 177 (1960).
[11] Osawa, H., and K. Satake, *J. Biochem. (Tokyo)*, **42**, 905 (1956).
[12] Bishop, J., J. Leaky, and R. Schweet, these PROCEEDINGS, **46**, 1030 (1960).
[13] Yoshida, A., and T. Tobita, *Biochim. et Biophys. Acta*, **37**, 513 (1960).
[14] Shimura, K., H. Fukai, J. Sato, and R. Saeki, *J. Biochem. (Tokyo)*, **43**, 101 (1956).
[15] Muir, H., A. Neuberger, and J. Perrone, *Biochem. J.*, **52**, 87 (1952).
[16] Kruh, J., J. Dreyfus, and G. Schapira, *J. Biol. Chem.*, **235**, 1075 (1960).
[17] Loftfield, R. B., and E. A. Eigner, *J. Biol. Chem.*, **231**, 925 (1958).

ANALYSIS OF SEQUENCE PATTERNS IN RIBONUCLEASE, II. PRIMITIVE GROUPS, THEIR COORDINATIONS, AND PERIODICITY

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A previous paper[1] introduced a new method, *vectorial analysis*, for investigating sequence patterns in proteins. In its primary application to beef pancreatic ribonuclease,[2] the analysis proceeded as follows:

1. The amino acids of ribonuclease were classified in three groups to provide a basis for discerning analogies among suitable nonidentical subsequences.

2. In certain pairs of analogous subsequences, the constituent residues were ordered in the same direction in the molecular chain. In other pairs, the orders of the residues were mutually inverted. Each of these order relations was described by an *analogue sequence vector*, which was written as an arrow along each of the analogous subsequences and directed to conform to the order of the residues regardless of the peptide-bond orientation. With a selected subsequence as vectorial reference, it was possible to deduce a consistent set of vectors, together constituting a *vector map*, that covered most of the chain. This incomplete *analogue map* showed several points of vector inversion, or *vector breaks*.

3. Attention was next directed to 30 distinct kinds of repeating dipeptides, called *repeaters*, which had been ignored in the foregoing analysis. Two classes of re-