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SEQUENTIAL BIOSYNTHESIS OF THE PEPTIDE CHAINS OF HEMOGLOBIN

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Measurements in living cells and cell-free systems have recently been used to study the synthesis of the peptide chains of hemoglobin. The results of Bishop, Leahy, and Schweet[1] in a cell-free system indicate clearly that the NH_2-terminal amino acid is incorporated very early in the synthesis of the polypeptide chain. Schapira *et al.*[2] have demonstrated that a significant difference exists in the specific activity of arginine in various tryptic peptides of rabbit hemoglobin from cells incubated for a short time with C^{14}-arginine.

In a previous communication,[3] it was concluded that in living cells the polypeptide chains of rabbit hemoglobin grow sequentially from the free amino end toward the free carboxyl end. That conclusion was based on the time-course of labeling of various peptide fragments isolated from rabbit globin after incubation of whole rabbit reticulocytes with radioactive leucine. Since most of the data presented previously were limited to time measurements, they did not constitute a rigorous proof of the sequential assembly of amino acids into hemoglobin although they were in complete agreement with such a model. Here we present a more complete demonstration of the sequential assembly model by showing that the time-course labeling data are in agreement with the actual sequence of amino acids in rabbit hemoglobin.

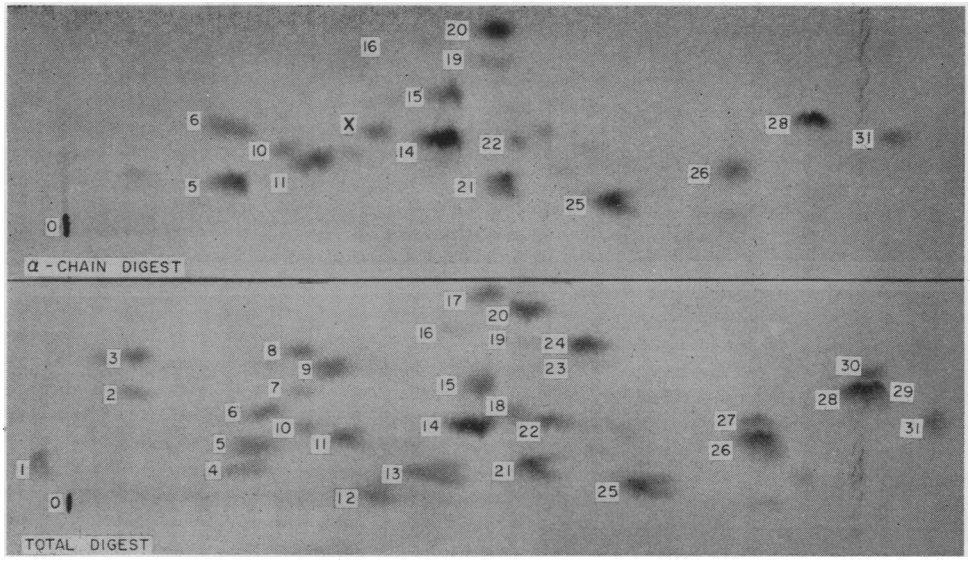

FIG. 1.—Peptide maps of tryptic digest of total rabbit globin (bottom) and column-separated α chain (top). The point of application of digest to the paper is marked by 0. The positive electrode is to the left. This figure is identical to Figure 3 of reference 1, except for the identification of peptide X.

The complete amino acid sequence of human hemoglobin has been determined through the efforts of workers in two laboratories.[4, 5] In addition, the compositions of the tryptic peptides of the α chain of horse and rabbit hemoglobins have been reported.[6, 7] The compositions of the peptides obtained by tryptic digestion of human, horse, and rabbit hemoglobin α chains were remarkably similar, and it was possible to match corresponding peptides of the three species on the basis of the total number of amino acids in each peptide and their amino acid compositions. In the present paper, we present the results of matching corresponding tryptic peptides from both α and β chains of rabbit and human hemoglobin.

General Experimental Considerations.—Figure 1 is identical to Figure 3 of reference 3 and represents the separation of peptides used for the time-course labeling reported previously. That separation was done on about 2 mg, i.e., approximately 0.1 μmole, of peptide digest of individual chains by a method involving paper electrophoresis and chromatography at right angles. For the purposes of the present experiment, the method was modified to allow the separation of peptides prepared from quantities of single hemoglobin chain as large as 5 μmoles (85 mg). The yield of high purity peptides was 1 μmole or more. The preparation of α and β chains of rabbit globin and their tryptic digestion was conducted as before.[3]

Paper Electrophoresis.—For a single peptide separation, 60 to 90 mg of the tryptic digest of a single chain of rabbit globin were applied along a 12-in. line on prewet Whatman No. 3MM paper. Cooled-plate electrophoresis was carried out for a time long enough to move the fastest peptide approximately 30 in. from the origin. The paper was then dried, placed upon a clean glass plate, and marked with a pencil along its center in the direction of electrophoresis. To raise the paper from the plate, glass rods were then placed under it on either side of the pencil line. The long edges of the paper were then carefully wet with a stream of $2^1/_2$ per cent formic acid from a plastic wash bottle. The wetting was done symmetrically so as to wash the peptides from both sides in toward the center of the paper. The outer edges were then carefully blotted and the paper hung up to dry. After the paper had dried, it was trimmed along lines 2 in. on either side of the pencil line.

Chromatography.—By making use of a sewing machine which made a zigzag stitch, a 12-in.

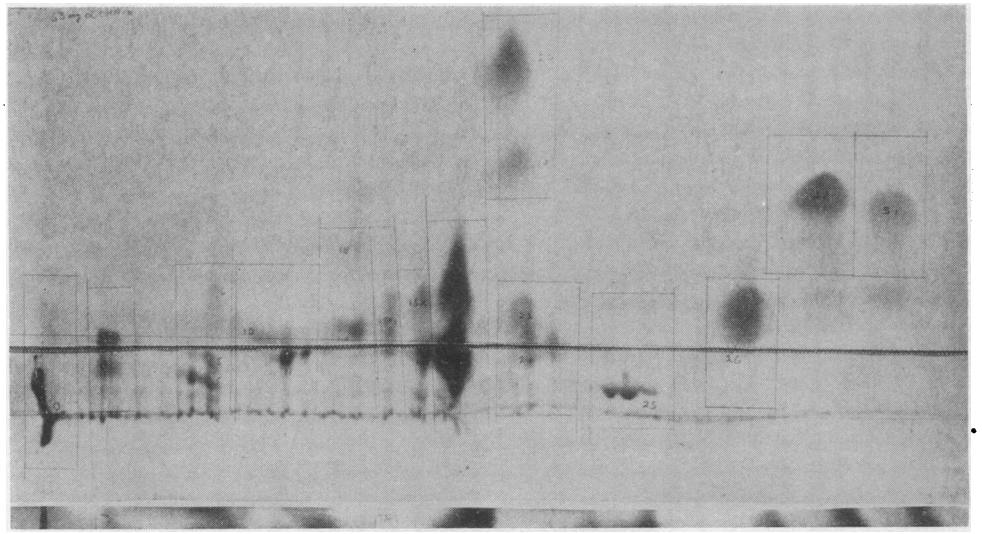

FIG. 2.—Separation of peptides from tryptic digest of 68 mg of α chain. The bottom band is a "guide strip" sliced off the edge of the paper after the first electrophoretic separation.

wide sheet of Whatman No. 3MM filter paper was sewn alongside a 4-in.-wide strip of paper prepared as above. The papers were then trimmed to a length of 33 in. and stapled into the shape of cylinders 16 in. high. These were chromatographed at room temperature as before.[3] With the quantities of peptide employed, it was found necessary to continue the chromatography until the top of the paper was thoroughly wet (60 to 72 hr) in order to insure a good separation.

A Third Dimension of Separation by Means of Paper Electrophoresis at a New pH.—After drying, the chromatograms were dipped in 0.025 per cent ninhydrin in acetone, dried, and allowed to stand in the dark overnight. The blue spots which appeared (Fig. 2) were outlined by pencil marks, numbered by reference to the analytical peptide map shown in Figure 1 and then cut out and thoroughly washed free of excess ninhydrin with acetone. These paper areas were then sewn on to a piece of Whatman No. 3MM filter paper, 36 in. long and 18 in. wide by means of zigzag

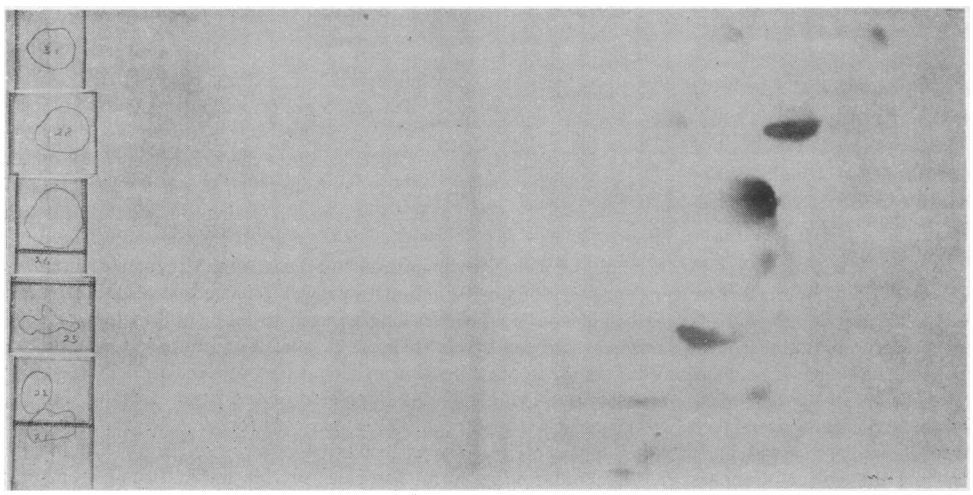

FIG. 3.—A third separation of peptides by means of paper electrophoresis at pH 1.9. This separation was performed on each peptide. The positions of the spots, as originally stitched onto the paper before electrophoresis began are marked at the left (see text for further explanation).

stitching (Fig. 3). After this sewing, the original filter paper behind the numbered areas was cut away, leaving a single layer of filter paper connecting two pieces of clean Whatman No. 3MM paper.[8] The resultant hybrid sheet was then folded along the centers of the numbered areas, placed on a clean glass plate over glass rods, and wet with a solution of 2.5 per cent formic acid and 8.7 per cent acetic acid.[9] The wetting was done symmetrically from both sides of the strips so as to wash the peptides toward the center line. When the advancing liquid fronts had met, the paper outside the strips was thoroughly blotted. Electrophoresis was carried out at 5,000 volts for one hr in a tank of Varsol (Standard Oil Company), after which the paper was dried, dipped in 0.025 per cent ninhydrin, and allowed to stand in the dark for 12 hr. The resulting spots (Fig. 3) were outlined with pencil markings in the shape of a wedge, numbered, and cut out. Each paper wedge was washed free of excess ninhydrin with acetone, dried, and eluted with 6 *N* HCl into a small test tube. The eluate (approximately 0.5 ml) was sealed in the test tube by closing off the tube top in an oxygen flame. The ampoules thus formed were heated for 18 hr in an oven at 105°C to hydrolyze the peptides. They were then opened and the contents dried over soda lime in a vacuum desiccator.

The experimental methods described above are a relatively fast and simple way of preparing peptides in quantities large enough for reliable analysis. The peptides isolated may be conveniently indexed with respect to the corresponding peptides on a conventional analytical two-dimensional electrophoresis-chromatography separation.

Amino Acid Analysis.—The contents of each vial were dissolved in 0.10 ml of water. Of the resulting solution, 0.010 ml was used for amino acid analysis by the paper electrophoresis method previously described.[8] This procedure served to give a qualitative and semiquantitative analysis of each peptide and indicated those peptides which were present in sufficient quantity to permit analysis in the Beckman Spinco Model 120 Amino Acid Analyzer.

Results.—From a comparison of the amino acid analyses obtained from the tryptic peptides of rabbit hemoglobin with those previously reported for human hemoglobin, it was possible to match corresponding peptides in terms of two separate parameters. The first of these was the total of amino acids found in a given peptide. The second was the actual amino acid composition of the individual peptide. In a number of cases, the match between rabbit peptides and human peptides was perfect, both with regard to the total length of the peptide and to its exact composition. In a number of other cases, the length corresponded precisely, but there were one or more changes in composition. In a few cases, the lengths did not correspond and other criteria had to be used to position the peptides. These will be discussed in detail below.

The degree of correspondence between rabbit and human peptides is shown in Table 1, which is arranged so that the left-to-right ordering of the peptides corresponds to their spatial sequence in the polypeptide chain from free amino end to free carboxyl end. In the conventional nomenclature,[10] the tryptic peptides of the human α chain are numbered from amino to carboxyl end α TR I, II, III–XIV, while those of the β chain are numbered β TR I, II, III–XV.

In several cases, owing to incomplete digestion by trypsin, rabbit peptides have been found which contain two lysines. Thus, for example, rabbit peptide 21, containing the amino acids of rabbit peptides 10 plus 26, corresponds to human α TR I plus α TR II. The same situation is found in human hemoglobin peptide maps and probably results from the slow rate of hydrolysis at a lysine residue preceded by an aspartic acid. Another example of partial digestion is represented by rabbit peptides 25 and 18 (Tables 1A and 1B) which correspond respectively to human α TR VIII plus α TR IX and β TR VIII plus β TR IX. In both of these cases, there is a lysine-lysine sequence in human hemoglobin. Assuming the same sequence to exist in rabbit hemoglobin, if trypsin splits after the second lysine, then

TABLE 1A

AMINO ACID COMPOSITION OF α CHAIN PEPTIDES

Chemical Order (Refs. ŧ, 5, 10)	I		II		III		IV		V		VI		VII		VIII		IX			X		XI		XII		XIII		XIV	
	R	H	R	H	R	H	R	H	R	H	R	H	R	H	R	H	R^1	R^2	H	R	H	R	H	R	H	R	H	R	H
Lys	1	1	1	1	—	1	—	—	1	1	—	1	1	1	1	1	1	1	1	—	—	1	1	(2)	1	—	1	—	—
His	—	—	—	—	—	—	1	1	—	—	—	2	1	1	—	—	—	2	3	—	—	—	—	(3)	3	—	—	—	—
Arg	—	—	—	—	—	—	1	1	—	—	—	—	—	—	—	—	—	—	—	1	1	—	—	—	—	—	—	1	1
Asp	1	1	1	1	—	—	—	—	—	—	—	1	—	—	—	—	—	3	6	—	—	2	2	(2)	1	—	—	—	—
Thr	—	—	1	1	—	—	—	—	2	2	—	1	—	—	—	—	1	1	1	—	—	—	—	(3)	2	—	2	—	—
Ser	1	1	—	—	—	—	1	—	—	1	—	2	—	—	—	—	1	2	2	—	—	—	—	(2)	2	—	3	—	—
Glu	—	—	—	—	—	—	3	3	—	—	—	1	—	—	—	—	1	—	—	—	—	—	—	(3)	1	—	—	—	—
Pro	1	1	—	—	—	—	—	—	1	1	—	1	—	—	—	—	—	1	1	—	—	1	1	(2)	2	—	—	—	—
Gly	—	—	—	—	—	1	4	3	1	—	—	1	1	2	—	—	—	2	—	—	—	—	—	(4)	—	—	—	—	—
Ala	1	1	—	—	—	2	2	4	—	—	—	1	1	—	—	—	1	4	7	—	—	—	—	—	5	—	1	—	—
½ Cys	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	(1)	1	—	—	—	—
Val	1	1	—	1	—	—	1	1	—	—	—	1	—	—	—	—	1	1	3	—	—	2	2	—	2	—	2	—	—
Met	—	—	—	—	—	—	—	—	1	1	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
Isol	—	—	1	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	(2)	—	—	—	—	—
Leu	1	1	—	—	—	—	—	1	1	1	—	1	—	—	—	—	1	5	4	1	1	—	—	(1)	7	—	2	—	—
Tyr	—	—	—	—	—	—	1	1	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	(1)	—	—	—	1	1
Phe	—	—	—	—	—	—	—	—	2	2	—	2	—	—	—	—	—	—	—	—	—	1	1	(4)	1	—	1	—	—
Try	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
Total	7	7	4	4	—	5	15	15	9	9	—	16	4	4	1	1	7	22	29	2	2	7	7	(29)	28	—	12	2	2
Rabbit peptide number (Fig. 1)	10		26		—		5		19, 20		—		33		36		11	14		31		6		15		—		30	
Time order number (ref. 3), encircled	②								③								⑤	⑥		⑦									
	21 ①														25 ④														

TABLE 1B

AMINO ACID COMPOSITION OF β CHAIN PEPTIDES

Chemical Order (Ref. 4, 5, 10)	—I— R	H	—II— R	H	—III— R	H	—IV— R	H	—V— R	H	—VI— R	H	—VII— R	H	—VIII— R	H	—IX— R	H	—X— R	H	—XI— R	H	—XII— R	H	—XIII— R	H	—XIV— R	H	—XV— R	H
Lys	1	1	1	1	—	—	—	—	1	1	1	1	1	1	1	1	1	1	1	1	—	—	—	1	1	1	1	1	—	—
His	1	1	—	—	—	—	—	—	—	—	—	—	1	1	—	—	1	1	1	1	—	1	—	2	—	—	—	1	1	—
Arg	—	—	—	—	1	1	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—
Asp	—	—	—	—	1	2	—	—	4	3	—	—	—	—	—	—	3	3	2	1	—	2	—	1	—	—	1	1	—	—
Thr	—	1	1	1	—	—	1	1	—	1	—	—	—	—	—	—	—	—	—	2	—	—	—	—	1	1	—	—	—	—
Ser	2	—	1	1	—	—	—	—	2	2	—	—	—	—	—	—	1	1	2	1	—	—	—	—	—	—	—	—	—	—
Glu	2	2	—	—	3	2	1	1	1	1	—	—	—	—	—	—	1	—	1	1	—	1	—	—	4	3	—	—	—	—
Pro	—	1	—	—	—	—	1	1	1	2	—	—	—	—	—	—	—	—	—	—	—	1	—	—	1	2	—	—	—	—
Gly	—	—	1	1	3	3	—	—	1	2	—	—	1	2	—	—	1	2	1	1	—	—	—	2	—	—	1	1	—	—
Ala	—	—	2	2	1	1	—	—	2	1	—	—	1	–	—	—	2	2	2	1	—	—	—	1	2	2	4	4	—	—
½ Cys	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	1	—	—	—	—	—	—
Val	1	1	1	1	3	3	2	2	1	1	1	1	—	—	—	—	1	1	1	—	—	1	—	3	—	—	3	3	—	—
Met	—	—	—	—	—	—	—	—	+	1	—	—	—	—	—	—	—	—	+	—	—	—	—	—	—	—	—	—	—	—
Leu	1	1	1	1	1	1	2	2	2	1	—	—	—	—	—	—	3	4	2	2	—	1	—	4	—	—	1	1	—	—
Tyr	—	—	—	—	—	—	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1	—	—	1	1
Phe	—	—	—	—	—	—	—	—	3	3	—	—	—	—	—	—	1	1	1	1	—	1	—	1	1	1	—	—	—	—
Try	—	—	1	1	—	—	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total	8	8	9	9	13	13	10	10	19	19	2	2	4	4	1	1	15	16	15	13	—	9	—	16	12	12	12	12	2	2
Rabbit peptide number (Fig. 1)	13		24		1		17		2, 3		32		33		36		9		7, 8		—		—		4		27b		30	
Time order number (ref. 3), encircled	①		②		③		④		⑤		—		—		—		⑥		—		—		—		—		⑨		—	
															18 ⑦												28 ⑨			

Chemical order refers to the listing (across the top) of the human peptides, H, beginning with the NH_2-terminal peptide (I). The rabbit peptides, R, are matched to the human peptides as described in the text. The compositions of rabbit peptides 10, 26, 5, 19, 20, 33, 36, 11, 31, 6, and 30 of the α chain agree exactly with those reported by Diamond and Braunitzer,[7] while peptide 14 disagrees with their analysis in apparently having one fewer histidine and one extra alanine. Peptide 15 agrees neither with their results nor with any known human sequence; it may perhaps represent a mixture of peptides.

Our failure to detect half-cystine residues in the β-chain of rabbit hemoglobin should not be taken as an indication of their absence. They would not be likely to be observed under the conditions of our experiments.

rabbit peptides 11 and 9 are produced with subsequent partial liberation of one of the lysines as the free amino acid (Peptide 36). However, if trypsin splits between the two lysines, then rabbit peptides 25 and 18 are produced. These peptides contain a lysine at either end. A similar situation in human hemoglobin peptide maps has been reported by Baglioni.[11] Another type of incomplete digestion occurs near the carboxyl end of the β chain of rabbit globin where the terminal peptide, Tyr-His, is not completely split off, giving rise to rabbit peptide 28, corresponding to α TR XIV plus α TR XV in human hemoglobin. A comparable case of incomplete digestion of a COOH-terminal Tyr-His peptide has been reported in the γ chain of human fetal hemoglobin by Schroeder *et al.*[12] These peptide sequences resulting from incomplete tryptic digestion of rabbit globin provide additional evidence for the order of alignment which has been proposed by analogy with the human peptides.

In Table 1A, rabbit peptide 15 has been placed to correspond with human peptide XII although it is clear that the agreement is not good. However, the size of peptide 15 and its cysteine content make it difficult to place elsewhere. Moreover, recent measurements based on leucine label[13] place both peptides 15 and X (Val,

TABLE 2

COLOR STAINS FOR SPECIFIC AMINO ACIDS

Peptide	Chain	Human peptide correspondence	Met	Try	His	Tyr	Arg	Remarks
1	β	βT3	—	—	—	—	X	
2 ⎫	β	βT5	X	—	—	—	—	Peptide 2 = 3
3 ⎭								
4	β	βT13	—	—	—	X	—	
5	α	αT4	—	—	X	X	X	
6A	α	(αT1 + αT2)	—	—	—	—	—	Loss of amide from 21?
6B	α	αT11	—	—	—	—	—	
7 ⎫	β	βT10	X	—	X	—	—	Peptide 7 = 8
8 ⎭								
9	β	βT9	—	—	X	—	—	
10	α	αT1	—	—	—	—	—	
11	α	αT9 (a)	—	—	—	—	—	
X	α	?	—	—	—	—	—	(Val, leu, asp) arg
12	β	—	—	—	—	—	—	
13	β	βT1	—	—	(X)	—	—	
14	α	αT9 (b)	—	X	X	—	—	
15	α	Region of αT12–13	—	—	X	X	—	Positioned by cysteine
16	α	?	—	—	—	—	—	(Thr_2 Ser_{0-1} Gly_{1-2} Leu_1 Phe_1) Lys
17	β	βT4	—	X	—	X	(X)	
18	β	βT8 + βT9	—	—	—	—	—	
19 ⎫	α	αT5	X	—	—	—	—	Peptide 19 = 20
20 ⎭								
21	α	(αT1 + αT2)	—	—	—	—	—	
22	α	—	—	—	—	—	—	
23	β	—	—	—	—	—	—	
24	β	βT2	—	X	—	—	—	
25	α	(αT8 + αT9a)	—	—	—	—	—	
26	α	αT2	—	—	—	—	—	
27a	β	βT14	—	—	X	—	—	
27b	β	?	—	—	—	—	—	(Phe, ala, gly, thr)lys
28	β	βT14 + βT15	—	—	X	X	X	
30	α + β	αT14 + βT15	—	—	X	X	X	
31	α	αT10	—	—	—	—	X	
32	β	βT6	—	—	—	—	—	
33	α + β	αT7 + βT7	—	—	X	—	—	
36 (34)	α + β	αT8 + βT8	—	—	—	—	—	

Color staining procedures were obtained from *Paper Chromatography and Paper Electrophoresis* by R. J. Block, E. L. Durrum, and G. Zweig, Academic Press, Inc. (New York: 1958), pp. 128–139.

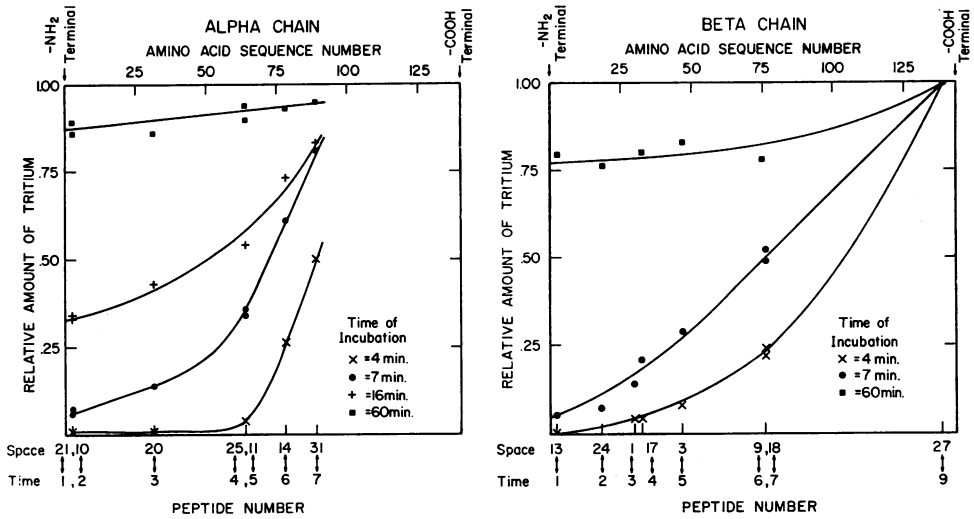

FIG. 4.—This figure represents the data of Figure 5, reference 3, replotted to indicate the chemical sequence position of each rabbit peptide, where known. The peptides are positioned by assuming that the leucines have the same sequence numbers in rabbit and human hemoglobins. If more than one leucine is present in the human peptide, an arithmetic average sequence number is used to indicate the peptide position. Space peptide number refers to the numbering scheme of Figure 1. Time peptide number indicates the order of assembly reported in reference 3.

Leu, Asp, Arg) in the region of human peptides XII and XIII, a 40 amino acid section which should correspond to rabbit peptides 15, 16, 22, and X.

Table 2 shows the correspondence between the tryptic peptides of rabbit hemoglobin numbered as in Figure 1 and the tryptic peptides of human hemoglobin. All the rabbit peptides listed gave ninhydrin stains, and the observed color stains for specific amino acids are indicated. Rabbit peptides 6 and 27, which appeared as a single spot on the original fingerprint, were found to consist of two peptides after being subjected to electrophoresis at pH 1.9. One of these peptides (6A) has the same composition as peptide 21, the difference in position possibly being due to loss of an amide group from peptide 21. In the three cases where a peptide contained methionine, a doubling of the spot was found after the chromatographic separation, presumably due to partial methionine sulfoxide formation (peptides 2 and 3, 7 and 8, 19 and 20).

Discussion.—The work reported in this and the preceding paper was done in an effort to determine the sequence of assembly of the polypeptide chains in rabbit hemoglobin. At the bottom of Table 1 is indicated the previously reported ordering of the tryptic peptides[3] based on the time-course of labeling after incubation of living cells with radioactive leucine. It is important to note that for those peptides previously placed in a time sequence the time sequence numbers at the bottom of Table 1 (in circles) and the chemical sequence numbers (Roman numerals) at the top of Table 1 both increase consistently from left to right. These results are summarized in Figure 4, where it may be seen that of the peptides previously ordered in time of synthesis, 7 of the 9 from the α chain and 8 of the 9 from the β chain had been placed in the correct chemical order. The three remaining rabbit peptides (22, 16, and 12) have not yet been isolated in sufficient quantity to obtain amino acid

compositions. The correspondence of ordering is sufficiently good to establish for rabbit hemoglobin the model of sequential growth of polypeptide chains from amino end to carboxyl end.

Evidence now accumulating from many sources indicates that this mechanism of protein synthesis is very general if not universal. Cell-free studies by Bishop, Leahy, and Schweet[1] suggest that polypeptide chain synthesis begins at the amino end of the chain in rabbit hemoglobin. Studies on intact cells of *Escherichia coli* by Goldstein and Brown[14] indicate that most if not all of the proteins of these bacteria terminate their synthesis at the carboxyl end of the polypeptide chain. In addition, evidence specifically indicating sequential polypeptide chain growth from amino end to carboxyl end has been reported by Yoshida and Tobita[15] for a bacterial amylase and recently by Canfield and Anfinsen for egg white lysozyme.[16]

Summary.—In the case of rabbit hemoglobin made by living cells, the notion that protein synthesis occurs by stepwise sequential addition of amino acids to a polypeptide chain growing from the NH_2-terminal toward the COOH-terminal end has been verified for 5 peptides of the α chain and 7 peptides of the β chain.

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