The Amino Acid Sequence of the α Chain of Human Fetal Hemoglobin*

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The 141 residues of the α^F chain of human hemoglobin F have been shown to have the same amino acid sequence as those of the α^A chain of human hemoglobin A.

Because both human adult and fetal hemoglobins contain polypeptide chains that are N-terminal in the sequence val-leu, Schroeder and Matsuda (1958) suggested that these chains (since termed α^A and α^F chains) have identical sequence in the two proteins. Shelton and Schroeder (1960) showed that the sequence of α^A and α^F chains was identical in the first six residues from the N-terminus. Other evidence of identity was obtained from tryptic peptide patterns of the two hemoglobins (Hunt, 1959) and from subunit hybridizations (Jones *et al.*, 1959). Nevertheless, the final proof of identity requires a comparison of the entire amino acid sequence of the two chains.

The goal of the present investigation was the determination of the amino acid sequence of the α^F chain with the material that became available during the investigation of the γ^F chains (Schroeder *et al.*, 1963). We have considered that this end would be attained by the isolation of peptides in sufficient number to match the entire reported sequence of the α^A chain (Braunitzer *et al.*, 1961; Hill and Konigsberg, 1962).

EXPERIMENTAL

The experimental procedures have been detailed by Schroeder *et al.* (1963). Hemoglobin F and α^F chains were products of the procedures for the isolation of γ chains. Chemical and enzymatic methods, the isolation of peptides, and the determination of the sequence of peptides was carried out as described. The details of the enzymatic hydrolyses, however, need be given.

The tryptic peptides from the α^F chains were isolated from two hydrolysates. One of these was a hydrolysate of hemoglobin F and the other of α^F chains that had been isolated by chromatography and then oxidized.

A 1-g sample of hemoglobin F in 50 ml of water at *p*H 8 was denaturated for 4 minutes at 90°. It was hydrolyzed for 90 minutes at 38° and *p*H 8 by the addition of 5-mg samples of trypsin at the beginning and after 60 minutes of reaction. After the *p*H had been reduced to 6.5, the insoluble portion was centrifuged off and washed with water. The washings were combined with the soluble peptides.

A 420-mg sample of oxidized α^F chains was dissolved in 25 ml of water. When the *p*H was taken to 8, the material precipitated and then coagulated into a single clump. The hydrolysis was carried out for 24 hours at room temperature and *p*H 8 with addition of 2.5-mg samples of trypsin at 0, 3, and 12 hours of reaction. Although the coagulum was broken up mechanically during the reaction, enzymatic action was incomplete on a small portion after 24 hours. Consequently when the *p*H was taken to 6.5 the insoluble material was of two kinds, a finely divided white portion and some of the original. These were washed and the washings were added to the soluble portion.

When 465 mg of oxidized α^F chains was used for chymotryptic digestion, the same problem of coagulation forced unplanned variation in the schedule of hydrolysis. Hydrolysis was begun at *p*H 8 and 30° in 25 ml of water to which 0.8 ml of 0.4 M calcium chloride and 7.5 mg of α-chymotrypsin were added. The reaction was slow and erratic because of small surface area. Consequently, after about 1.5 hours the temperature was raised to 40° and after 2 hours an equal amount of chymotrypsin was added. At the end of 4 hours of hydrolysis, when the reaction was stopped, only traces of material had not dissolved. Likewise, virtually all was in solution after the reagents for chromatography had been added (Schroeder *et al.*, 1962).

RESULTS

Amino Acid Composition of the α^F Chain

As has previously been pointed out (Schroeder *et al.*, 1963), pure α^F chains may be obtained from the chromatographic separation of the chains of globin F. When samples were analyzed after 22 and 70 hours of acidic hydrolysis, the results of Table I were obtained. The data are in excellent agreement with those that are reported for the α^A chain (Braunitzer *et al.*, 1961; Hill *et al.*, 1962) with the exception of serine and threonine. For them the correction for destruction is somewhat less than commonly experienced and hence the extrapolated value is influenced. However, nine residues of threonine and eleven of serine were detected in peptides. The correct value of cys/2 may be fortuitous but the detection of cysteic acid in peptides from the oxidized chain substantiates the result. One tryptophyl residue was detected in peptide form.

Amino Acid Composition of the Tryptic Peptides

The amino acid composition of the soluble tryptic peptides is presented in Table II. The designations follow the scheme that has been employed before (Schroeder *et al.*, 1963) in that the peptides are listed in order from the N-terminus and are preceded by the letter "T" to indicate their origin in a tryptic hydrolysate. Both acidic and LAP[1] hydrolysis were used for T-3 which contained tryptophan. Tryptophan was largely lost in the acidic hydrolysis and survived the LAP hydrolysis but LAP did not completely release glycine and lysine. LAP hydrolysis of T-4 gave an excellent value for tyrosine which was low with acid hydrolysis. A satisfactory value for methionine in T-5 was obtained only after oxidation to the sulfone.

The amino acid composition of these tryptic peptides agrees with that of the corresponding peptides from the α^A chain (Braunitzer *et al.*, 1961; Guidotti *et al.*, 1962).

Amino Acid Sequence of the α^F Chain

The amino acid sequence in the α^F chain is presented in Figure 1. In presenting the basis for this formula-

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[1] Abbreviations used in this paper: LAP, leucine aminopeptidase; PTH, phenylthiohydantoin.

TABLE I

AMINO ACID COMPOSITION OF α^F CHAINS

Amino Acid	Residues per Chain 22 hr	70 hr	Avg or Extrap. Value	Reported for α^A Chain[a]
Lysine	10.6	11.2	10.9	11
Histidine	9.6	10.3	10.0	10
Arginine	2.9	3.1	3.0	3
Aspartic acid	11.6	12.2	11.9	12
Threonine	1.4	8.3	8.4	9
Serine	9.5	8.6	9.9	11
Glutamic acid	4.8	4.9	4.9	5
Proline	6.8	7.2	7.0	7
Glycine	6.8	7.1	7.0	7
Alanine	20.3	20.9	20.6	21
Cystine/2	1.0	0.8	0.9	1
Valine	12.0	13.1	13.1	13
Methionine	1.9	1.9	1.9	2
Isoleucine	0.0	0.0	0.0	0
Leucine	17.3	18.0	17.7	18
Tyrosine	2.8	2.7	2.8	3
Phenylalanine	6.7	6.9	6.8	7
Tryptophan				1

[a] Braunitzer *et al.* (1961) and Hill *et al.* (1962).

tion, the conventions and abbreviations previously employed (Schroeder *et al.*, 1963) will be used.

T-1,2: val-leu-ser-pro-ala-asp-lys-thr-aspNH$_2$-val-lys.—In a 90-minute tryptic digest, peptide T-1,2 is in greater yield than T-1 and T-2 and is more readily purified. Edman degradation gave the entire sequence, thus:

val–leu–ser–pro–ala–asp–lys–thr–aspNH$_2$–val–lys

Because the N-terminal sequence of the α^F chain is

val–leu–ser–pro–ala–asp–

and T-1,2 is the only tryptic peptide with this sequence, it must be at the N-terminus of the α^F chain.

T-3: ala-ala-try-gly-lys.—Again the Edman degradation gave the entire sequence: ala-ala-try-gly-lys. No chymotryptic peptide has been found to link T-1,2 to T-3. Hydrolysis on the C-terminal side of tryptophan would be expected but oxidation no doubt destroyed tryptophan. The peptide with altered tryptophan has not been detected.

T-4: val-gly-ala-his-ala-gly-glu-tyr-gly-ala-glu-ala-leu-glu-arg.—The Edman degradation gave the sequence val–gly–ala–his–(ala$_3$, gly$_2$, glu$_3$, tyr, leu, arg) and the following chymotryptic peptides completed the sequence:

C-2 1.03 1.04 0.99 0.85
ala–gly–glu–tyr
Dowex-50-*p*H 3.8; Dowex-1-(II)-*p*H 2.7

C-3 1.00 1.00 1.01 1.00 1.00
gly–ala–glu–ala–leu
Dowex-50-*p*H 3.6; Dowex-1-(II)-*p*H 3.4

C-4 1.04 1.03 0.94 0.96
glu–arg–metSO$_2$–phe 20%
Dowex-50-*p*H 4.35; Dowex-1-(III)-*p*H 4.2

The composition and sequence of these peptides are such that they must have derived from T-4. Peptide C-1 links T-4 to T-3 although not unequivocally

C-1 0.89 1.01 1.00 0.89 0.99 0.95
gly–lys–val–gly–ala–his 12%
Dowex-50-*p*H 4.35; Dowex-1-(III)-*p*H 8.3

because of the gly-lys sequence in T-7.

T-5: met-phe-leu-ser-phe-pro-thr-thr-lys.—The sequence met–phe–leu–ser–phe–pro–(thr$_2$, lys) by degradation in effect completes the sequence of T-5. Chymotryptic peptide C-5 further verifies it.

C-5 0.91 0.85 1.00 0.90 0.90 1.25
ser–phe–pro–thr–thr–lys 26%
Dowex-50-*p*H 4.05; Dowex-1-(II)-*p*H 8.0

Peptide C-4 already described above links T-5 to T-4. The glu-arg sequence is unique in α chain and must come from T-4. The met–phe sequence is also unique in α chain and must come from T-5.

T-6: thr-tyr-phe-pro-his-phe-asp-leu-ser-his-gly-ser-ala-gluNH$_2$-val-lys.—The Edman degradation was successful for seven steps to give thr–tyr–phe–pro–his–phe–asp–(leu, ser$_2$, his, gly, ala, gluNH$_2$, val, lys). Chymotryptic peptide C-6 completed the sequence:

C-6 0.99 1.00 0.82 1.20 1.00 0.82 1.20 0.98 0.97 1.01
asp–leu–ser–his–gly–ser–ala–gluNH$_2$–val–lys
Dowex-50-*p*H 4.00; Dowex-1-(II)-*p*H 7.2

TABLE II

AMINO ACID COMPOSITION OF SOLUBLE TRYPTIC PEPTIDES OF THE α^F CHAIN[a]

	T-1,2	T-3[b]	T-3[c]	T-4[c]	T-5	T-6	T-7	T-8	T-9	T-10	T-11	T-14
Tryptophan		0.39	1.00									
Lysine	2.07	1.01	0.38		1.09	1.00	1.03	Present[d]	1.04		1.02	0.06
Histidine				0.93		2.02	0.97		2.92			
Arginine				1.07						1.04		1.00
Cyteic acid												
Aspartic acid	2.00				0.09	0.99			5.95		1.95	
Methionine sulfone					0.89				0.98			
Threonine	0.85				1.83	0.80			0.94		0.08	
Serine	0.80				0.89	1.74			1.86		0.18	0.08
Glutamic acid				2.85		1.01					0.06	0.08
Proline	1.03				1.07	0.95			1.06		1.15	
Glycine		1.00	0.36	2.91	0.08	1.06	2.00			0.05	0.14	0.15
Alanine	1.02	1.92	1.98	4.03	0.11	0.96			7.09		0.19	0.17
Valine	1.89			0.98	0.09	0.93			3.10		1.97	
Methionine												
Leucine	0.98			1.06	1.00	0.98			4.09	0.96	0.19	0.07
Tyrosine				0.95		0.90						0.81
Phenylalanine					1.83	1.92					0.90	
Dowex-50[e]	4.1	4.5		4.3	4.25	4.75	4.45	3.9	4.0	4.4	3.9	4.6
Dowex-1[e]	III-8.5	II-7.8			II-7.8	II-6.6	III-8.5	II-8.0	II-4.2	III-9.0	II-6.4	

[a] In terms of residues per peptide. A blank space indicates less than 0.05 residue. [b] Acidic hydrolysis. [c] Hydrolysis with leucine aminopeptidase (LAP). [d] By paper chromatography and electrophoresis. [e] The data refer to the *p*H of emergence on Dowex-50 or Dowex-1; the approximate volume of emergence may be inferred from Fig. 1 of Schroeder *et al.* (1963).

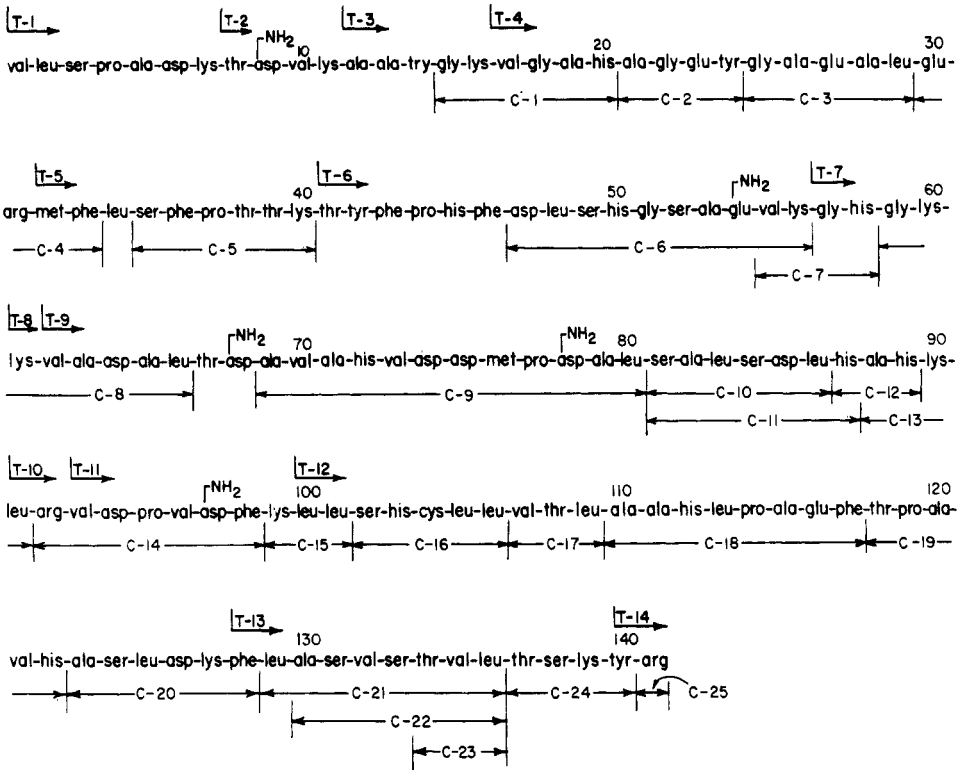

FIG. 1.—The amino acid sequence of the α chain of human hemoglobin F. The positions and designations of the peptides that are discussed in the text are given.

No peptide was isolated which would link T-6 to T-5.

T-7: gly–his–gly–lys.—The sequence followed directly from the degradation: gly–his–gly–lys. The following chymotryptic peptides clearly relate to T-7.

C-7 0.89 1.03 1.12 0.97 val–lys–gly–his 17%
Dowex-50-*p*H 4.4; Dowex-1-(III)-*p*H 8.3

C-8 1.03 1.06 1.06 1.00 0.99 1.00 0.99 0.98 gly–lys–lys–val–ala–asp–ala–leu 12%
Dowex-50-*p*H 4.35; Dowex-1-(III)-*p*H 7.9

Peptide C-7 provides the link between T-6 and T-7. The gly–his portion is unique in the α chain and must come from T-7. The val–lys portion could come from either T-2 or T-6. We assume that the val–lys of C-7 comes from T-6.

T-8: Lysine.—Free lysine was isolated from a tryptic digest of hemoglobin F. Analysis of equal portions of the lysine zone, one hydrolyzed and the other unhydrolyzed, showed equal amounts of lysine. Lysine was also shown to be present in a tryptic digest of α^F chains by electrophoresis and paper chromatography.

T-9: val–ala–asp–ala–leu–thr–aspNH_2–ala–val–ala–his–val–asp–asp–met–pro–aspNH_2–ala–leu–ser–ala–leu–ser–asp–leu–his–ala–his–lys.—The Edman degradation gave the first five residues as val–ala–asp–ala–leu–. When 2.2 μmoles of T-9 were hydrolyzed in 10 ml of refluxing 0.25 M acetic acid for 16 hours and the hydrolysate was chromatographed on a column of Dowex-50, these peptides were isolated:

AA-1 0.97 1.05 0.86 1.03 0.97 0.98 0.97 0.84 0.98 ala–leu–thr–aspNH_2–ala–val–ala–his–val 37%
Dowex-50-*p*H 3.7

AA-2 0.80 1.89 1.07 0.99 (leu, his, ala, lys) 26%
Dowex-50-*p*H 4.6

The chymotrypytic digest of oxidized α^F chains yielded peptides C-9 to C-13.

C-9 0.89 0.99 0.89 0.99 0.99 1.01 1.01 0.92 1.03 1.01 ala–val–ala–his–val–asp–asp(metSO_2, pro, aspNH_2, 0.89 0.80 ala, leu) 17%
Dowex-50-*p*H 3.8; Dowex-1-(II)-*p*H 3.7

C-10 0.77 1.00 1.00 0.77 1.06 1.00 ser–ala–leu–ser–asp–leu
Dowex-50-*p*H 3.65; Dowex-1-(II)-*p*H 3.0

C-11 1.70 1.06 1.88 1.00 0.95 (ser, ala, leu, asp, his) 13%
Dowex-50-*p*H 3.85; Dowex-1-(II)-*p*H 5.0

C-12 0.85 1.09 0.85 his–ala–his
Dowex-50-*p*H 4.45; Dowex-1-(III)-*p*H 6.3

C-13 0.99 0.93 1.08 1.00 ala–his–(lys, leu) 33%
Dowex-50-*p*H 4.55; Dowex-1-(III)-*p*H 8.6

Examination of the above peptides shows that AA-1 overlaps residues 4 and 5 of T-9 and also a good portion of C-9. Thus, the sequence of the first fourteen residues of T-9 is established. Peptides C-10 to C-13, and AA-2 complete the sequence of the last twelve residues of T-9.

In order to resolve the sequence of the methionine, proline, and asparagine (residues 76 to 78), approximately 3 μmoles of oxidized T-9 were digested with papain.[2] After the digest had been evaporated with a stream of air at 40°, it was further hydrolyzed with LAP.[3]

The following peptide was isolated by chromatography.

0.91 0.92 1.13 1.03
metSO_2–pro–aspNH_2–ala
Dowex-50-*p*H 3.6

[2] To the peptide in 200 μl of water were added 20 μl of acetate buffer (0.2 M, *p*H 5.1), 20 μl of 0.1 M potassium cyanide, and 5 μl of papain solution (15 mg/ml.) and the mixture was incubated at 40° for 24 hours.

[3] Conditions for LAP hydrolysis have been given (Schroeder *et al.*, 1963).

The PTH procedure readily gave the above results and completed the sequence of T-9. Peptide C-8 described under T-7 links T-9 to T-7 and T-8.

T-10: leu–arg.—Leucine was shown to be N-terminal by the Edman degradation. Peptide C-13 provides the link between T-10 and T-9. However, peptide T-12 also has an N-terminal leucyl residue; this uncertainty in sequence has not been eliminated.

T-11: val–asp–pro–val–aspNH$_2$–phe–lys.—The entire sequence val–asp–pro–val–aspNH$_2$–phe–lys resulted from the PTH procedure. Peptide C-14 provides the link between T-11 and T-10.

C-14 0.99 1.00 1.04 1.08 1.00 1.04 1.01
arg–val–asp–pro–val–aspNH$_2$–phe
Dowex-50-*p*H 4.2; Dowex-1-(II)-*p*H 4.8

Of the three arginyl residues in α^F chain, that in T-4 has been shown to be N-terminal to methionine and another from T-14 will be shown to be the C-terminal residue of α^F chain. Therefore the arginine in C-14 must come from T-10.

T-12: leu–leu–ser–his–cys–leu–leu–val–thr–leu–ala–ala–his–leu–pro–ala–glu–phe–thr–pro–ala–val–his–ala–ser–leu–asp–lys.—Peptide T-12 was not isolated from the soluble portion of the tryptic digest and therefore is assumed to form part of the insoluble material from a tryptic digest of α^F chains. The composition is based on fragments from a chymotryptic digest of the whole chain. None of the chymotryptic peptides assigned to T-12 fits into any of the soluble peptides. The peptides were arranged in order by comparison with α^A chains. The possibility of difference in sequence within these fragments as compared to similar parts of α^AT-12 has been eliminated. The possible deviation between α^FT-12 and α^AT-12 would then be in the order of the fragments.

The following chymotryptic peptides were assigned to T-12.

C-15 1.03 0.99 0.99
lys–leu–leu
Dowex-50-*p*H 4.35; Dowex-1-(III)-*p*H 8.0

O-C-16 0.96 1.00 0.90 1.00 1.00
ser–his–cySO$_3$H–leu–leu 34%
Dowex-50-*p*H 3.85; Dowex-1-(II)-*p*H 3.9

C-17 1.00 0.88 1.17
val–thr–leu
Dowex-50-*p*H 3.70; Dowex-1-(I)-*p*H 6.2

C-18 0.96 0.96 0.98 1.01 1.05 0.96 0.99 0.92
ala–ala–his–leu–pro–ala–glu–phe
Dowex-50-*p*H 4.05; Dowex-1-(II)-*p*H 4.3

C-19 0.91 1.00 1.05 1.03 0.98
thr–pro–ala–val–his
Dowex-50-*p*H 3.95; Dowex-1-(I)-*p*H 6.5

C-20 1.11 0.76 0.97 1.01 0.99 0.80
ala–ser–leu–asp–lys–phe 13%
Dowex-50-*p*H 4.05; Dowex-1-(II)-*p*H 5.6

T-13: phe–leu–ala–ser–val–ser–thr–val–leu–thr–ser–lys.—T-13 is also part of the insoluble material from a tryptic digest of α^F chains. Its amino acid composition and sequence likewise are based on fragments from the chymotryptic digest of the whole chain.

Peptide C-20 (above) provides the N-terminal phenylalanine of T-13; N-terminal phenylalanine is not present in any other tryptic peptide. The remainder of the sequence is given by these chymotryptic peptides.

C-21 0.99 1.30 1.02 1.01 1.02 0.76 1.01 0.99
leu–ala–ser–val–ser–(thr, val, leu)
Dowex-50-*p*H 3.6; Dowex-1-(II)-*p*H 6.0

C-22 1.12 0.95 0.99 0.95 0.93 0.99 1.00
ala–ser–val–ser–thr–(val, leu)
Dowex-50-*p*H 3.55; Dowex-1-(II)-*p*H 6.8

C-23 0.88 1.00 1.02
thr–val–leu
Dowex-50-*p*H 3.75; Dowex-1-(II)-*p*H 5.7

C-24 1.10 0.68 1.01 0.88
thr–ser–lys–tyr
Dowex-50-*p*H 4.30; Dowex-1-(III)-*p*H 5.6

T-14: tyr–arg.—T-14 is the only peptide that is N-terminal in tyr. Therefore T-14 is linked to the C-terminus of T-13 by C-24. Also, free arginine was isolated from the chymotryptic digest of α^F chain and identified by paper electrophoresis and paper chromatography. Hydrolysis of a bond C-terminal to arginine by chymotrypsin has not been observed. Therefore we may conclude that arginine is the C-terminus of α^F chain.

About twenty other chymotryptic peptides were isolated and investigated. By means of amino acid composition and/or PTH degradation they substantiated the above conclusions but they have not been described because they were smaller fragments of the above peptides.

Discussion

As mentioned above, the goal of this investigation was to show that the sequences of tryptic and chymotryptic peptides from the α^F chain agreed with some part of the reported sequence of the α^A chain. This end has been achieved.

Of the fourteen possible tryptic peptides, the twelve soluble ones from α^F chains have been shown to have amino acid compositions identical to equivalent peptides from the α^A chain. In many instances, the entire sequence of the individual tryptic peptide was determined by the Edman degradation (see T-1,2); in others, the sequence resulted from a combination of data from the tryptic peptide itself and from chymotryptic peptides from the hydrolysate of the whole chain (for example, T-4). The sequences of T-11 and T-12 (the tryptic peptides of the "core") were derived solely from nonoverlapping chymotryptic peptides from the hydrolysate of the entire chain. Although the overlapping sequences are not sufficient to place all residues unambiguously in their assigned positions, all peptides from both hydrolysates may be fitted exactly into the reported sequence of the α^A chain. One is, therefore, justified in drawing the conclusion that the sequences of the α^A and α^F chains are identical.

Because the α^A and α^F chains are identical in amino acid sequence, the differences in the properties of hemoglobins A and F necessarily result from the differences in the sequences of the β^A and γ^F chains. Whether the amino acid sequence per se confers the distinctive properties or whether it influences, in gross or subtle manner, not only the secondary and tertiary structure of the β or γ chain itself but also the secondary and tertiary structure of the associated α chains will be unanswered until the three-dimensional structure is determined.

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Chemical Characterization and Subunit Hybridization of Human Hemoglobin H and Associated Compounds*

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Two abnormal hemoglobin components have been detected in association with thalassemia-hemoglobin H disease. These components, as well as the major hemoglobin component, have been chemically characterized by determination of the amino acid composition, N-terminal amino acid sequence, tryptic peptide patterns, sedimentation coefficients, and subunit hybridization. The abnormal component in larger amount has a subunit formula of β_4; the abnormal component in smaller amount has a subunit formula of γ_4. The major hemoglobin component could not be distinguished chemically from normal hemoglobin A. Subunit hybridization studies of hemoglobins indicate that the affinities of the various subunits for one another are not equal.

Human hemoglobin H was first reported by Rigas *et al.* (1955) and was also observed independently by Gouttas *et al.* (1955) and by Motulsky (1956) about the same time. This abnormal hemoglobin has been described from hematological, chemical, and genetic points of view by these and other authors (Rigas *et al.*, 1956; Minnich *et al.*, 1958; Bergren and Sturgeon, 1960; Huehns *et al.*, 1960; Koler and Rigas, 1961; Rigas and Koler, 1961; Buhler and Rigas, 1962; Benesch and Benesch, 1962). The chemical properties and mode of inheritance of the abnormal hemoglobins associated with thalassemia-hemoglobin H disease are quite unusual in comparison to other hemoglobins. However, knowledge of the gross polypeptide structure of the hemoglobin components that are associated with this disease has resulted in explanations for the unusual chemical and genetic properties of these hemoglobins. The purpose of this paper is to present in detail the experimental results which have been briefly described earlier (Jones *et al.*, 1959a; Jones and Schroeder, 1960; Sturgeon *et al.*, 1961) and which have led to the elucidation of the gross chemical structure of two abnormal hemoglobin components that are associated with thalassemia-hemoglobin H disease.

EXPERIMENTAL

Preparation of Hemoglobin Solutions.—Samples of blood were obtained from members of two apparently unrelated families that have been described by Rigas *et al.* (1956) and by Bergren and Sturgeon (1960). The blood was preserved in Alsever's solution and transported either by air mail from Portland to Pasadena or in ice from Hollywood to Pasadena. Solutions of hemoglobin were prepared from the cells by the procedure of Clegg and Schroeder (1959) with only minor modification: the volume of toluene was only 0.1 that of the cells.

Preparation of Radioactive Hemoglobins.—Radioactive hemoglobins were prepared by the procedure of Borsook *et al.* (1952) as modified by Vinograd and Hutchinson (1960). The radioactive amino acid in all instances was L-leucine that was uniformly labeled with carbon-14. After incubation the cells were washed six times with 2–4 volumes of 0.9% NaCl and hemolyzed as described. The radioactive hemoglobin solutions were first dialyzed against several changes of 0.01 M L-leucine (nonradioactive) in distilled water or chromatographic developer at 4° and then against one of the chromatographic developers (without added leucine).

Procedures for the Separation and Isolation of Hemoglobin Components.—The separation and isolation of hemoglobin components were carried out chiefly by means of the chromatographic procedures that have been described in detail by Allen *et al.* (1958), Clegg and Schroeder (1959), and Jones and Schroeder (1963).

The chromatographic fractions that contained a desired hemoglobin were pooled, and the solutions were concentrated by centrifugation (Vinograd and Hutchinson, 1960).

Because the abnormal components that are associated with hemoglobin H move at or near the solvent front during chromatography with developer No. 4, of Allen *et. al.* (1958), considerable use has been made of developer No. 6, which is a weak developer. Its composition has been given by Schnek and Schroeder (1961).

Further purification by starch-grain electrophoresis was necessary in order to separate one of the abnormal components (γ_4) from nonheme proteins after the initial isolation by chromatography. The general procedure of Kunkel (1954) under the specific conditions of Schnek and Schroeder (1961) was used.

Procedure for Amino Acid Determination.—The amino acid composition of acid hydrolysates of hemo-

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