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

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Received April 1, 1963

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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TABLE I

BUFFER SOLUTIONS FOR SEDIMENTATION STUDIES

*p*H at 25°	Composition of Solution (g/l)
6.74	4.60 $NaH_2PO_4 \cdot H_2O$; 1.18 Na_2HPO_4; 0.65 KCN
7.07	6.54 $K_2HPO_4 \cdot 3H_2O$; 1.94 KH_2PO_4
9.7	3.0 NaOH; 10.2 NaCl; 7.7 glycine
10.00	3.0 NaOH; 10.2 NaCl; 7.4 glycine
10.50	3.0 NaOH; 10.2 NaCl; 6.5 glycine

globin components was determined with an automatic amino acid analyzer (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.) according to the procedure of Spackman *et al.* (1958). A quantity of 2–15 mg of each hemoglobin component was placed in a 12 × 100 mm Pyrex test tube and dried to constant weight by heating at 110°. Two ml of doubly glass-distilled 6 N hydrochloric acid was added, the air was evacuated with a water aspirator, and the tube was sealed. Acid hydrolysis was effected by heating these tubes at 110 ± 0.5° for either 22 or 70 hours. Following hydrolysis, the hydrochloric acid was evaporated by a stream of air while the tubes were warmed to 40° in a water bath.

Ultraviolet Spectra.—The ultraviolet spectrum of several hemoglobins in aqueous solution was examined and automatically recorded with a Cary Model 14 recording spectrophotometer.

Determination of the Sequence of the N-Terminal Amino Acid Residues.—The determination of the N-terminal residues and of the N-terminal sequences of the hemoglobins that had been isolated by chromatography on Amberlite IRC-50 was carried out by the DNP-method. The procedures did not deviate in significant degree from those that Rhinesmith *et al.* (1957a,b; 1958) have described in detail.

Tryptic Peptide Pattern Analysis.—Peptide patterns or "fingerprints" of tryptic peptides were carried out essentially as described by Ingram (1958, 1959) with only minor modifications during the hydrolytic and chromatographic procedures. Hemoglobin solutions after dialysis against distilled water were digested at 40° in a volume of 4–5 ml at a concentration of 2–25 mg/ml after the usual heat denaturation at *p*H 8.[1] During the hydrolyses, additional trypsin was added after 60 minutes and the digestion was stopped after 90 minutes.

The soluble peptides were separated by electrophoresis on Whatman 3MM paper with a pyridine–acetic acid buffer at *p*H 6.4 essentially as described by Ingram (1958) with the modifications of Zuckerkandl *et al.* (1960). Ascending chromatography was carried out either with the original developer of Ingram (1958), *n*-butanol–glacial acetic acid–water, 3:1:1 (v/v/v), or with a developer that consisted of *iso*-amyl alcohol–pyridine–water, 7:7:6 (v/v/v), (Wittman and Braunitzer, 1959; Baglioni, 1961).

The detection of the peptides was generally made by dipping the dried papers into a 0.5% solution of ninhydrin in acetone and allowing the color to develop for at least 2 hours at room temperature before heating in an oven at 60–80° for 3–5 minutes. For the detection of tryptophyl peptides, the papers (either treated with ninhydrin or untreated) were sprayed with a solution of 0.4% 4-dimethylaminocinnamaldehyde in ethanol–6 N hydrochloric acid, 9:1 (v/v).[2] Arginyl and histidyl peptides were detected with reagents that are described by Block *et al.* (1958).

[1] In these digestions, 0.1 ml of 0.4 M calcium chloride was added, but it has since been concluded that it is without significant influence on the digestion.

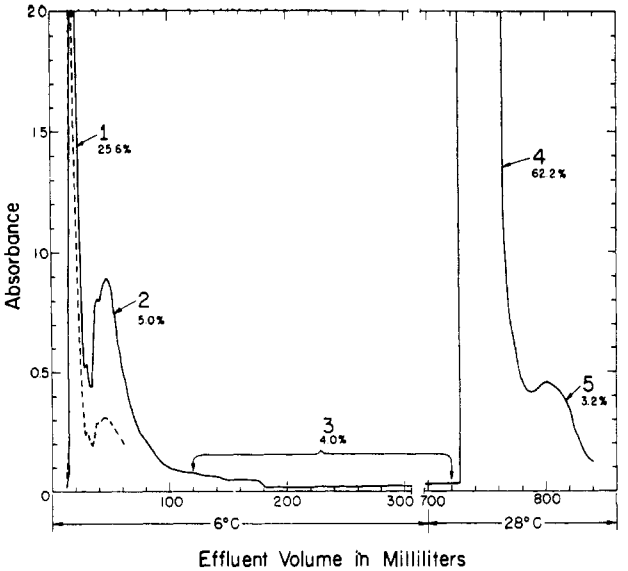

FIG. 1.—Amberlite IRC-50 chromatography of 100 mg of hemoglobin from subject P.T. with thalassemia-hemoglobin H disease. Developer No. 5 was used for the separation on a 1 × 35-cm column. The absorbance was measured at 280 mμ (dotted line) and at 415 mμ (solid line).

Determination of Sedimentation Coefficients.—The measurement of sedimentation coefficients was carried out in a Spinco Model E analytical ultracentrifuge that was equipped with a double-cell rotor. In all experiments, as a control, one of the cells was filled with normal adult hemoglobin which had been treated in the same manner as the sample under study (Hasserodt and Vinograd, 1959). Hemoglobin solutions had concentrations of 0.5–0.8%. The composition and *p*H of the buffer solutions which were used for the sedimentation experiments are listed in Table I.

Procedures for Subunit Hybridization.—Hybridization experiments (Singer and Itano, 1959; Vinograd *et al.*, 1959; Vinograd and Hutchinson, 1960) were carried out at 4° at either *p*H 4.60–4.70 or *p*H 11.0–11.2. For the hybridization at low *p*H, the solvent was either 0.1 or 0.2 M sodium acetate that had been adjusted with glacial acetic acid to the proper *p*H. Hybridization experiments at high *p*H were carried out in solutions that contained 6.25 g trisodium phosphate dodecahydrate, 4.68 g anhydrous disodium phosphate, and 8.78 g sodium chloride per liter.

The mixture of hemoglobins to be hybridized (which in various experiments ranged from 1 to 50 mg per ml in concentration) was then dialyzed against two changes of the appropriate buffer at 4° over a period of 12–48 hours. Finally, it was dialyzed against at least two changes of the selected chromatographic developer and then chromatographed.

RESULTS

Chromatography and Starch-Block Electrophoresis.—Extensive chromatographic studies were made on a number of specimens from three individuals with thalassemia-hemoglobin H disease. The most satisfactory separation of components was obtained by chromatography with developer No. 5. Figure 1 illustrates the results of the chromatography of hemoglobin

[2] Personal communication from K. N. F. Shaw.

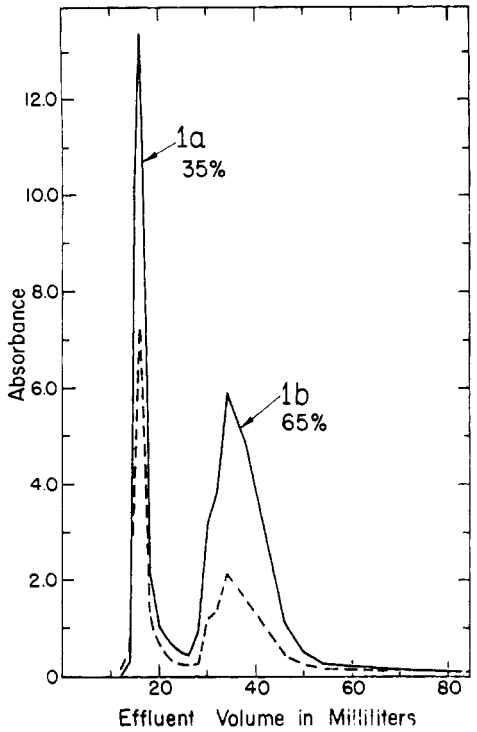

FIG. 2.—Rechromatography of zone 1 from chromatogram shown in Figure 1. Developer No. 6 was used for the separation on a 1 × 35-cm column. The absorbance was measured at 280 mμ (dotted line) and at 415 mμ (solid line).

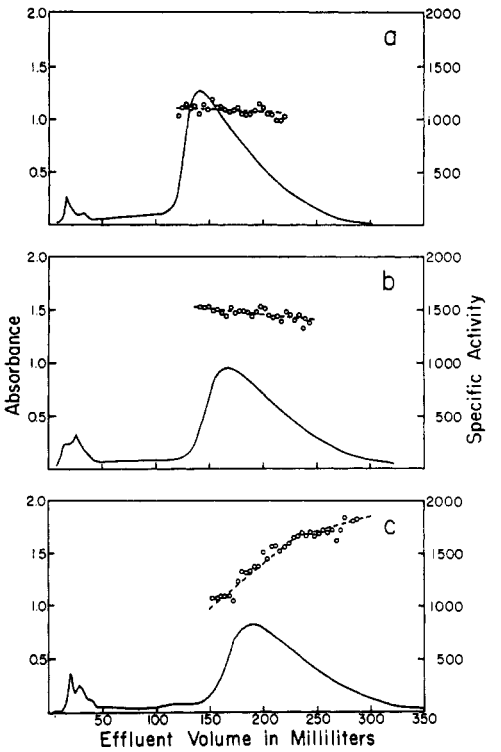

FIG. 3.—Chromatograms of mixtures of approximately equal amounts of radioactive hemoglobin A with (a) nonradioactive hemoglobin from zone 4 (Figure 1); (b) nonradioactive hemoglobin A; and (c) nonradioactive *gorilla* hemoglobin. The hemoglobins were chromatographed as ferrihemoglobin cyanide using developer No. 2 at 6° on a 1 × 35-cm column. Dotted line, absorbance at 542 mμ; small circles, specific activity in cpm/mg hemoglobin.

from subject P.T. (specimen CH-12a, Table II). Five zones or regions were obtained. When zone 1 was rechromatographed with developer No. 6 (Schnek and Schroeder, 1961) two hemoglobin components (zones 1a and 1b, Figure 2) were apparent. The ratio of absorbance at 280 and 415 mu revealed that zone 1a contained nonheme protein(s) in addition to the hemoglobin component. The chromatographic behavior of zone 1a was the same as that of a sample of hemoglobin Bart's (Ager and Lehmann, 1958).

Zone 2 of Figure 1 was chromatographically similar to the F_{II} component of umbilical cord blood and to the A_{Ic} component of the normal adult hemoglobin (Allen *et al.*, 1958).

About 4% of the total hemoglobin was present in the region marked 3. Although Clegg and Schroeder (1959) have observed two peaks in this region in chromatograms of normal adult hemoglobin, no definite peaks were evident in samples from the individuals with hemoglobin H.

Zone 4 was chromatographically indistinguishable from the A_{II} component of the normal adult hemoglobin. This conclusion comes from the results of mixed chromatograms with radioactive hemoglobin as a reference according to a previously published procedure (Jones and Schroeder, 1963). When the component from zone 4 of Figure 1 and an equal amount of radioactive hemoglobin A_{II} were chromatographed, the radioactivity as shown in Figure 3a was uniformly distributed throughout the main zone. These results are identical with those (Figure 3b) from a mixture of nonradioactive hemoglobin A_{II} from a normal individual and radioactive hemoglobin A_{II}. The value of this technique in demonstrating slight differences in chromatographic behavior of hemoglobins is shown in Figure 3c which depicts a chromatogram of a mixture of gorilla hemoglobin and radioactive human hemoglobin A_{II} (Zuckerkandl and Schroeder, 1961). In this case

TABLE II

PERCENTAGE OF COMPONENTS IN HEMOGLOBIN FROM THREE SUBJECTS WITH THALASSEMIA-HEMOGLOBIN H DISEASE

Subject	Specimen No.	Dates of Specimen	Dates of Chrom.	Per Cent of Zones[a] 1 a	1 b	2	3	4	5
R.C.	UO-1a	7-22-1958	8-7-1958	6.3	38.4	3.0	3.0	46.0	3.3
	UO-1b	11-28-1958	12-1-1958	5.2	30.3	3.5	3.7	52.5	4.7
	UO-1c[b]	1-5-1959	2-20-1959	—	44.0	—	—	56.0	—
H.D.	UO-2	12-3-1958	12-7-1958	6.8	28.6	3.6	3.2	52.4	5.4
P.T.	CH-12a	5-14-1959	5-16-1959	9.0	16.6	5.0	4.0	62.2	3.2
	CH-12b	8-5-1959	8-10-1959	11.5	14.2	5.1	3.8	60.4	5.0
	CH-12c	2-10-1960	11-17-1960	10.8	15.4	3.2	3.3	63.6	3.6
	CH-12d	12-22-1960	1-6-1961	9.6	15.7	3.6	4.0	61.4	5.7

[a] See Figures 1 and 2 for zone numbers. [b] This chromatographic separation was with developer No. 1 only, whereas all other separations were first with developer No. 5 and then with developer No. 6.

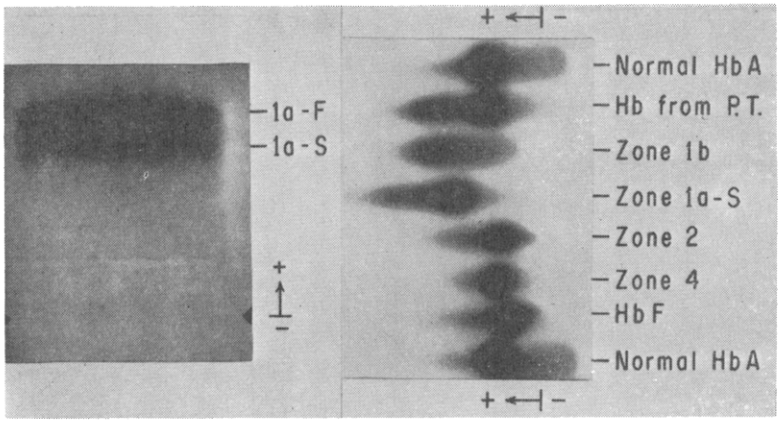

FIG. 4.—Starch-block electrophoreses at *p*H 8.6. (a) Zone 1a from a chromatogram similar to that of Figure 2. (b) Comparison of various components with each other and fetal and adult hemoglobins.

the radioactivity is not uniformly distributed throughout the main zone. Its distribution indicates that the gorilla hemoglobin moves more rapidly than does hemoglobin A_{II}.

Zone 5 of Figure 1 was chromatographically similar to the A_{III} components of Clegg and Schroeder (1959); however, this region generally could not be resolved into the two components A_{IIIa} and A_{IIIb}.

Table II lists the percentages of these various components in specimens from three individuals with thalassemia-hemoglobin H disease. Subject R.C. is No. 7 in the family diagram published by Rigas *et al.* (1956) and is the brother of the propositus (subject H.D.) in whom hemoglobin H was first discovered. The third subject, P.T., and her family have been carefully studied by Sturgeon and co-workers (Sturgeon *et al.*, 1961; Bergren and Sturgeon, 1960). Clearly the relative percentage of each component is rather constant in repeated specimens from subject P.T. The spleen was surgically removed from subject R.C. on 7/23/58. The change in the percentage of zone 1b from 7/22/58 to 11/28/58 is probably due to the splenectomy as noted by Rigas and Koler (1961). Of special interest is specimen CH-12c. Although the sample was 9 months old when it was examined, the results accord with those from the fresh samples (CH-12a, CH-12b, and CH-12d). This apparent stability of the hemoglobin components that are associated with hemoglobin H disease is in disagreement with the observations of Rigas *et al.* (1956) and Rigas and Koler (1961). The stability of our samples *in vitro* appears to have been due to our practice of storing the freshly hemolyzed solution in chromatographic developer which is buffered at about *p*H 7.0 and contains potassium cyanide.

The hemoglobin in zone 1a of Figure 2 was subjected to further purification by starch-block electrophoresis at *p*H 8.6. Figure 4a illustrates its separation into two hemoglobin components. The faster component (1a-F) had an electrophoretic mobility similar to zone 1b of Figure 2. Apparently the component of zone 1b was present also in zone 1a-F. Zone 1b will be identified with hemoglobin H as shown below. The apparent presence of hemoglobin H in zones 1a and 1b may stem from the isomerism that hemoglobin H exhibits under certain conditions as Benesch *et al.* (1962) have

TABLE III
AMINO ACID COMPOSITION[a] OF HEMOGLOBIN COMPONENTS FROM THALASSEMIA-HEMOGLOBIN H DISEASE

	Zone 1a-S, Fig. 4 (γ_4)			Zone 1b, Fig. 2 (β_4)			Zone 2, Fig. 1	Zone 4, Fig. 1		Zone 5, Fig. 1
	22 hr	22 hr	70 hr	22 hr	22 hr	70 hr	22 hr	22 hr	70 hr	22 hr
Ala	6.10	6.34	6.32	7.68	8.13	7.74	8.90	9.45	9.47	9.29
Arg	3.67	3.38	3.32	3.37	3.20	3.20	3.21	3.02	3.47	3.07
Asp	10.34	10.43	10.31	9.97	10.36	10.13	9.51	9.94	9.98	9.61
Cys/2	1.27	0.81	0.72	1.19	1.51	1.22	1.07	0.58	0.76	0.78
Glu	10.45	10.84	10.59	9.42	9.97	9.89	6.91	7.22	7.20	7.15
Gly	5.48	5.61	5.56	5.62	5.82	5.63	4.36	4.52	4.48	4.47
His	6.24	6.11	6.19	8.03	7.88	7.44	8.11	8.28	8.40	7.96
Ileu	3.01	2.89	3.01	0.12	0.15	0.32	0.26	0.02	0.02	0.02
Leu	12.76	13.04	13.05	13.41	14.08	13.73	13.54	14.05	13.89	13.86
Lys	10.05	9.51	9.60	9.07	9.18	8.99	8.64	9.13	9.18	8.73
Met	1.70	1.70	1.68	0.82	0.94	0.93	1.27	1.33	1.31	1.29
Phe	7.60	7.66	7.93	7.65	7.86	7.76	7.10	7.40	7.33	7.28
Pro	3.16	3.16	3.12	4.64	4.94	4.77	4.64	5.09	4.84	4.83
Ser	5.08	5.63	4.40	2.67	2.84	2.29	3.79	4.36	3.29	4.22
Thr	6.27	6.53	5.89	4.56	4.80	4.27	5.12	5.36	4.77	5.17
Tyr	2.29	2.10	2.12	2.40	2.63	2.58	1.96	3.13	2.87	2.52
Val	8.84	8.84	9.12	11.51	11.83	12.01	9.98	10.46	10.93	10.18
Recovery[b]	89%	90%	88%	87%	91%	88%	84%	88%	88%	86%

[a] Values are given in grams of amino acid per 100 g protein. [b] These recoveries are not corrected for partial destruction of serine and threonine, complete loss of tryptophan, nor for the presence of heme. See text for further discussion of recovery.

TABLE IV

AMINO ACID COMPOSITION OF HYDROLYSATES OF HEMOGLOBINS F_{II} AND A_{II} AND THE α, β, AND γ CHAINS[a]

Amino Acid	Experimentally Determined Compositions of Hemoglobin Components						Composition of Chains from Literature[h]		
	F_{II}[b]	A_{II}[c]	$^1/_4\beta_4$[d]	α[e]	γ[f]	$^1/_4\gamma_4$[g]	γ	α	β
Ala	64.2	70.2	15.1	20.0	12.1	11.6	11	21	15
Arg	11.8	12.3	3.0	3.1	2.8	3.3	3	3	3
Asp	50.0	49.4	12.9	11.9	13.2	12.9	13	12	13
Cys/2	3.9	3.8	2.1	0	2.0	1.1	1	1	2
Glu	34.3	32.4	11.2	5.0	12.1	11.9	12	5	11
Gly	38.8	39.6	12.8	7.0	12.4	12.2	13	7	13
His	34.0	35.5	8.4	9.4	7.6	6.6	7	10	9
Ileu	7.6	—	—	—	3.8	3.7	4	0	0
Leu	69.8	70.4	17.8	17.5	17.5	16.3	17	18	18
Lys	45.8	41.4	10.4	10.3	12.6	11.0	12	11	11
Met	7.9	5.9	1.0	1.9	2.1	1.9	2	2	1
Phe	29.6	29.5	7.9	6.9	7.9	7.7	8	7	8
Pro	22.8	28.3	7.1	7.1	4.3	4.5	4	7	7
Ser	43.6	31.1	4.9	10.7	11.2	9.9	11	11	5
Thr	39.6	31.3	7.0	8.7	12.2	9.5	10	9	7
Tyr	10.2	11.9	2.4	3.5	1.6	2.0	2	3	3
Val	51.3	60.3	16.7	13.5	12.2	12.6	13	13	18

[a] Values are in residues per molecular weight. [b] Analyses from Schroeder *et al.* (1961). [c] Average of 22- and 70-hour hydrolyses of zone 4, Fig. 1, except for serine and threonine which are extrapolated values. [d] Analysis of 22-hour hydrolysis of zone 1b, Fig. 2, from Table III, in residues per 16,500 mw. [e] $^1/_2(A_{II}-2\beta) = \alpha$ chain in residues per 16,500 mw. [f] $^1/_2(F_{II}-2\alpha) = \gamma$ chains in residues per 16,500 mw. [g] Average of 22- and 70-hour hydrolysis of zone 1a-S, Fig. 4, from Table III in residues per 16,500 mw. [h] Amino acid composition of γ, α, and β chains as reviewed by Schroeder (1963).

shown. The conditions of chromatography are within the range that is conducive to isomerization. The slower component (1a-S) had an electrophoretic mobility similar to hemoglobin Bart's. This slower component (1a-S) was observed only on starch-block electrophoresis of samples from subject P.T., and not in any appreciable amounts from subjects H.D. and R.C. Zones 1a-S, 1b, 2, and 4 have been compared with one another, with fetal hemoglobin, and with normal adult hemoglobin by starch-block electrophoresis as shown in Figure 4b.

From more complete chemical studies which are presented later, it will be shown that zone 1a-S is γ_4 (hemoglobin Bart's) and zone 1b is β_4 (hemoglobin H). The symbols zone 1a-S and γ_4 will be used interchangably in the rest of the paper, as will zone 1b and β_4.

Amino Acid Analysis of Hemoglobin Components.—The amino acid composition of the different components of thalassemia-hemoglobin H disease is shown in Table III. The recoveries of grams amino acid residues per 100 g of sample are also included in Table III. These recoveries were not corrected for losses due to total destruction of tryptophan and partial destruction of serine, threonine, and tyrosine, nor for the presence of 4 hemes per mole of hemoglobin. Corrections for these losses are about 6% and raise all recoveries above 90%.

The amino acid composition of zone 1a-S is more like the composition of hemoglobin F than of hemoglobin A, but not identical with either (Schroeder *et al.*, 1961; Schroeder *et al.*, 1950). The amino acid composition of this component does agree very well with that determined for the γ chain of hemoglobin F_{II}, the major component of fetal blood as seen in Table IV.

Zone 1b is similar, but not identical in amino acid composition, to hemoglobin A. The small amount of isoleucine is equivalent to less than one-half residue of isoleucine per 66,000 mw, and presumably has its source in incomplete separation from the zone 1a or in the presence of a small amount of nonheme protein. This conclusion is supported by a recent analysis of zone 1b that had been further purified by starch-block electrophoresis. Such material contained no trace of isoleucine.[3] In Table IV the data are considered in terms of the number of residues of each amino acid per 66,000. If the data from zone 1b are divided by 4, it is apparent that the results are identical within experimental error with the amino acid composition of the β chain of hemoglobin A.

As we have noted, zone 2 is chromatographically similar to the minor component A_{Ic} or to hemoglobin F_{II} in this particular chromatographic system. The amino acid composition of A_{Ic} from a normal adult is essentially indistinguishable from the amino acid composition of A_{II} and contains only a trace of isoleucine. The amino acid composition of zone 2 suggests that it is composed in the main of the minor component A_{Ic}. However, isoleucine is present, and if this is considered to come from hemoglobin F_{II} then approximately one-sixth of zone 2 is composed of hemoglobin F_{II}.

The amino acid compositions of zones 4 and 5 of Figure 2 are not significantly different from those of hemoglobins A_{II} and A_{IIIb}, respectively (Jones, 1961).

The amino acid composition of the α, β, and γ chains of human hemoglobins A and F may be calculated from the amino acid analyses of hemoglobin F_{II}, A_{II}, and the main component of hemoglobin H (1b, Figure 2) because the α chains of hemoglobin A and F are identical (Hunt, 1959; Jones *et al.*, 1959b; Schroeder, *et. al.* 1963). Such a calculation has been made and the results are listed in Table IV (see legend for calculations). It is apparent from this table that the agreement between these experimental results and the results obtained by others and derived from different materials is good.

Ultraviolet Spectra.—Jope (1949) apparently was the first to note the difference in the ultraviolet absorption spectra of the human adult and fetal hemoglobin. The difference has been attributed to the greater content of tryptophan in fetal hemoglobin. The spectra of components A_{II} and F_{II}, and zones 1a-S (γ_4) and 1b (β_4), between 250 and 300 mμ, reveal rather striking differences as shown in Figure 5. Hemoglobin A_{II} and zone 1b have inflections only in the region of 290

[3] R. T. Jones, R. D. Koler, and D. A. Rigas, unpublished data.

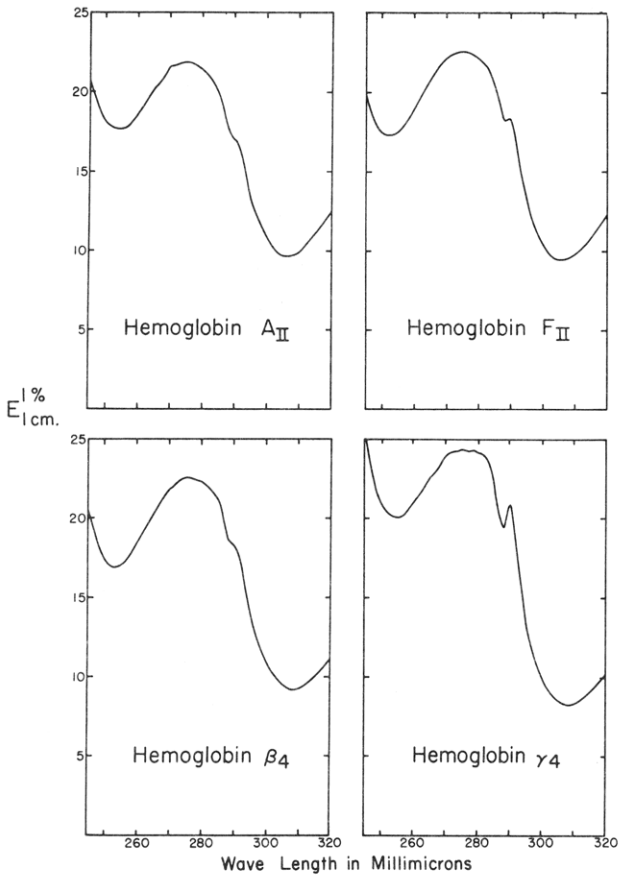

FIG. 5.—Ultraviolet absorption spectra of hemoglobin A, hemoglobin F, hemoglobin β_4 (zone 1b, Figure 2), and hemoglobin γ_4 (zone 1a-S, Figure 4). The absorbance was measured through a 1-cm path of a 1% solution. The major components from adult hemoglobin (A_{II}) and from fetal cord blood hemoglobin (F_{II}) were obtained by chromatography as described elsewhere (Allen *et al.*, 1958).

mμ, whereas hemoglobin F_{II} and zone 1a-S have a maximum. This prominent maximum in zone 1a has been noted on three preparations from subject P.T., but it is absent in zone 1a from subjects R.C. and H.D. It must be concluded that zone 1a of subjects R.C. and H.D. does not contain an appreciable amount of hemoglobin γ_4.

N-Terminal Amino Acid Sequences.— The results of the determination of N-terminal amino acid sequences of components from zones 1a-S, 1a, 1b, and 4 (Figures 1, 2, and 4) are listed in Table V. The data in Table V have been calculated on the basis of the following assumptions: (1) that 88 mg of air-dried DNP-protein is equivalent to one micromole; (2) that, in short hydrolyses, DNP-valine and DNP-val-leu arise from

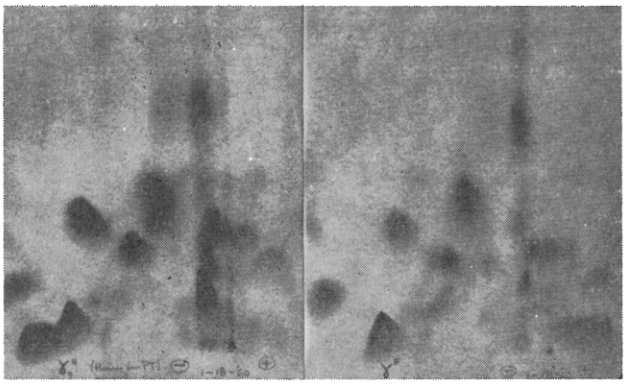

FIG. 6.—Peptide pattern of soluble peptides from tryptic hydrolysis of hemoglobin γ_4 (zone 1a-S, Fig. 4a) on the left is compared with a pattern obtained with γ chain from fetal hemoglobin on the right. Reactions for tryptophan, histidine, and arginine peptides were identical on the two patterns. Pyridine–acetic acid–water was used for chromatography.

the α chain, DNP-val-his-leu and DNP-val-his from the β chain, and DNP-glycine from the γ chain; and (3) that, in long hydrolyses, DNP-valine arises from the β chain. The basis for these assumptions has been described elsewhere (Rhinesmith *et al.*, 1957a, 1958; Schroeder and Matsuda, 1958). The number of chains of each type in a given molecule is then obtained by summation of the appropriate figures as presented in the right-hand three columns of Table V.

Two determinations of the N-termini of the component in zone 1a (Figure 2) from subject P.T. were made. Material for the first determination had been purified only by chromatography whereas that for the second was purified by chromatography and starch-block electrophoresis. Only DNP-glycine was obtained in significant yield; however, it was equivalent only to one to two chains per 66,000 mw. A difference is not unexpected because electrophoresis as already noted is required to remove nonheme protein(s). Further comment on this point will be made.

Material for these studies of the hemoglobin component in zone 1b (Figure 2) was isolated from two samples from subject R.C. and one from subject P.T. DNP-val-his-leu and DNP-val-his were the principal compounds to be isolated from this hemoglobin component. The yields of these DNP-peptides and the absence of other N-terminal DNP-compounds are concordant with the conclusion that there are four β chains per 66,000 mw. Buhler and Rigas (1962) have reached the same conclusion.

The N-terminal sequences of the main component (zone 4, Figure 1) appear to be identical with those of normal hemoglobin A (Rhinesmith *et al.*, 1958) except for unusually high yields of the N-terminal peptides of the β chain.

TABLE V

SEQUENCES OF N-TERMINAL AMINO ACIDS OF HEMOGLOBIN COMPONENTS BY THE DNP-METHOD[a]

Hemoglobin Zone from (Subject)		α		β			γ	Chains per 66,000 mw		
		val-leu	val	val-his-leu	val-his	val	gly	α	β	γ
1a	(P.T.)	0.1		0.1	0.1		1.09			1.09
1a-S	(P.T.)	0.1				0.1	1.96			1.96
1b	(R.C.)	0.2	0.2			3.26			3.26	
1b	(R.C.)	0.2	0.2	1.65	0.97	0.53			3.15	
1b	(P.T.)	0.2	0.2	1.47	0.84	0.84			3.15	
4	(P.T.)	1.47	0.17	1.31	0.63			1.64	1.94	

[a] Values in moles of DNP-compound per mole of DNP-protein. Duration of hydrolysis was 1 hour in each instance except those of valine from the β chains and glycine from the γ chains, which were 22 hours and $^1/_4$ hour, respectively.

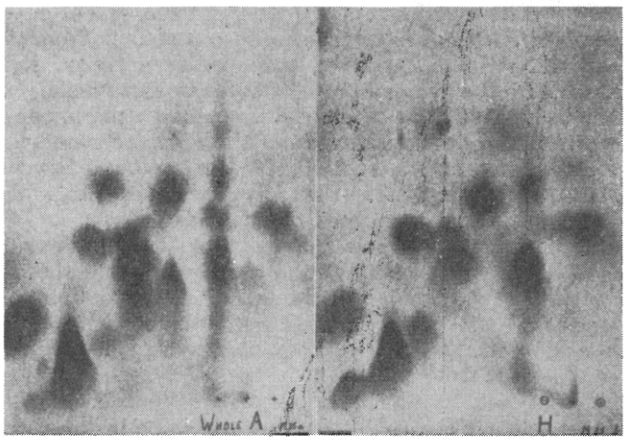

FIG. 7.—Peptide pattern of soluble peptides from tryptic hydrolysis of hemoglobin β_4 (zone 1b, Fig. 2) on the right is compared with a pattern obtained from normal adult hemoglobin on the left. Reactions for tryptophan, histidine, and arginine peptides of hemoglobin β_4 corresponded to those of the β chain of hemoglobin A. Pyridine–acetic acid–water was used for chromatography.

Peptide Patterns of Tryptic Hydrolysates.—Peptide patterns or "fingerprints" of the soluble tryptic peptides of the hemoglobin components in zones 1a-S, 1b, 2, and 4 (Figures 1, 2, and 4) have been studied, and the results are illustrated in Figures 6, 7, and 8. The pattern for zone 1a-S appears to be identical with that from the γ chains of hemoglobin F, and the pattern from zone 1b is identical with that from the β chains of hemoglobin A. Peptide spots from the α chain of human hemoglobins could not be detected in either of these two abnormal components. The peptide patterns of zones 2 and 4 (Figure 1) were indistinguishable from that of the normal hemoglobin A_{II} when the solvent system described by Ingram (1958) was used. Paired peptide patterns gave comparable results after staining with specific reagents; these agreed with observations of other investigators.

Sedimentation Coefficient as a Function of pH.—The sedimentation coefficients of hemoglobin components in zone 1a-S and zone 1b (Figure 2) were compared with the coefficients of hemoglobin A_{II} or whole normal adult hemoglobin and are listed in Table VI as experiments 1 and 2. Both components gave values that were several per cent higher than that of the control. This result for zone 1b was not unanticipated because Rigas *et al.* (1956) had reported that the sedimentation constant of hemoglobin H was somewhat greater than that of hemoglobin A. If the frictional coefficients of these compounds are similar to that of

TABLE VI

SEDIMENTATION COEFFICIENTS ($s_{20,w}$)[a] OF HEMOGLOBIN γ_4 (ZONE 1a-S) AND HEMOGLOBIN β_4 (ZONE 1b)

Expt. No.	Hemoglobin Components: Experimental	Hemoglobin Components: Control	pH	$s_{20,w}$: Experimental	$s_{20,w}$: Control	$\Delta s_{20,w}$
1	γ_4	A	6.74	4.97	4.86	0.11
2	β_4	A	7.07	4.63	4.39	0.24
3	β_4	A	9.70	3.93	4.09	−0.16
4	β_4	A	10.0	3.96	4.07	−0.11
5	β_4	A	10.5	2.81	3.77	−0.96
6	β_4		7.07	4.51		
7	β_4		10.5	2.81		

[a] Sedimentation coefficients are expressed in Svedberg units.

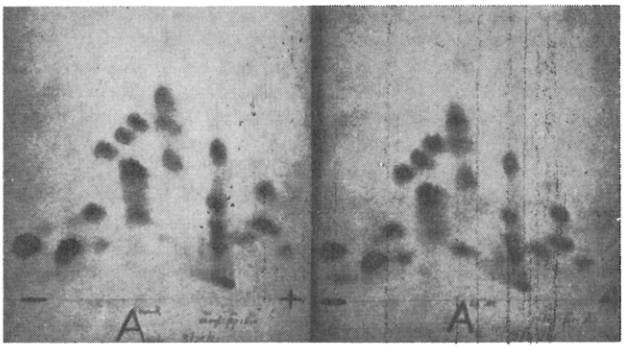

FIG. 8.—Peptide pattern of soluble peptides from tryptic hydrolysis of hemoglobin from zone 4 (Fig. 1) on the right compared with a pattern of normal adult hemoglobin on the left. Reactions for tryptophan, histidine and arginine peptides were identical on the two patterns. *Iso*-amyl alcohol-pyridine-water was used for the chromatography. The peptide pattern of hemoglobin from zone 2 (Fig. 1) was indistinguishable from this pattern.

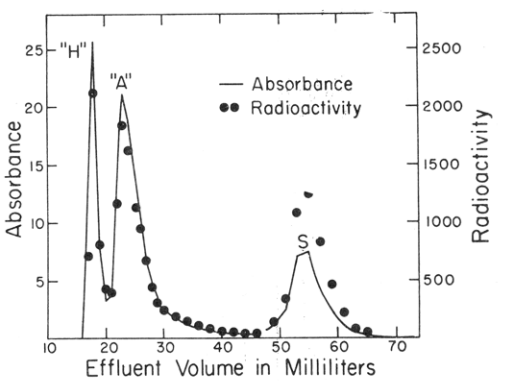

FIG. 9.—Chromatogram of products resulting from subunit hybridization of hemoglobin β_4 (zone 1b, Figure 2) and radioactive hemoglobin S. The solid line represents the absorbance at 415 mμ. The solid circles represent the radioactivity in cpm/ml effluent. See text for description of "H" and "A" zones.

hemoglobin A, then the sedimentation coefficients indicate that the molecular weights are approximately 60,000–70,000. These estimates of molecular weight are the basis for the conclusion that zone 1a-S and zone 1b have the substructure formulas γ_4 and β_4, respectively. This conclusion is certainly supported in the case of zone 1b or β_4 by the results of the determination of the N-terminal sequences. However, on the same basis one is led to the conclusion that only two chains are present in zone 1a-S. On the other hand, the results of the peptide patterns would throw doubt on such a conclusion. Why zone 1a-S gives what appears to be relatively low yields in the N-terminal determination is uncertain, although low yields have also been noted from the γ chains after isolation from hemoglobin F_{II}.[4] Another possibility, however, cannot be ignored. It has recently been concluded (Schroeder *et al.*, 1962) that hemoglobin F_I differs from hemoglobin F_{II} in the presence of a single N-terminal acetyl group on a γ chain. Perhaps zone 1a-S, instead of having the structure γ_4, actually is composed of two γ chains and two acetylated γ chains. Since this possibility arose it has not been possible to test it because the decease of subject P.T. has made further material of this type unavailable.

In order to determine whether subunit hybridization experiments might be practical, the dissociation of

[4] W. A. Schroeder, unpublished data.

hemoglobin β_4 in alkaline solution was studied. The results are seen in experiments 3 to 5, inclusive, of Table VI. If the results are interpreted as Hasserodt and Vinograd (1959) have done, them hemoglobin β_4 is more completely dissociated at *p*H 10.5 than is hemoglobin A. Experiments 6 and 7 were carried out to determine whether the dissociation were reversible. In experiment 6 the hemoglobin β_4 was dialyzed against buffer at *p*H 10.5 for 16 hours and then against buffer at *p*H 7.07 for 16 hours. In the case of experiment 7 the dialyses were carried out in the reverse order. Comparison of these results with the results of experiments 2 and 5 clearly shows that the dissociation is reversible.

Subunit Hybridization.– The first hybridization of hemoglobin β_4 was made with a mixture of equal parts of it and of radioactive hemoglobin S at *p*H 11.0 and 3° for 24 hours. Some pertinent data for this experiment have been briefly described earlier (Jones *et al.*, 1959a).

The products of this hybridization were chromatographed with developer No. 1 to give the chromatogram that is shown in Figure 9. As we consider the results of this experiment let us assume that the hybridization of equal amounts of hemoglobin β_4 and hemoglobin S can be represented by equation (1) where

$$3\beta_4^{H-A} + 3\alpha_2{}^*\beta_2{}^{S*} \longrightarrow \underbrace{2\beta_4^{H-A} + \beta_4^{S*}}_{\text{zone "H"}} + \underbrace{2\alpha_2{}^*\beta_2{}^{H-A}}_{\text{zone "A"}} + \underbrace{\alpha_2{}^*\beta_2{}^{S*}}_{\text{zone S}} \quad (1)$$

the asterisk indicates radioactivity.

Of the three zones that are apparent in Figure 9 one clearly was hemoglobin S.

Zone "A" was shown to be hemoglobin A by chromatographic behavior, starch-block electrophoresis, sedimentation coefficient, and N-terminal determinations. The results of the N-terminal studies were especially valuable in confirming the basic validity of equation (1). Because the radioactive label which was originally present in both the α and β chains of the radioactive hemoglobin S reactant was found to be present only in the α chain of the radioactive hemoglobin A product, the β chain of the hemoglobin A must have arisen from the nonradioactive hemoglobin β_4.

Zone "H" of Figure 9 was separated into two components by rechromatography with developer No. 4. One of these components was identical with the original hemoglobin β_4, whereas the second component, from its chromatographic behavior and specific activity, was assumed to be hemoglobin $\beta_4{}^{S*}$ by analogy to hemoglobin $\beta_4{}^{A}$. Although there is no other experimental confirmation of the structure of the assumed hemoglobin $\beta_4{}^{S*}$, it should be noted that Huisman and Sebens (1960) have reported the isolation of hemoglobin $\beta_4{}^{S}$ from the umbilical cord blood of Negro babies.

Equation (1) assumes that none of the components is lost, that the affinity of the α chain is the same for the β^{H-A} and β^{S} chains, and that no other recombinant forms to any significant extent. Because approximately twice as much hemoglobin A as hemoglobin S was isolated from the hybridization experiment, it may be concluded that hemoglobin β_4 is composed only of β^{A} chains. This conclusion is valid only if the ratio of β^{A} to β^{S} chains which precipitated in part during this experiment was the same as the ratio of β^{A} to β^{S} chains that were available for combination with α chains. Otherwise a decrease in the number of β^{S} chains relative to β^{A} chains could force the hybridization past the point of theoretically uniform distribution of the α chains between β^{A} and β^{S} chains. The results of the above experiment appeared to satisfy equation (1) at least as far as the stable products $A_{II}{}^*$ and $S_{II}{}^*$ are concerned (also see Jones *et al.*, 1959a).

Likewise, the hybridization of one part hemoglobin β_4 with two parts hemoglobin $S_{II}{}^*$ can be represented by equation (2).

$$2\beta_4^{A} + 4\alpha_2{}^*\beta_2{}^{S*} \longrightarrow \beta_4^{A} + \beta_4^{S*} + 2\alpha_2{}^*\beta_2{}^{A} + 2\alpha_2{}^*\beta_2{}^{S*} \quad (2)$$

This equation was tested by hybridizing 25 mg of hemoglobin β_4 with 50 mg of radioactive hemoglobin $S_{II}{}^*$ at *p*H 4.65 for 24 hours. Although some hemoglobin precipitated, each of the products in equation (2) was detected. The ratio of the hemoglobin A* that formed to hemoglobin S* that remained was 0.82. Therefore either the hybridization was incomplete or more β^{A} chains than β^{S} chains were precipitated during hybridization. Of course, it is also possible that the α chains have a greater affinity for the β^{S*} chains than for the β^{A} chains under the conditions of the hybridization.

TABLE VII

HYBRIDIZATION STUDIES OF HEMOGLOBINS γ_4, β_4, F, AND A

Experiment	Hybridization Mixture[a]	Amount of Reactants[b] (mg)	*p*H	Duration[c] (hours)	Percentage of Products: Front Zones[d]	Percentage of Products: F	Percentage of Products: A	Ratio of Products A to F: Exptl.	Ratio of Products A to F: Theoretical[e]	Estimation of *K*: Equation (5)[f]
A	$\gamma_4 \times$ A*	13 × 26	4.7	24	28	5	67	13	1	2×10^3
B	$\gamma_4 \times$ A*	13 × 26	11.2	24	27	8	65	8.1	1.0	5×10^2
C	$\gamma_4 \times$ A*	6.6 × 13.2	4.7	24	27	16	57	3.6	1.0	45
D	$\beta_4 \times$ F	7.6 × 8.2	4.7	14	38	23	39	1.7	1.5	1.5
E	$\beta_4 \times$ F*	14.5 × 29	4.7	24	20	25	55	2.2	1.0	11
F	$\gamma_4 \times$ F*	5.0 × 10.0	4.7	24						

[a] The molecular formulas of hemoglobin F and A are $\alpha_2\gamma_2$ and $\alpha_2\beta_2$, respectively. The formula γ_4 corresponds to zone 1a-S and formula β_4 corresponds to zone 1b. The subunit hybridization procedure is indicated by an "×." The asterisk indicates the presence of C-14L-leucine in the hemoglobin. [b] The amount of the reactants is listed in the same order as given in the preceding column. [c] This is the time during which the mixture was dialyzed against the hybridizing solution. [d] The components were isolated by chromatography with developer No. 5. The percentages are relative to the total hemoglobin from the chromatogram. The fast zone is comprised of β and γ chains, presumably only as β_4 and γ_4. In each hybridization experiment a small amount of precipitate was formed. A comparison of the ratios of the reactant to the percentage of the products indicate that the precipitate contained substantially more γ and β chains than α chains. An amino acid analysis of the precipitate from experiment E confirmed this conclusion. [e] The experimental ratio of the products A to F was calculated from the relative percentages of each. The theoretical ratio is based upon equation (5) and assumes $K = 1$ and that the affinity of the α chain for γ and β chains is equal. [f] The K for equation (5) is estimated from the relative percentages of A and F products and by assuming that the losses of chains during the hybridization are in the proportions of the chains in the final products.

Hybridization studies of zone 1a (Figure 2) were also made with radioactive hemoglobin A_{II}*. The pertinent data of these hybridizations are shown as experiments A, B, and C in Table VII. Because of the proportions of the reactants, equation (3) was

$$2\gamma_4 + 4\alpha_2{}^*\beta_2{}^* \longrightarrow \beta_4{}^* + \gamma_4 + 2\alpha_2{}^*\gamma_2 + 2\alpha_2{}^*\beta_2{}^* \quad (3)$$

used to calculate the percentage of complete hybridization that had been achieved. In the most successful experiment (experiment C), the hybridization was of the order of only 44% of that to be expected on the basis of equation (3), even though the duration of hybridization was 24 hours. The apparent incompleteness of these hybridizations prompted other experiments and a re-evaluation of previous experiments. These will now be discussed.

Recombination of Subunits in the Hybridizations of Hemoglobins A, F, β_4, and γ_4.—If we write an equation similar to equation (3) for the hybridization of hemoglobins F and β_4, thus,

$$2\beta_4 + 4\alpha_2\gamma_4 \rightarrow \beta_4 + \gamma_4 + 2\alpha_2\gamma_2 + 2\alpha_2\beta_2 \quad (4)$$

it is apparent that the products of equations (3) and (4) are identical. If the same proportion of products may be obtained from the two starting mixtures, then equations (3) and (4) may be combined into equation (5). Equation (5) has the

$$2\alpha_2\gamma_2 + \beta_4 \overset{K}{\rightleftharpoons} 2\alpha_2\beta_2 + \gamma_4 \quad (5)$$

form of an equilibrium expression. Because all components of the mixture are available, the equilibrium, if, indeed, it is an equilibrium, can be studied from both sides of the reaction. The results of experiment C (Table VII) would indicate that if $K = 1$ in equation (5) then equilibrium had not been reached, or if equilibrium had been reached then $K > 1$ or approximately 45.

Equation (5) from the left-hand side has been used in the study of the identity of the α^A and α^F chains (Jones *et al.*, 1959b). From the results it may be concluded that K is about 1. The data are given as experiment D in Table VII. However, the duration of experiment D was less than that of experiments A and C, and the results cannot therefore be compared exactly. So that exact comparison could be made, experiment E (Table VII) was performed in which not only was the duration equal to that of experiments A and C but the mole ratio of the α, β, and γ chains was one, as it had been in experiments A and C. The results[5] of experiment E show the presence of about twice as much hemoglobin A as hemoglobin F instead of the equal quantities that would be expected if $K = 1$. Therefore, K cannot be unity; and if equilibrium has been reached K is approximately 11, and therefore in qualitative agreement with experiments A and C.

This conclusion is supported not only by the ratio of hemoglobin A to hemoglobin F but also by an examine ation of the rapidly moving zone. If $K = 1$ in equation (5), then the rapidly moving zone should contain equal amounts of hemoglobin γ_4* and β_4. Because experiment E was made with radioactive hemoglobin F, the specific activity of the rapidly moving zone should be about one-half that of the original radioactive hemoglobin F if the amount of γ_4* equals the amount of β_4. Actually the specific activity of this zone (about 2000 cpm/mg) was almost as high as the original radioactive hemoglobin F (2100 cpm/mg). This observation indicates that the rapidly moving zone probably contains substantially more radioactive hemoglobin γ_4* than hemoglobin β_4.

Although experiments C and E, Table VII, indicate that the K for equation (5) is greater than unity, the two values of K are not identical. This disagreement might indicate that equilibrium had not yet been reached under the conditions of the experiments. Relatively slow rates of reaction in the hybridization process could account for this incomplete equilibrium.

In order to estimate the extent of exchange of the γ-chain subunits in hybridization reactions, experiment F, Table VII, was made. Five mg of zone 1a-S (pure γ_4) was mixed with 10 mg of radioactive hemoglobin F and the mixture was subjected to conditions of hybridization identical to those used in experiments C and E. The reactants were separated with developer No. 5. The specific activity of the radioactive hemoglobin F (1880 cpm/mg) recovered from the hybridization was compared with the specific activity of radioactive hemoglobin F (2267 cpm/mg) from a control experiment in which only the radioactive hemoglobin F was subjected to the hybridizing conditions and chromatography. Assuming approximately equal distribution of the radioactive label in the α and γ chains of this hemoglobin, the ratio of specific activities of the hemoglobin F which had been hybridized in the presence and absence of nonradioactive hemoglobin γ_4 indicates that the exchange of γ-chain subunits was about 70% complete. The results of this experiment support the contention that the rates of reactions of subunit hybridizations, at least those of hemoglobins with γ chains, are relatively slow.

If the results of experiment F, Table VII, are used to estimate the extent to which equilibrium had been reached in experiment C, then the K for experiment C can be corrected to about 16. This value is in good agreement with the value of 11 for K from experiment E. Until a more extensive experimental study of subunit hybridizations described by equation (5) can be made, the present experiments indicate that the K for equation (5) is greater than unity and probably in the order of ten to twenty.

The observation that the exchange of γ-chain subunits in experiment F was at least 70% complete indicates that at least two-thirds of the chains in zone 1a-S are normal γ chains. If zone 1a-S had a structure such as $\gamma_2(Ac\gamma)_2$ or $\gamma_3(Ac\gamma)$, the hybridization with hemoglobin F should have led to structures such as $\alpha_2\gamma(Ac\gamma)$ or $\alpha_2(Ac\gamma)_2$. The former is hemoglobin F_1 (Schroeder *et al.*, 1962), and both it and any $\alpha_2(Ac\gamma)_2$ should have been detectable but were not found when the hybridization mixture from experiment F was rechromatographed. This result suggests that the low yield of DNP-glycine in the study of the N-terminal amino acids of zone 1a-S must have been due to some factor other than the presence of N-terminal acetyl groups.

Discussion

It is clear that at least two abnormal hemoglobin components are associated with thalassemia-hemoglobin H disease. The subunit formula for these components may be represented as γ_4 (zone 1a-S) and β_4 (zone 1b). Recently Huehns *et al.* (1960) have concluded that hemoglobin δ_4 is present among the abnormal hemoglobins associated with this disease. The presence of such a component has also been inferred from hybridi-

[5] Although a small precipitate formed during experiment E, its amino acid composition indicated that the ratio of $\alpha:\beta:\gamma$ chains was approximately 1:5:6. This small and essentially equal loss of γ and β chains could not have been responsible for the observed ratio of hemoglobin A to F.

zation studies which have not been included in the present report (see Jones, 1961).

It is appropriate to use the term "hemoglobin β_4" synonymously with hemoglobin H because the present study demonstrates that it is the only abnormal hemoglobin in appreciable amount in two of the individuals in whom Rigas *et al.* (1955) discovered hemoglobin H. Our evidence for the gross structure β_4 is based upon amino acid analysis, N-terminal amino acid sequence, tryptic peptide patterns, sedimentation coefficient, and subunit hybridization. However, not until the exact sequence of each amino acid residue in the chains of hemoglobin H is established can we be certain that hemoglobin H is chemically identical with the β chain of hemoglobin A. The over-all conformation of the β chains in hemoglobin H probably is not identical with the conformation of the β chains which are in association with α chains in hemoglobin A.

The presence of only γ chains of fetal hemoglobin in zone 1a-S can be concluded from the amino acid analysis, tryptic peptide patterns, and subunit hybridization experiments. This component was also shown to be chromatographically identical with hemoglobin Bart's. Although N-terminal glycine was detected by the DNP analysis of zone 1a-S, the low yield is difficult to explain at the present time. The results of subunit hybridization of zone 1a-S with radioactive hemoglobin F as well as the formation of a component which is chromatographically identical with zone 1a-S from hybridizations of hemoglobin β_4 and hemoglobin F, are strong evidence that zone 1a-S is composed of the normal γ chains of hemoglobin F. Although the presence of some γ chains with N-terminal acetyl groups has not been ruled out, it does appear from consideration of all the data, including the sedimentation coefficient, that zone 1a-S is composed of molecules of four identical subunits and that these subunits are probably identical with the γ chains of fetal hemoglobin. Thus, the designation of this hemoglobin as γ_4 which was proposed in our initial report of this component (Jones and Schroeder, 1960) can be used synonymously with hemoglobin Bart's. Hunt and Lehmann (1959) and Kekwick and Lehmann (1960) have reported the same chemical structure for hemoglobin Bart's on the basis of "fingerprint" and sedimentation studies. Again, until the exact sequence of each amino acid residue has been determined, the identification of these chains in zone 1a-S and hemoglobin Bart's with the normal γ chain of fetal hemoglobin is not established beyond question.

The other hemoglobins which are found in addition to hemoglobins β_4 and γ_4 appear to be the normal minor components, possibly a small amount of fetal hemoglobin, and normal adult hemoglobin.

The amino acid analysis, N-terminal sequences, tryptic peptide pattern, and chromatographic behavior of the major adult component (zone 4, Figure 1) are normal in all respects. The possibility of the substitution of one or more amino acid residues by residues which impart similar electrophoretic and chromatographic properties to this component would, therefore, seem to be remote. The possibility cannot be entirely excluded, however, if, as has been postulated by several authors (Jones and Schroeder, 1960; Ingram and Stretton, 1959; Huehns *et al.*, 1960; Sturgeon *et al.*, 1961), hemoglobins β_4 and γ_4 result from deficient production of either normal or abnormal α chains. Two genetic factors may be operating in individuals with thalassemia-hemoglobin H disease. One factor is the presence of thalassemia. The other factor may be either a "silent" or overt hemoglobinopathy which affects the structure of the α chain. Hemoglobin Q is an overt α-chain hemoglobinopathy that occurs with hemoglobin H (Vella *et al.*, 1958; R. T. Jones and R. L. Hill, unpublished data). The present data clearly show that thalassemia-hemoglobin H disease may manifest itself in different ways. Thus, in subjects R.C. and H.D. hemoglobin γ_4 is absent, and the percentage of hemoglobin β_4 is greater than the total of hemoglobins β_4 and γ_4 in subject P.T. (Table II).

Dance *et al.* (1963), in a publication that appeared after the present paper was submitted, have presented some data on the abnormal hemoglobins in hemoglobin H disease. The chemical data of the two studies agree with and extend each other. The most striking difference is in the quantities of the various components in the several subjects. In the subjects that Dance *et al.* have studied, the hemoglobins β_4 and γ_4 were present to 7.5 and 0.2% in one subject and 14 and 2% in another. On the other hand, in subjects R.C. and H.D., hemoglobin β_4 was of the order of 35–45% (Table II; note that in these subjects zone 1a as well as zone 1b apparently contained hemoglobin β_4), whereas γ, if present was certainly less than 1%. In subject P.T., the percentage of β_4 was about 15 and of γ_4 about 10. This extreme variation would seem to support the contention that more than one disease state is being observed among these various subjects.

The simultaneous occurrence of hemoglobin Bart's with hemoglobin H has been observed by several groups (Ramot *et al.*, 1959; Jones and Schroeder, 1960; Huehns *et al.*, 1960; Sturgeon *et al.*, 1961). Huehns *et al.* (1960) have postulated that the wide variations in the proportions of hemoglobin H and hemoglobin Bart's may be explained by assuming different rates of synthesis of the α, β, and γ chains at various ages and by assuming that during the final assembly of hemoglobin the α chains have a greater affinity for β chains than for γ chains. The proportions of the recombination products from the hybridization of hemoglobins γ_4, β_4, A, and F indicate that, at least *in vitro*, the affinities of tne various subunits for complementary subunits are unequal. Although the processes by which the various subunits assemble to form the final hemoglobin *in vivo* may be substantially different from the mechanism *in vitro*, unequal affinities between the subunits which combine *in vivo* may exist and may be important in the biological formation of hemoglobins. However, it is apparent from equation (5) that *in vitro*, when the number of α chains is less than the total number of γ plus β chains, the affinity of the γ chains for one another relative to the affinity of the β chains for one another will influence the final distribution of the α chains between hemoglobins A and F. The stability of γ_4 relative to β_4 may also be important in the formation of hemoglobins *in vivo* and thus the relative proportions of these hemoglobins in individuals with thalassemia-hemoglobin H disease. It is possible that the relative proportion of the hemoglobins formed *in vivo*, as well as the proportion of products from subunit hybridizations, is influenced not only by a greater affinity of α chains for β chains than for γ chains but also by a greater stability of γ_4 than β_4.

Acknowledgments

The authors wish to thank Drs. D. A. Rigas, R. D. Koler, P. Sturgeon, and W. R. Bergren for obtaining samples of blood and for their valuable help and discussions during the course of this work. A sample of hemoglobin Bart's was obtained from Dr. H. Lehmann. Mrs. Joan Balog Shelton carried out the determinations of the N-terminal sequences. Special thanks are due to Dr. J. R. Vinograd for the gift of

some radioactive hemoglobins, for assistance in the preparation of other samples, for supervising the determination of sedimentation constants which was done with the aid of Mrs. Janet Morris, and for valuable discussions.

This investigation has been supported in part by a grant (H-2558) from the National Institutes of Health, United States Public Health Service, through the use of equipment and chemicals that had been purchased under the grant.

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