As in the usual Michaelis–Menten equation, equation 9 predicts a linear relationship between the inverse of the initial velocity (hydrolysis or methanolysis) and the inverse of the substrate concentration, $(S)_0$. By carrying out the analysis of the velocity-concentration relationship predicted by equation 9, two apparent Michaelis constants can be defined as

$$d(1/v_0^*)/d(1/(S)_0)/(1/v_0^*)_{(S)_0=\infty} = K_5(1 + (W)/K_2 + (A)/K_4)/(1 + (W)/K_2' + (A)/K_4') = K_m \text{ (methanolysis)} \quad (10)$$

$$d(1/v_0)/d(1/(S)_0/(1/v_0)_{(S)_0=\infty} = K_1(1 + (W)/K_2 + (A)/K_4)/(1 + (W)/K_2' + (A)/K_4') = K_m \text{ (hydrolysis)} \quad (11)$$

Comparison of equations 10 and 11 indicates that K_m(methanolysis) is equal to K_m(hydrolysis) *at fixed concentrations of methanol and water.* Since Q_A and Q_w, of equations 7 and 8, respectively, are not equal, the condition K_m (hydrolysis) = K_m (methanolysis) is a necessary and sufficient condition for the maintenance of quasi-equilibrium.[6]

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[CONTRIBUTION NO. 2508 FROM THE DIVISION OF CHEMISTRY AND CHEMICAL ENGINEERING, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA]

Further N-Terminal Sequences in Human Hemoglobins A, S and F by Edman's Phenylthiohydantoin Method

BY J. ROGER SHELTON AND W. A. SCHROEDER

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By means of the Edman phenylthiohydantoin method, the N-terminal sequences in the peptide chains of several human hemoglobins have been found to be as follows: α^A and α^F, val-leu-ser-pro-ala-aspNH_2-; β^A, val-his-leu-thr-pro-glu-; β^S, val-his-leu-thr-pro-val-; and γ^F, gly-his-phe.

Introduction

By Sanger's DNP-method, normal adult human hemoglobin (hemoglobin A) has been shown to contain four N-terminal valyl residues and two kinds of polypeptide chains. The two chains termed α chains terminate in val-leu[1] and the two termed β chains in val-his-leu.[1b,2] Human fetal hemoglobin (hemoglobin F) also contains two chains N-terminal in val-leu (α chains), and, in addition, two chains N-terminal in glycine (γ chains).[3] The α^A, α^S and α^F chains are identical.[4–6] (The superscripts denote the hemoglobin that was the source of the peptide chain.) All attempts to extend the above N-terminal sequences by careful partial hydrolysis of the DNP-proteins were unsuccessful because of the lability of the adjacent peptide bonds. Only a step-wise degradation procedure such as the Edman method[7] seemed capable of extending the N-terminal sequences. When the Edman method as modified by Fraenkel-Conrat[8] was applied to the several hemoglobins the above sequences were verified and extended.

Experimental

Preparation of Hemoglobin Solutions.—Solutions of hemoglobin from the blood of normal adults and sickle cell anemics and from the umbilical cord blood of new-born infants were prepared as described by Clegg and Schroeder[9] with the exception that immediately prior to use, the hemoglobin was dialyzed against water instead of developer. For most experiments with hemoglobin A, solutions so prepared were used without chromatographic purification to remove minor components.[9,10] However, the main component in sickle-cell hemoglobin and Zone F_{II}[10] were isolated chromatographically and used as hemoglobins S and F, respectively. Solutions with concentrations of about 10 mg./ml. were used. The results were unaltered when globin replaced hemoglobin in the degradation procedure.

Preparation of Single Polypeptide Chains of Hemoglobin.—Ingram[11] has successfully applied the method of Wilson and Smith[12] for the separation of the peptide chains of horse globin to the separation of the chains of human globin. The isolation of the chains was carried out in these Laboratories without alteration of procedure. The purity of the α chains was 95–100% and that of the β^A, β^S and γ^F chains about 75%.

Paper Strips.—The Fraenkel-Conrat modification[8] of the Edman procedure was used with only minor modifications. Strips of Whatman No. 1 filter paper (1 × 7 cm.) in which a small hole had been punched near one end were used as carriers for hemoglobin and the peptide chains of hemoglobin. Each strip would absorb about 0.08 ml. of solution without difficulty. The strips carrying the isolated peptide chains were oven dried at 90° for 5 minutes prior to the first treatment with phenyl isothiocyanate.

Formation of PTC-Protein and Subsequent Degradation.—Essentially, the only modification of these steps in the procedure[8] lay in the method of exposing the strips to reagents. The strips were not placed in beakers[8] or laid in petri dishes.[8] Rather, racks of glass rods with glass hooks were used. On the glass hooks, the strips were suspended above the reagents by means of the small hole in each strip. Suspension in this way permitted good access by the reagent to both sides of the strip. The time of exposure to acid during the degradation was varied from step to step as experience suggested; the time at each step is given in the results below.

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(1) (a) H. S. Rhinesmith, W. A. Schroeder and L. Pauling, THIS JOURNAL, **79**, 609 (1957). (b) G. Braunitzer, *Z. physiol. Chem.*, **312**, 72 (1958).

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(4) J. R. Vinograd, W. D. Hutchinson and W. A. Schroeder, *ibid.*, **81**, 3168 (1959).

(5) J. A. Hunt, *Nature*, **183**, 1373 (1959).

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(7) P. Edman, *Acta Chem. Scand.*, **4**, 277, 283 (1950).

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(10) D. W. Allen, W. A. Schroeder and J. Balog, *ibid.*, **80**, 1628 (1958).

(11) V. M. Ingram, *Nature*, **183**, 1795 (1959).

(12) S. Wilson and D. B. Smith, *Can. J. Biochem. Physiol.*, **37**, 405 (1959).

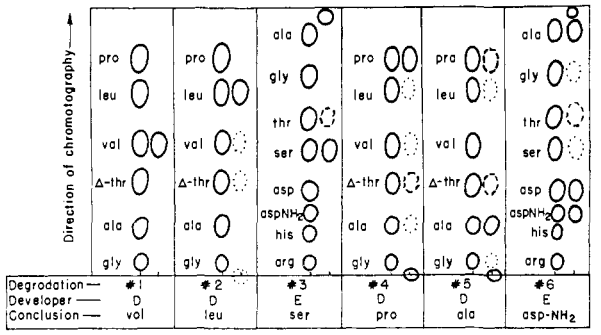

Fig. 1.—Drawings of final chromatograms for products from six degradations of α chains. At each step the sample is on the right and the standards on the left. Quantity: ———, spot prominent; – – – –, spot apparent; . . . . . , spot only faintly visible.

Isolation and Identification of PTH-Amino Acids.—The PTH-amino acids were extracted from the strips in test tubes by two changes of 8 ml. of 1:1 ethyl ether–ethanol. After the solvents had been evaporated from the extracts, the residue was taken up in acetone, spotted on starched Whatman No. 1 filter paper and chromatographed by ascending paper chromatography. Several reference PTH-amino acids were always chromatographed in one dimension side by side with the PTH-amino acid to be identified. Some sequence of developers A and C of Sjöquist[13] and of developers D, E and F of Edman and Sjöquist[14] on separate samples was sufficient in most cases to identify positively any PTH-amino acid. The erratic results that frequently were observed with developer C could be prevented by placing the lower part of the chromatographic tank (the part that contained the developer) in a water-bath at 30° during the chromatogram. The 2-butanol–phthalate buffer solvent of Landmann, Drake and Dillaha[15] was used to separate PTH-aspartic acid and PTH-glutamic acid. The reference PTH-amino acids were prepared according to Edman[7] and Edman and Lauber[16] with the exception of serine, threonine and cystine which were prepared according to Levy and Chung.[17] The iodine-azide spray described by Sjöquist[13] was used to locate the PTH-amino acids on the paper.

Results

In the figures are shown the final chromatograms of the several that were required to identify the PTH-amino acid at each degradation of the several chains. From Fig. 1, it is readily seen that the products from the α chain become increasingly complex at each degradation. After the first degradation, PTH-threonine and PTH-Δ-threonine were always present as were PTH amino acids from one or more preceding steps. PTH-Δ-threonine was the stronger spot if the time of acid treatment at a given degradation was 15 hr. whereas PTH-threonine predominated if the time of acid treatment was 7 hr. or less. In the chromatogram of the products of degradation No. 4, the PTH-serine remains at the origin with developer D. In degradation No. 6, both PTH-aspartic acid and PTH-asparagine were present in approximately equal yields. Because of the presence of both asparagine and aspartic acid in the sixth degradation, the conclusion is drawn that asparagine rather than aspartic acid occupies the sixth position in the sequence. The preceding five degradations could easily have been responsible for the hydrolysis of the amide group from the asparagine of a part of the molecules. Although many attempts were made to extend the sequence, no dominant spot appeared on the chromatograms of the seventh or ensuing degradations.

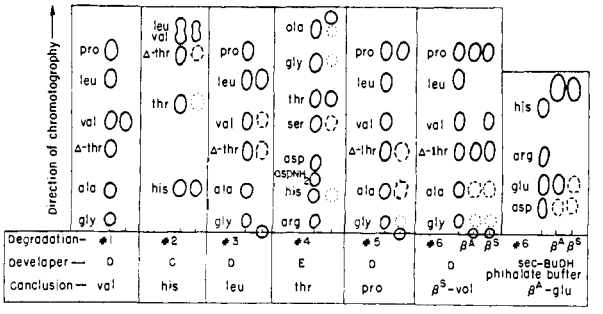

Fig. 2.—Drawings of final chromatograms for products from six degradations of β^A and β^S chains. The products from the first five degradations are identical. For sample position, etc., see legend of Fig. 1.

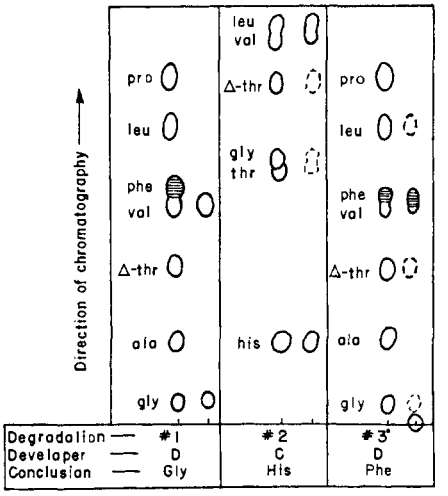

Fig. 3.—Drawings of final chromatograms for products from three degradations of hemoglobin F. Confirmatory chromatograms for the sequence relating to the γ-chain only are shown here. For sample position, etc., see legend of Fig. 1. The shaded portion of the spot of PTH-phenylalanine shows the position of the yellow color that this PTH-amino acid produces.

A comparison of Figs. 1 and 2 clearly shows that the products from the degradation of the β chain become more heterogeneous at an earlier step than in the degradation of the α chain. This is due in part to the poor yield of PTH-histidine in the second degradation of the β chain and in part to contamination of the β chain with α chain. However, if we compare the products of the sixth degradation of the β^A and β^S chains, an obvious difference is seen only in the valine and glutamic acid regions.

Figure 3 shows the chromatograms relating to the sequence in the γ chains. Although PTH-valine and PTH-phenylalanine have very similar R_f values with developer D, they are readily distinguished by the yellow center in the spot of PTH-phenylalanine.

(13) J. Sjöquist, *Acta Chem. Scand.*, **7**, 447 (1953).
(14) P. Edman and J. Sjöquist, *ibid.*, **10**, 1507 (1956).
(15) W. A. Landmann, M. P. Drake and J. Dillaha, THIS JOURNAL, **75**, 3638 (1953).
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(17) A. A. Levy and D. Chung, *Biochem. and Biophys. Acta*, **17**, 454 (1955).

TABLE I

N-TERMINAL SEQUENCES IN CERTAIN HUMAN HEMOGLOBINS AS DETERMINED ON THE WHOLE HEMOGLOBIN AND THE INDIVIDUAL POLYPEPTIDE CHAINS

Hb or chain	Residue no. 1	2	2	4	5	6
	(a) Whole hemoglobin					
Hb A	val[a]	leu[a]	ser	pro	ala	
		his[a]	leu[a]			
Hb F	val[a]	leu[a]	ser	pro	ala	
	gly[a]	his	phe			
	(b) Individual polypeptide chains					
α^{A} and F	val(7)[b]	leu(7)	ser(7)	pro(15)	ala(7)	aspNH$_2$(15)
β^{A}	val(7)	his(15)	leu(7)	thr(7)	pro(15)	glu[c](15)
β^{S}	val(7)	his(15)	leu(7)	thr(7)	pro(15)	val[c](15)

[a] Sequence previously established by DNP-method. [b] The figure in parentheses is the time in hours that was found to be most satisfactory for the degradation to the phenylthiohydantoin. [c] Not the predominating amino acid at this step. See text.

These results are summarized in Table I. They are based upon a series of 50 runs for various hemoglobins and 20 runs for the various individual chains.

Discussion

For the first experiments, whole hemoglobin was used because the individual chains were not yet available. Although the results in Table I were eventually obtained with hemoglobin, the first results were not very satisfactory. Contrary to expectations from the results by the DNP-method, threonine in the dehydrated form of PTH-Δ-threonine appeared as early as the second residue, and, in addition, histidine was difficult to detect. Extraneous spots in small amount were present on the chromatograms. In the course of the experiments, many variations in procedure were tried. Glass fiber paper was substituted in vain for Whatman No. 1 paper in the hope that some of the extraneous zones might be eliminated. Alterations in the procedure of coupling with the phenylisothiocyanate had little effect on the results. Other solvents were used for the extraction of the PTH-amino acid, A 1:4 water–acetone solution extracted PTH-histidine faster than the ether–alcohol solvent, but because the water–acetone was less convenient to evaporate prior to the preparation of the sample for spotting on the paper, this procedure was abandoned. Basically, the only vital variation lies in the period of reaction with acetic and hydrochloric acids to bring about the degradation and cyclization. It could be applied only to the individual chains from which one amino acid at a time could be removed. By repeated runs, the most satisfactory times of cyclization at each step were determined in order to obtain as complete a degradation as possible at each step with a minimum of random splitting.

The most evident extraneous product of the degradation is PTH-Δ-threonine, a result which suggests a very considerable lability of bonds to this amino acid in the protein. Under the conditions that were finally employed, however, this random splitting was kept to a minimum and did not interfere with the identification of the amino acid at each degradation. When threonine was actually present in the sequence (fourth residue of the β chains), it was readily detected. With the final procedure, it has been possible to carry the identifications unequivocally through the fifth residue of the α and β chains and to obtain good evidence for the sixth residue. Only the first three residues of the γ chains have been identified. This sequence was established with whole hemoglobin F_{II} only because application of the procedure to the γ chain itself gave almost no yield of PTH-amino acid. The cause of this behavior is as yet unexplained. However, the sequence gly-his-phe- in the γ chains agrees with less definite preliminary results of gly-(his, phe) by the DNP-method.[18]

The yield of PTH-amino acid from each degradation except the N-terminal is difficult to ascertain. After extraction of the PTH-amino acid and evaporation of the solvent, the N-terminal PTH-amino acid was taken up in a suitable quantity of absolute ethanol and the spectrum was taken between 235 and 280 mμ. By inserting the maximum reading obtained in the region of 270 mμ in the equation of Fraenkel-Conrat,[8b] the yield of PTH-amino acid was calculated. After the first degradation, however, the product contains not only the PTH-amino acid from the particular degradation but also products of random splitting and products arising from incomplete reaction in preceding degradations. Nevertheless, by comparing the intensity of the spots on the chromatogram, the yield could be estimated rather roughly. N-Terminal PTH-valine was obtained in 90–100% yield. PTH-Histidine was obtained in about 60% yield. PTH-Glycine and PTH-proline gave yields of approximately 70%. All other yields seemed to be better than 75%.

Hunt and Ingram[19] and Hill and Schwartz[20,21] have recently revised the sequence of the peptides that differs in tryptic hydrolysates of hemoglobins A and S. The sequences now reported are:

Hemoglobin A val-his-leu-thr-pro-glu-glu-lys
Hemoglobin S val-his-leu-thr-pro-val-glu-lys

Inasmuch as the difference in hemoglobins A and S is known to occur in the β chain,[4,11] they have suggested that the above sequences are N-terminal in the β chains. If true, this difference would be in the sixth residue and should be apparent in a degradative procedure such as has been used in the present investigation. However, by the sixth residue there has been a sufficient accumulation of extraneous zones due to random splitting and incomplete preceding degradations that a definitive answer at this point is difficult to obtain. Proline appears to be one of the more difficult amino acids to remove completely by the Edman method and hence its presence in the fifth position results in a poor yield in the sixth degradations. The statement that glutamic acid and valine, respectively, are the sixth amino acids in the β^{A} and β^{S} chains is based upon the comparison of the results from the careful simultaneous degradation of the two kinds of chains: the difference is apparent though not striking at the sixth residue but becomes more obvious in the seventh degradation. By this point, how-

(18) G. Matsuda, unpublished experiments.
(19) J. A. Hunt and V. M. Ingram, *Nature*, **184**, 640 (1959).
(20) R. L. Hill and H. C. Schwartz, *ibid.*, **184**, 641 (1959).
(21) R. L. Hill, personal communication.

ever, it is impossible to tell whether there is a glu-glu sequence in the β^A chain or val-glu in the β^S chain because of the complexity of the material from the degradation. The evidence of this investigation, though less conclusive than might be desired, leads to the conclusion that hemoglobins A and S differ in the sixth amino acid residue from the N-terminus of the β chains.

NOTE ADDED IN PROOF.—Among the peptides in a tryptic hydrolysate of hemoglobin A, we have found one for which all evidence points to the N-terminus of the α chains as the source. The sequence is val-leu-ser-pro-ala-asp-lys-thr-aspNH_2-val-lys. It contains aspartic acid, however, instead of asparagine in the sixth position from the N-terminus as reported in the present paper for α chains. The assignment of asparagine to the sixth position of the α chains is based on indirect evidence whereas in the peptide the sixth position is definitely occupied by aspartic acid. Experiments to resolve the discrepancy are in progress. N. Hilschmann and G. Braunitzer[22] have concluded on the basis of indirect evidence that the N-terminal sequence of the β^A chains is val-his-leu-thr-pro-glu-glu-lys-(ser, ala, ala, thr, val, leu)-(try, gly, lys)-.

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(22) N. Hilschmann and G. Braunitzer, *Z. physiol. Chem.*, **317**, 285 (1959).

[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY,[1] PHILADELPHIA 18, PENNSYLVANIA]

The Isolation of a Red Protein from Milk[2]

BY MERTON L. GROVES

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A method is described for the preparation of the red protein of milk. It was separated from casein by acid extraction, then fractionated with ammonium sulfate and DEAE cellulose chromatography. The isolated red protein is homogeneous by electrophoresis and the ultracentrifuge. It has a molecular weight of 86,100 and contains two atoms of iron. The molecular weight and absorption spectra of the red protein are similar to those given by the complex of iron with conalbumin and the B_1-metal combining protein of plasma. It is a glycoprotein with an isoelectric point at about *p*H 7.8. It is possible to remove the iron at *p*H 2 with Dowex 50. Addition of ferric ions to the apoprotein appears to restore the complex.

The occurrence of a red protein in bovine milk was reported in 1939 by Sörensen and Sörensen[3] who found it to be present in a small concentration and were able, by careful fractionation of the whey protein, to prepare it in a partially purified form. Polis and Shmukler[4] at this Laboratory also partially purified a red protein during the course of preparation of lactoperoxidase by column chromatography of whey protein fractions. In none of this work was the red protein purified sufficiently to permit accurate characterization, however, it was reported to contain iron, an unusually high content of tryptophan and some phosphorus and carbohydrate. Recently, Johansson[5] isolated a salmon-colored component from human milk whey by calcium phosphate chromatography and like the red protein of bovine milk, it contains a small amount of iron.

In the present study, the red protein was prepared from casein rather than from the whey. During the preparation of α_z-casein[6] by the acid extraction of casein, it was found that the red protein was also extracted. The isolation and characterization of the red protein is described.

Preparation of the Red Protein.—Three preparations of the pure red protein were made and the method used for their preparation varied only slightly. They will be referred to as preparations A, B and C.

The casein from 15 gallons of fresh unpasturized skim milk was precipitated at 25° by the addition of 1 *N* HCl to *p*H 4.6. After filtration through a cloth bag and draining overnight at 2°, the casein was either stored at about −20° until needed or worked up immediately by washing four to five times with distilled water in a 20 gallon crock. The supernatant was removed by decantation. Resuspension of the acid precipitated casein in distilled water was always accompanied by a drop in *p*H which was adjusted to 4.6–4.7 by the addition of 0.1 *N* sodium hydroxide. The amount of alkali required decreased on successive washings until it became negligible. Thorough washing of the casein is necessary to remove the whey proteins that are carried down with the occluded water of the casein.

Acid Extraction of the Casein.—The washed casein was suspended in 16 l. of water and the *p*H adjusted to 4.0 with 1 *N* acetic acid. After 2 hr. of stirring during which the *p*H was kept at 4.0, the casein was removed by filtration on large Buchner funnels. The filtrate, which contains about 2% of the original protein, was then adjused to *p*H 6.0 and a casein fraction was precipitated.[6] It was removed by centrifugation. A proteolytic enzyme[7] was associated with this fraction. On saturation of the filtrate with ammonium sulfate, the protein which precipitated was filtered off by gravity. It contained the red protein and phosphatase among other proteins. The above fractionation was carried out in the presence of liberal amounts of toluene as a preservative.

Ammonium Sulfate Fractionation.—The fraction containing the red protein was next dialyzed free of ammonium sulfate at 2° giving a brown solution with some precipitate. After lyophilization, water was added to give a 2% protein concentration, the *p*H adjusted to 7.6 and a saturated solution of ammonium sulfate also adjusted to *p*H 7.6 was then added. The red protein precipitated between 40 and 65% of saturation and for preparation B this fraction contained 2.59 g. protein. In earlier experiments, the fractionation with ammonium sulfate was repeated. On the second fractionation of preparation A, the red fraction precipitated

(1) Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

(2) Presented at the 135th Meeting of the American Chemical Society, Boston, Mass., April 1959.

(3) M. Sörensen and S. P. L. Sörensen, *Compt. rend. trav. lab. Carlsberg*, **23**, 55 (1939).

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(7) R. C. Warner and E. Polis, THIS JOURNAL, **67**, 529 (1945)