(filtrate) = 10,460 (88% recovery based on G5′P–NH_2). The nucleotides were fractionated in a 2 cm. diameter × 4 cm. high Dowex-1-chloride column: H_2O, 134 ml., $TOD_{2.8}^{280}$ = 244; 0.025 *M* NH_4Cl, 1.876 ml., $TOD_{2.8}^{280}$ = 1,820 (C5′-P)[34]; 0.003 *N* HCl + 0.03 *M* LiCl,[35] 700 ml., $TOD_{2.8}^{280}$ = 7,196 (CDP, 68%),[36] 2 *N* HCl, 64 ml., $TOD_{2.8}^{280}$ = 38; total recovery, 9,298 (89%). The CDP fraction was worked up in a manner similar to that described for GDP; yield, 200 mg. of amorphous, white lithium salt. This material gave a single spot by electrophoresis and was active in the polynucleotide phosphorylase exchange assay.[32]

Anal. Cytidine:labile P:total P, 1.00:0.97:1.95. Theory, 1.00:1.00:2.00. Equivalent weight based on phosphate analysis = 486 which corresponds to $Li_2HCDP \cdot 4H_2O$[37] (theory 487).

(34) This is actually a double peak, but the only ultraviolet absorbing material which could be detected by chromatography or electrophoresis was C5′P. The double peak effect is attributed to simultaneous elution of inorganic phosphate and C5′P. A similar effect has been found with UMP and inorganic phosphate (see ref. 5).

(35) On some runs a small peak (3%) was eluted with 0.003 *N* HCl. This material has been tentatively identified as P¹P²-dicytidine 5′-pyrophosphate (*cf.* G. W. Kenner, C. B. Reese and A. R. Todd, *J. Chem. Soc.*, 546 (1958)). This peak did not appear with 0.003 *N* HCl in the run described here, nor could the presence of P¹P²-dicytidine 5′-pyrophosphate be detected by electrophoresis.

(36) The yield of CDP (68%) using dioxane diphosphoric acid was only slightly higher than runs made with 85% phosphoric acid (58%). However, the run described here was made during hot, humid weather and experiments with ADP have indicated that special precautions are necessary to exclude moisture under these conditions. Therefore, it is probable that the yield can be considerably increased.

Adenosine 5′-Diphosphate.—Dioxane diphosphoric acid was freshly prepared and dried for 3 days at room temperature *in vacuo* over P_2O_5. Dicyclohexylguanidinium adenosine 5′-phosphoramidate[3] was dried over P_2O_5 *in vacuo* overnight. *o*-Chlorophenol was redistilled. All glassware was dried in an oven and cooled in a desiccator over P_2O_5. The following operations were carried out in a dry box.[38] Dioxane diphosphoric acid (300 mg.) and A5′P–NH_2 (100 mg.) were dissolved in 3 ml. of *o*-chlorophenol contained in a 12 ml. conical centrifuge tube. The tube was fitted with a mercury seal stirrer and removed from the dry box. The remaining operations were carried out in the room. The reaction mixture was stirred rapidly for 3 hrs. at 0°. Petroleum ether (b.p. 30–60°, 8 ml.) was added. The solid was removed by centrifugation and washed 3 × 2 ml. of dry ether. The white powder was dissolved in 4 ml. of 1 *N* NH_4OH and diluted to 10.0 ml. with water; $TOD_{2.8}^{260}$ = 2,400. The mixture was fractionated on a 1 cm. diameter × 6 cm. high Dowex-1-chloride column: H_2O, 88 ml., $TOD_{2.8}^{260}$ = 26; 0.003 *N* HCl + 0.015 *M* LiCl (two peaks), 246 ml., $TOD_{2.8}^{260}$ = 70 (A5′P–NH_2); 212 ml., $TOD_{2.8}^{260}$ = 303 (A5′P); 0.003 *N* HCl + 0.03 *M* LiCl, 345 ml., $TOD_{2.8}^{260}$ = 1,975 (ADP, 83%); 2 *N* HCl, 74 ml., $TOD_{2.8}^{260}$ = 48. Total recovery = 2.410 (100%). ADP was isolated as its lithium salt in the usual way; 38 mg.

This procedure offers a considerable improvement in yield over that obtained using 85% phosphoric acid.[4]

(37) Data are not sufficiently accurate to rule out other formulations such as $Li_2CDP \cdot 3H_2O$.

(38) Rigidly anhydrous conditions are necessary, but the dry box is required only during humid weather.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE CARNEGIE LABORATORY OF CHEMISTRY, ALLEGHENY COLLEGE]

A Quantitative Study of the Number of N-Terminal Amino Acid Residues and the Number and Kind of N-Terminal Peptides in Horse Hemoglobin

BY MONTE LEVITT AND HERBERT S. RHINESMITH

RECEIVED AUGUST 7, 1959

Horse dinitrophenyl(DNP)globin has been hydrolyzed in refluxing 6 *N* hydrochloric acid. The amount of DNP-valine recovered after a 22 hour hydrolysis indicates that there are four N-terminal residues per molecule. Two kinds of N-terminal peptides have been found after a 15 minute hydrolysis. DNP-val-leu is released almost quantitatively from two chains, corresponding to the α-chains of human hemoglobin. Two β-chains have the N-terminal sequence val-glu-leu. Two DNP-peptides have been isolated almost quantitatively from the β-chains: DNP-val-glu and DNP-val-glu-leu; their movements and separation on chromatographic columns are discussed.

Introduction

Various values for the number of N-terminal valyl residues in horse hemoglobin have been published. Porter and Sanger[1] reported in 1948 that horse hemoglobin has six N-terminal valyl residues per molecule. This figure was also obtained by Ozawa and Satake[2] in 1955. In contrast, Schramm, Schneider and Anderer[3] in 1956 and Smith, Haug and Wilson[4] in 1957 reported four N-terminal valyl residues. Still another figure was obtained by Undenfriend and Velick[5] who in 1951 detected by their method only two N-terminal residues in horse hemoglobin.

In addition, various N-terminal sequences have been reported in the literature. Sanger[6] has reported crystallizing DNP-val-leu, DNP-val-glu-leu and DNP-val-glu(NH_2)-leu. Ozawa and Satake[2] have given the following N-terminal sequence for the six chains they reported: A chains, val-leu; B chains, val-gly; C chains, val-glu. Smith, Haug and Wilson[4] obtained evidence that the N-terminal sequence is val-leu and val-glu or val-glu(NH_2). Finally, Dévényi[7] reported that he had isolated val-leu-lys from horse hemoglobin.

In 1957, Rhinesmith, Schroeder and Pauling,[8] using normal adult human hemoglobin, made a quantitative study of various aspects of Sanger's DNP method and showed that the correction factor for operational, chromatographic and hydrolytic losses was appreciably lower than previously reported values of 20–30%. These authors reported a correction factor of 8% for the destruction of DNP-valine during a 22 hr. hydrolysis in refluxing 6 *N* hydrochloric acid and 5% for two chromatograms on silicic acid–celite adsorbent; the same

(1) R. R. Porter and F. Sanger, *Biochem. J.*, **42**, 287 (1948).

(2) H. Ozawa and K. Satake, *J. Biochem. (Japan)*, **42**, 641 (1955).

(3) G. Schramm, J. W. Schneider and A. Anderer, *Z. Naturforsch.*, **11b**, 12 (1956).

(4) D. B. Smith, A. Haug and S. Wilson, *Federation Proc.*, **16**, 766 (1957).

(5) S. Udenfriend and S. F. Velick, *J. Biol. Chem.*, **190**, 733 (1951).

(6) F. Sanger, *Nature*, **162**, 491 (1948).

(7) T. Dévényi, *Acta Physiol. Acad. Sci. Hung.*, **9**, 321 (1956).

(8) H. S. Rhinesmith, W. A. Schroeder and L. Pauling, THIS JOURNAL, **79**, 609 (1957).

corrections were employed in the present investigation.

The conflicting results reported in the literature indicate the desirability of a quantitative study of the N-terminal valyl residues and peptides in horse hemoglobin. In this paper the quantitative techniques developed in the study of human hemoglobin[8] have been applied to horse hemoglobin.

Experimental

Preparation and Purification of Horse DNP-Globin.—Horse blood was treated by the methods of Drabkin,[9,10] with slight modifications. The erythrocytes were hemolyzed in a mixture of water and toluene and the ruptured cells removed by centrifugation at 5,000 r.p.m. The oxyhemoglobin was further purified by centrifugation for 1 hr. at 15,000 r.p.m. to remove traces of stroma. It was then converted to carbonmonoxyhemoglobin and crystallized by dialysis against 2.8 *M* phosphate buffer at *p*H 6.8 saturated with carbon monoxide. The crystals were then washed with buffer and centrifuged for five minutes at 5,000 r.p.m. until the wash solution was colorless. The dialysis and washing were repeated a second time. The twice-crystallized material was dialyzed against frequently changed distilled water for several days until the molybdate test for phosphate ion was no longer positive. This stock solution of carbonmonoxyhemoglobin was stored at 0° under carbon monoxide.

The concentration of the carbonmonoxyhemoglobin solution was determined with the Beckman D.U. spectrophotometer, using the factor 1.14 g. cm. per l. at 541 mμ.[11] The concentration was approximately 4 g. per 100 ml. of solution.

Twice-crystallized carbonmonoxyhemoglobin was converted to DNP-hemoglobin by an adaption of the method of Levy and Li[12] as previously reported.[8] The *p*H was maintained at 8.15 during the dinitrophenylation.

Air-dried DNP-globin was prepared from DNP-hemoglobin by the method of Anson and Mirsky[13] as described in an earlier paper.[8]

Determination of the N-Terminal Residues in Horse DNP-Globin.—For hydrolysis, a 0.1-g. sample of air-dried horse DNP-globin was suspended in 10 ml. of doubly-distilled 6 *N* hydrochloric acid and refluxed for 22 hr. on a hot plate. The solution was cooled to room temperature and extracted with 4 × 25 ml. of ether. The combined ether extracts were washed with 4 × 5 ml. of distilled water, each containing one drop of 6 *N* hydrochloric acid. The ether was evaporated and the residue was taken up in acetone and re-evaporated.

The separation and identification of the DNP-compounds extracted from the hydrolysis of horse DNP-globin was performed by chromatography by the method of Green and Kay,[14] with slight modification. The adsorbent was a mixture of Mallinckrodt analytical reagent silicic acid powder (Lot 2844) and Celite 545 in the ratio 4.7 to 1 by weight.

The residue from the ether extract was dissolved in 1AA-10A-L[15] and chromatographed with 9AA-4A-L.[16] Three zones were observed, corresponding to Groups I, III and IV in the scheme of Green and Kay.[14] The Group I zone was hydrolyzed and was found to contain no DNP-valine; its identity is not known. The Group III zone was chromatographed with 3F-8E-L and separated into two zones, the smaller and slower of which was DNP-val-leu; the other zone was dinitroaniline (DNA). The Group IV zone was chromatographed with 5AA-2A-L and was identified as DNP-valine.

The concentration of each zone was determined with the Beckman D.U. spectrophotometer against glacial acetic acid.

Determination of the N-Terminal Peptides in Horse DNP-Globin.—Samples of horse DNP-globin varying in weight from 0.2 to 0.4 g. were hydrolyzed for exactly 15 min. in refluxing 6 *N* hydrochloric acid. A 10-ml. portion of acid was used for each 0.1 g. of sample and the hydrolysis was carried out in a pre-heated oil-bath. At the end of the hydrolysis, the reaction was stopped by immersion in water and the hydrolysate was extracted with 4 × 25 ml. of ether. The combined ether extracts were washed with 4 × 5 ml. of distilled water, each containing one drop of 6 *N* hydrochloric acid. The ether was evaporated and the residue was taken up in acetone and re-evaporated.

The residue from the ether extract was dissolved in 1AA-10A-L and chromatographed with 9AA-4A-L to produce two zones. The more strongly adsorbed zone moved like Group I and was rechromatographed with 12AA-3A-L to yield two new zones which were identified by hydrolysis and subsequent identification of the parts. The slower and smaller zone was DNP-val-glu; the faster and larger zone was DNP-val-glu-leu. The less strongly adsorbed zone from the 9AA-4A-L chromatogram moved like Group III and was rechromatographed with 3F-8E-L. The two zones obtained were identified as DNP-val-leu and DNA.

The concentration of each zone was determined spectrophotometrically against glacial acetic acid.

Results and Discussion

The Number of N-Terminal Residues.—The results of seven determinations are listed in Table I. Analyses were performed on horse DNP-globin obtained from two different donors.

TABLE I

NUMBER OF N-TERMINAL RESIDUES BASED ON 0.1 G. OF DNP-GLOBIN HYDROLYZED 22 HR.

Run no.	Sample[a]	DNP-valine isolated, μmole	DNP-valine cor.,[b] μmole	No. of N-terminal residues[c]
1	A	3.72	4.28	3.69
2	B	3.64	4.18	3.61
3	C	3.66	4.22	3.63
4	C	3.98	4.58	3.95
5	C	3.96	4.55	3.92
6	C	3.72	4.28	3.69
7	C	3.81	4.38	3.78
	Av.	3.78	4.35	3.75

[a] Samples A and B were obtained from the same horse but were dinitrophenylated in separate runs. Sample C was obtained from a second horse. [b] Corrected 13% for hydrolytic, chromatographic and operational losses. [c] Based on a molecular weight of 68,000 for horse hemoglobin.

In calculating the number of N-terminal residues, the molecular weight of horse hemoglobin was taken as 68,000.[17] Sanger[1] has reported that the globin content of air-dried horse DNP-globin is 76%. Using the two figures just given and assuming the removal of the heme to lower the molecular weight 2,600, it can be calculated that 0.1 g. of air-dried horse DNP-globin contain 1.16 micromoles.

The same results can be obtained by another method. There are 97 groups in horse hemoglobin capable of attaching DNP-groups.[18] The net increase in molecular weight if dinitrophenylation is complete is 16,200. Calculating the loss in weight

(9) D. L. Drabkin, *J. Biol. Chem.*, **164**, 703 (1946).

(10) D. L. Drabkin, *Arch. Biochem. Biophys.*, **21**, 224 (1949).

(11) H. M. Winegarden and H. Borsook, *J. Cellular Comp. Physiol.*, **3**, 437 (1933).

(12) A. L. Levy and C. H. Li, *J. Biol. Chem.*, **213**, 487 (1955).

(13) M. L. Anson and A. E. Mirsky, *J. Gen. Physiol.*, **13**, 469 (1930).

(14) F. C. Green and L. M. Kay, *Anal. Chem.*, **24**, 726 (1952).

(15) The terminology employed for solvents and developers is that of Green and Kay[14]; that is, AA for acetic acid, A for acetone, L for ligroin, F for formic acid and E for ethyl acetate. The numerals are per cent. by volume.

(16) Due to the use of a different lot of silicic acid, it was necessary to increase the per cent. of acid in the developers to obtain movements comparable to those of Rhinesmith, Schroeder and Pauling.[8] Thus, 8AA-4A-L was changed to 9AA-4A-L, 4AA-2A-L to 5AA-2A-L and 2F-8E-L to 3F-8E-L.

(17) T. Svedberg and J. B. Nichols, THIS JOURNAL, **49**, 2920 (1927).

(18) There are 38 lysyl, 36 histidyl, 11 tyrosyl, 8 cysteinyl and 4 N-terminal valyl groups.

after deheming and correcting for 5% water content, it is again found that 0.1 g. of air-dried horse DNP-globin contains 1.16 micromoles.

The data included in Table I strongly suggest that there are four N-terminal residues in horse hemoglobin. The fact that the average of seven determinations is 3.75 end groups rather than 4.00 cannot be attributed to unhydrolyzed valine. The data in Table II show that 95% of the total N-terminal valine is released as DNP-valyl peptides after 15 min. of hydrolysis, and it is to be expected that the remainder would be released in 22 hr. No DNP-valyl peptides were found after 22 hr. even though it has been shown that DNP-val-leu from human DNP-globin can survive a 22 hr. hydrolysis.[8] Apparently horse DNP-globin enhances the hydrolysis of DNP-val-leu and possibly the destruction of DNP-valine.

TABLE II

N-TERMINAL PEPTIDES ISOLATED FROM 0.4 G. OF HORSE DNP-GLOBIN AFTER A 15 MIN. HYDROLYSIS

Peptide	μmoles isolated	μmoles cor.[a]		μmoles calcd. for 2 chains
DNP-val-leu	8.09	8.80		9.28
DNP-val-glu	2.57	3.13	8.81	9.28
DNP-val-glu-leu	4.67	5.68		

[a] DNP-val-leu corrected 4% for each chromatogram. DNP-val-glu and DNP-val-glu-leu corrected 6% per chromatogram. No correction made for hydrolysis.

The N-Terminal Peptides.—Listed in Table II are the results of a quantitative study of a 0.4-g. sample of DNP-globin hydrolyzed 15 min. in refluxing 6 *N* hydrochloric acid. DNP-valyl peptides from both chains were recovered in 95% of theoretical amounts. The isolation of DNP-val-glu and DNP-val-glu-leu indicates that the N-terminal sequence of the β-chains is val-glu-leu.

The β-chain-DNP-peptides exhibit an interesting chromatographic behavior. DNP-val-glu and DNP-val-glu-leu move together in a single zone with 9AA-4A-L and are adsorbed to the same extent as Group I DNP-acids. The two DNP-peptides may be separated by chromatographing them with 12AA-2A-L or 12AA-3A-L; the latter developer is preferable as it produced a separation sooner. Even with 12AA-3A-L, the adsorption of the two peptides is so similar that 14 to 18 V's of developer,[19] depending on the concentration, are needed to separate quantitatively the two peptides.

Contrary to what might be predicted from the work of Schroeder and Honnen[20] on the chromatographic behavior of DNP-peptides, DNP-val-glu is more strongly adsorbed than DNP-val-glu-leu. This behavior is analogous to the movements of the N-terminal peptides from the β-chains in human hemoglobin where it was found[21] that DNP-val-hist was more strongly adsorbed than DNP-val-hist-leu. It thus appears in the two tripeptides DNP-val-glu-leu and DNP-val-hist-leu that the C-terminal amino acid, leucine, has a significant effect on the chromatographic behavior.

The relative quantities of DNP-val-glu and DNP-val-glu-leu recovered after a 15 min. hydrolysis indicate that the glu-leu bond is more resistant to acid hydrolysis than the bond between leucine and the next amino acid. Evidence obtained from the hydrolysis of DNP-val-glu-leu indicates that the glu-leu bond is also more resistant than the val-glu bond.

Finally, this investigation supports the following conclusions about horse hemoglobin. There are four N-terminal valyl residues per molecule. The α-chains have the N-terminal sequence val-leu and the β-chains have the N-terminal sequence val-glu-leu. Ninety-five per cent. of the N-terminal valine in both the α-chains and the β-chains is released as DNP-valyl peptides after acid hydrolysis for 15 min.

Acknowledgments.—This investigation has been supported in part by a Grant from The Research Corporation and by Grants from The American Heart Association and the Northwestern Pennsylvania Heart Association.

(19) A V is the volume of developer needed to wet the column of adsorbent.

(20) W. A. Schroeder and L. R. Honnen, THIS JOURNAL, **75**, 4615 (1953).

(21) H. S. Rhinesmith, W A. Schroeder and N. Martin, *ibid.*, **80**, 3358 (1958).

MEADVILLE, PENNSYLVANIA