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The N-Terminal Amino Acid Residues of Normal Adult Human Hemoglobin: A Quantitative Study of Certain Aspects of Sanger's DNP-Method

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A quantitative redetermination of the N-terminal valyl residues of normal adult human hemoglobin by the DNP-method of Sanger has led us to question the validity of results previously reported. Our experimental results indicate that there are 3.6 N-terminal valyl residues per molecule, based on a molecular weight of 66,700 for human hemoglobin. The essential difference between this value and those of other investigators lies in a correction factor for operational, chromatographic and hydrolytic losses (13%) which is appreciably lower than any previously reported value. This low value is justified by a detailed study of losses with DNP-valine and two peptides, DNP-val-gly and DNP-val-leu, the latter an important hydrolytic product of human hemoglobin itself. On the basis of these results an integral value for the number of end groups in human hemoglobin can be achieved only by revising the molecular weight. If, on the other hand, the number of N-terminal valyl residues in human hemoglobin is non-integral, it may well indicate that normal adult human hemoglobin contains more than one kind of molecule.

Hemoglobins from several species were among the first proteins to which Sanger applied his DNP method for the identification of N-terminal amino acid residues of proteins. Porter and Sanger[2] in 1948 concluded that human hemoglobin has five N-terminal valyl residues per molecule. Havinga[3] in 1953 reported the same results and indicated an identical value for sickle-cell-anemia hemoglobin.

In the quantitative application of Sanger's method, five aspects of the procedure greatly influence the reliability of the final results; these factors are (1) the quantitative dinitrophenylation of the protein, (2) the quantitative hydrolysis of the DNP-protein, (3) the equivalence between the free and the DNP-protein, (4) the partial destruction of the N-terminal DNP-amino acids during hydrolysis of the DNP-protein, and (5) the operational loss of DNP-amino acids during extraction and chromatography of the protein hydrolysates. Of these factors, the fourth is by far the most difficult to assess correctly, and it is of vital importance in the final calculation of the number of N-terminal residues per molecule. Thus, Porter and Sanger estimated that the destruction of DNP-valine during hydrolysis amounted to 20 to 35% depending upon the time and conditions of hydrolysis. Corrections of this degree are relatively unimportant if only one or two N-terminal residues per molecule are present, but when there are five N-terminal residues, as reported for human hemoglobin, corrections of 20 to 35% are equivalent to more than one residue per molecule.

In the case of human hemoglobin an additional factor (6), unrelated to the DNP-method, exists, because the exact molecular weight of human hemoglobin is still open to some question. Since this weight is the reference standard for calculation of the end group values any reported discrepancies might well be considered.

The present investigation was begun in order to compare the N-terminal residues of normal human and sickle-cell-anemia hemoglobin. As the initial phase of the problem, work was begun on the easily accessible normal human hemoglobin in order to ascertain whether our techniques for the DNP procedure were satisfactory. It soon became evident that our results would not agree with those of Porter and Sanger because the number of N-terminal valyl residues we obtained was definitely less than five and possibly as low as three. Furthermore, it was apparent that the destruction of DNP-valine during the hydrolysis of the DNP-protein was much less under our conditions than had been found by Porter and Sanger or by Masri and Singer,[4] who, during the course of this work, reported that probably only four valyl residues were N-terminal both in normal adult human hemoglobin and sickle-cell-anemia hemoglobin. Huisman and Drinkwaard[5] reported the same values as Sanger and Havinga for normal and sickle-cell-anemia hemoglobin and in addition found five N-terminal valyl residues for hemoglobins C and E. Brown[6] in 1956 added results on hemoglobin from patients suffering from multiple myeloma, chronic myelogenous leukemia and Hodgkin's disease to the list of human hemoglobins for which five N-terminal valyl residues are reported to be present.

Because of the discrepancies between our initial results and those reported in the literature, we felt the need for a more exhaustive study of the several quantitative phases of the method as outlined above. In this paper we present the results of these investigations with particular emphasis upon the destruction of the DNP-amino acids during the hydrolysis of the DNP-protein and upon certain phases of the dinitrophenylation problem.

Experimental

Outline of Procedure.—The work involved in determining the number of N-terminal residues in normal adult human hemoglobin comprises five major operational steps. (1) Purified, crystalline human hemoglobin was dinitrophenylated to give DNP-hemoglobin, from which the hemes were then removed to produce DNP-globin. (2) Samples (0.1000 g. each) of air-dried DNP-globin were hydrolyzed under fixed conditions and the μmoles of N-terminal DNP-valine were determined by extraction and quantitative chromatography. (3) The amount of DNP-valine present in 0.1000 g. of air-dried DNP-globin was obtained by correcting the experimental value obtained in (2) for (a) operational and chromatographic losses, (b) loss of DNP-valine destroyed during hydrolysis and (c) any DNP-valine still in the form of unhydrolyzed DNP-valyl peptides. (4) The

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

(3) E. Havinga, *Proc. Natl. Acad. Sci. U. S.*, **39**, 59 (1953).

(4) M. S. Masri and K. Singer, *Arch. Biochem. Biophys.*, **68**, 414 (1955).

(5) T. H. J. Huisman and J. Drinkwaard, *Biochim. Biophys. Acta*, **18**, 588 (1955).

(6) H. Brown, *Arch. Biochem. Biophys.*, **61**, 241 (1956).

weight of hemoglobin equivalent to 0.1000 g. of DNP-globin was obtained by comparing the amino acid content of the two proteins. (5) The data of (3) and (4) together with a value for the molecular weight of human hemoglobin were used to calculate the number of N-terminal residues per molecule of human hemoglobin.

Preparation and Purification of Hemoglobin.—In some experiments use was made of hemoglobin solutions prepared by hemolysis of red blood cells and purified further only by high speed centrifugation. However, for most of the work crystalline hemoglobin was employed, prepared from red blood cells obtained from a commercial laboratory. The methods of Drabkin[7,8] were used with minor modifications. After two crystallizations by dialysis against 2.8 *M* phosphate buffer at *p*H 7 the crystals were dissolved, and the solution was dialyzed against distilled water for three days with several changes per day and against doubly distilled water for two days with two changes per day. A negative test for phosphate ion was obtained from the final dialyzing water. Before and after crystallizations the solutions were centrifuged at 78,000 × *g* (av.) for 30 min. The final solution was saturated with carbon monoxide to convert the oxyhemoglobin to carbonmonoxyhemoglobin, centrifuged at high speed, and stored in a brown glass bottle under carbon monoxide at 0°.

The homogeneity of the crystallized hemoglobin was tested by paper electrophoresis[9] and by starch block electrophoresis.[10] In each test the hemoglobin which had been crystallized behaved as though homogeneous. On the other hand, carbonmonoxyhemoglobin that had been prepared by hemolysis of cells but not crystallized exhibited the heterogeneity reported by Kunkel and Wallenius.

The concentration of the solutions was determined with the Beckman D. U. spectrophotometer, the factor used at 541 mμ being 1.14 g. cm. per l., as given by Winegarden and Borsook.[11] The stock solutions varied in concentration from 4.5 to 12.0 g. of hemoglobin per 100 ml. of solution.

Dinitrophenylation of Hemoglobin.—Proteins are commonly dinitrophenylated by Sanger's original procedure[12] in dilute aqueous ethanol in the presence of sodium bicarbonate. Under these conditions the protein usually precipitates immediately or rapidly. However, Sörm, Körbl and Matoušek[13] prepared water-soluble DNP-derivatives of horse serum albumin, ovalbumin, and insulin by omitting ethanol, even though Porter[14] had stated that the ε-amino groups of lysine in β-lactoglobulin and in several native serum globulins would not react with DNFB unless the protein was first denatured. More recently, Levy and Li[15] have described a method of dinitrophenylation in aqueous solution at 40° at constant *p*H. In the present work both Sanger's procedure and the Levy and Li method have been used to prepare the DNP-protein.

In dinitrophenylation by the Sanger procedure a twofold excess[16] of DNFB (0.1 ml.) was dissolved in 6 ml. of ethanol and added to 3 ml. of 10% hemoglobin solution previously treated with 0.1 g. of sodium bicarbonate. Addition of the alcoholic solution caused the immediate precipitation of the protein as a dark red gelatinous solid. At the end of 5 hr. of mechanical shaking the precipitate was greenish black in color and the supernatant liquid was light yellow. The solid was centrifuged and purified by washing exhaustively with acidified water as described in the procedure given below.

Because most of our DNP-hemoglobin was prepared by an adaptation of the Levy and Li method, a typical dinitrophenylation using this procedure is described below. A solution of 0.91 g. of purified hemoglobin in 100 ml. of distilled water was placed in a tall beaker and maintained at 40° in a constant temperature bath. The *p*H of the resulting solution (6.35) was brought to 9.0 by the addition of 3.25 ml. of 0.082 *N* potassium hydroxide. A twofold excess[16] of DNFB (0.35 ml.) was added and the solution was stirred vigorously to suspend the rather insoluble reagent in tiny droplets. The course of the reaction was followed by titrating the liberated hydrofluoric acid with standard alkali to maintain the *p*H at 9.0. At the end of the reaction the DNP-hemoglobin remained in solution in the reaction mixture, which was dark green in color. Addition of 1.5 ml. of *N* hydrochloric acid reduced the *p*H of the solution to 4.65 and precipitated the DNP-hemoglobin as a flocculent and gelatinous green precipitate. It was allowed to stand in the refrigerator overnight to coagulate before purification. Centrifugation gave a green solid and a bright yellow supernatant liquid. The solid was washed exhaustively with hydrochloric acid solution of *p*H 3.0[17] until the wash solutions showed no yellow color. The material was then air dried for direct use or was converted into DNP-globin as indicated below.

Preparation of DNP-Globin.—For removing the heme in DNP-hemoglobin, we used the method which Anson and Mirsky[18] devised for the preparation of water-soluble globin. The DNP-hemoglobin described above was suspended in 40 ml. of an ice-cold solution of 0.05 *N* hydrochloric acid. The suspension was then poured into an ice-cold mixture of 200 ml. of acetone and 2.0 ml. of *N* hydrochloric acid and the mixture was shaken mechanically for 1 hr. The fine yellow precipitate of DNP-globin was allowed to settle in the refrigerator overnight. The bulk of the solvent was decanted[19] and the product was washed exhaustively with acetone on a fritted glass suction funnel. It was finally rinsed several times with ether, removed from the funnel and allowed to equilibrate at room temperature. The air-dried product contained about 4.5% water, part of which it lost and gained reversibly with change in the relative humidity.

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

For the chromatography, the method of Green and Kay[20] was used for the separation and identification of the DNP-compounds which were extracted from the hydrolysates of DNP-globin or DNP-hemoglobin. The adsorbent was a mixture of Mallinckrodt analytical reagent silicic acid powder[21] and Celite 545 in ratio of 4.7 to 1 by weight. The columns were 9 × 150 mm. in dimension. The residue from the extract of the DNP-protein hydrolysate was dissolved in 2 ml. of 1AA-10A-L[22] and chromatographed with 8AA-4A-L for the group separation. Two zones were apparent on the chromatogram and that in Group IV was identified as DNP-valine by rechromatographing with 4AA-2A-L. The zone in Group III on rechromatographing with 2F-8E-L separated into two zones of which the smaller was the more strongly adsorbed. The smaller zone was identified as DNP-val-leu by hydrolysis and subsequent identification of the parts and the larger zone was found to be dinitroaniline (DNA) by further chromatography and spectrum.

For the spectrophotometric determination, a suitable aliquot of the sample was dissolved in glacial acetic acid and the optical density at the maximum was determined in the

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

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

(9) E. L. Durrum, *Science*, **113**, 66 (1951).

(10) H. G. Kunkel and G. Wallenius, *ibid.*, **122**, 288 (1955).

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

(12) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(13) F. Sörm, J. Körbl and L. Matoušek, *Collection Czechoslov. Chem. Commun.*, **15**, 295 (1950).

(14) R. R. Porter, *Biochim. Biophys. Acta*, **2**, 105 (1948).

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

(16) Calculated on the assumption that the molecule of hemoglobin contains about 100 groups which react with DNFB.

(17) Unless the wash solution is acid, the DNP-hemoglobin tends to peptize.

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

(19) This solution should be preserved if recovery of the dissolved heme is desired.

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

(21) Mallinckrodt silicic acid powder (Lot ANE-1) after equilibrium with atmospheric moisture and admixture with Celite in the proportion given was almost identical in its chromatographic properties with 2:1 Merck silicic acid (40446)-Celite 545 which was used by Green and Kay.[20]

(22) Abbreviations for solvents and developers follow Green and Kay,[20] that is, AA for acetic acid, A for acetone, L for ligroin, F for formic acid and E for ethyl acetate.

usual manner. Calibrated volumetric equipment was used in all cases. Since many DNP-valine determinations had to be made, the molecular extinction coefficient for purified DNP-L-valine was determined at 340 mμ in glacial acetic acid and reduced to the ratio (concentration in micromoles per 100 ml. of solution)/(optical density for a 1 cm. length of solution). The value of 6.2 obtained agreed exactly with that reported by Schroeder[23] for DNP-DL-valine.

Determination of the Molar Equivalence of Hemoglobin and DNP-Globin.—Before the number of N-terminal residues in a protein can be calculated from the quantity of the DNP-amino acid isolated, it is necessary to know the molecular weight of the DNP-protein. Usually this information is obtained indirectly by determining the equivalence between the free and DNP-protein. Thus, Porter and Sanger[2] compared the amide contents of the free protein and of DNP-globin and reported that the ratio was such that a given weight of air-dried DNP-globin from human hemoglobin contained 77% as much globin as an equal weight of globin itself. In the present work, this equivalence was determined by comparing the content of eight amino acids in DNP-globin with the known values for hemoglobin.

Thirty-mg. samples of the air-equilibrated DNP-globins were hydrolyzed by refluxing in doubly distilled 6 *N* hydrochloric acid for 22 hr. About one-tenth of the sample was then used for the estimation of aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine and valine by the ion-exchange chromatographic method of Moore and Stein.[24] The original ninhydrin method[25] was used for the colorimetric determinations. The values thus obtained for each amino acid were compared with the values previously found for hemoglobin.[26] If the results are expressed as the per cent. of globin in DNP-globin compared to hemoglobin, the following results from DNP-protein sample no. VII (Table I) are typical. On the basis of the individual amino acids, the percentages were: aspartic acid, 75; threonine, 78; serine, 76; glutamic acid, 79; proline, 91; glycine, 73; alanine, 73; and valine, 78. The high value for proline was obtained from all samples and is an indication that the determined content of proline in hemoglobin is incorrect.[27] The result from valine has been corrected for an assumed loss of four residues of valine per molecule by dinitrophenylation. If proline is excluded, the average of the results from sample no. VII is 76%. The average from sample no. III is 76%, from sample no. V 76%, and from sample no. VI 78%. On the basis of the excellent agreement of these results, the other samples were assumed to have the value 76%.

It should be emphasized that these values apply to air-equilibrated DNP-globin, which contains about 5% moisture. When the data are used to calculate a molecular weight of DNP-globin, it is an apparent molecular weight that includes the moisture content of the sample.

Destruction of DNP-Valine during Hydrolysis and Loss during Chromatographic Operations.—The destruction of N-terminal DNP-amino acid during the hydrolysis of a DNP-protein is generally taken into account by "hydrolyzing" a known amount of the free N-terminal DNP-amino acid either in the presence or absence of protein under the conditions that obtain during the hydrolysis of the DNP-protein and then determining the amount that was destroyed. In order to ascertain better the actual destruction we have not only made the customary recovery experiments with DNP-valine but have also extended them to include DNP-val-gly and DNP-val-leu, the latter being an important partial hydrolytic product of DNP-globin.

For these experiments, DNP-DL-valine, DNP-L-valine and DNP-L-val-gly were prepared from DL-valine (Winthrop Chemical Co.), L-valine (U. S. P. Reference Standard) and L-val-gly (Mann Research Laboratories) by the third procedure of Schroeder and LeGette,[28] and were chromatographed to remove dinitrophenol (DNP-DL-valine and DNP-L-valine with 4AA-2A-L and DNP-L-val-gly with 8AA-4A-L). The DNP-val-leu was prepared by hydrolyzing DNP-hemoglobin partially in refluxing 6 *N* hydrochloric acid for 2 hr. The ether-extractable portion of the hydrolysate was chromatographed on silicic acid–Celite with 2F-8E-L, by means of which the DNP-val-leu readily was separated from the DNP-valine, dinitroaniline, and a small amount of extraneous material.

Known amounts of these compounds were hydrolyzed for 22 hr. in refluxing 6 *N* hydrochloric acid either alone or in the presence of DNP-globin. The hydrolysates were extracted and the extracts were analyzed as described above.

Results and Discussion

Quantitative Determination of the Ether-extractable Compounds from the Hydrolysates.—The results of eleven analyses are presented in Table I. The first two determinations were made on DNP-hemoglobin and the remainder on DNP-globin. DNP-Globin was selected for most of the determinations primarily because of ease of manipulation and purification. Hemoglobin from four different donors was used and six dinitrophenylations were run under a variety of experimental conditions. Of the eight different DNP-protein samples which were hydrolyzed, two were in duplicate. Five runs were made on DNP-protein from uncrystallized hemoglobin and six on material from twice crystallized protein. The time of hydrolysis, with two minor deviations, was 22 hr. and the μmoles of DNP-valine reported represents material actually isolated with no corrections whatsoever applied. The average of 11 results is 3.39 μmoles with a standard deviation of 0.12 and a spread equal to 12% of the average. The results are independent of the source of the protein whether from crystalline hemoglobin or from uncrystallized material.

Dinitrophenylation of Hemoglobin.—Completeness of dinitrophenylation is a factor of great importance in the quantitative determination of the N-terminal residues of a protein by the Sanger method. The time required for complete reaction between DNFB and a protein varies widely with the solubility of the protein. Thus, the reaction with an insoluble protein like wool[29] is still incomplete after 72 hr., while collagen[30] takes from 3 days to 3 weeks. In contrast to these results, however, Levy and Li[15] showed that the dinitrophenylation of the water-soluble α-corticotropin was complete in 1.5 hr. at 40° and *p*H 8.0. Complete dinitrophenylation of the hemoglobin molecule probably requires the reaction of DNFB with the ε-amino groups of the 44 lysyl residues, the imidazole rings of the 36 histidyl residues, the hydroxyl groups of the 11 tyrosyl residues, the sulfhydryl groups of the 8 cysteinyl residues, and the N-terminal valyl residues. To ensure a complete reaction a twofold excess of DNFB was used and the time was varied from one to 14 hr. If one examines the uncorrected results in Table I it appears that the dinitrophenylation of the entire molecule must be complete in about one hr., because the values are essentially the same whether the dinitrophenylation is stopped after one hr. or continued for 14 hr. If the reaction had ever been stopped when the dinitrophenylation was only partially complete, con-

(23) W. A. Schroeder, THIS JOURNAL, **74**, 5118 (1952).

(24) S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

(25) S. Moore and W. H. Stein, *ibid.*, **176**, 367 (1948).

(26) W. A. Schroeder, L. M. Kay and I. C. Wells, *ibid.*, **187**, 221 (1950).

(27) If the proline content of hemoglobin as determined by P. C. van der Schaaf and T. H. J. Huisman (*Biochim. Biophys. Acta*, **17**, 81 (1955)) and by A. Rossi-Fanelli, D. Cavallini and C. de Marco, (*ibid.*, **17**, 377 (1955)) is used to calculate the percentage, the result is 80%.

(28) W. A. Schroeder and J. LeGette, THIS JOURNAL, **75**, 4612 (1953)

(29) S. Blackburn, *Biochem. J.*, **47**, 443 (1950).

(30) J. H. Bowes and J. A. Moss, *ibid.*, **55**, 735 (1953).

TABLE I

QUANTITIES OF ETHER-EXTRACTABLE COMPOUNDS FROM HYDROLYSATES OF 100 MG. OF DNP-GLOBIN

Run no.	Hemoglobin sample[a]	DNP-ation no.	DNP-ation time, hr.	DNP-Protein hydrolyzed		Hydrolysis time, hr.	μmole DNP-valine isolated[b]	μmole dinitroaniline isolated	μmoles DNP-val-leu isolated
1	A	i[c]	5	I	Hb	20.8	3.26	3.00	
2	B	ii	1	II	Hb	22.5	3.14		
3	B	ii	1	III	Globin	22.0	3.46		
4	B	ii	1	III	Globin	22.0	3.31		
5	C	iii	1.8	IV	Globin	22.0	3.35		
6	C	iv	12	V	Globin	22.0	3.41	4.92	0.13
7[d]	C	iv	12	V	Globin	44.0	3.44	4.70	.00
8	C	v	13	VI	Globin	22.0	3.50	3.36	.15
9	C	v	13	VI	Globin	22.0	3.52	3.38	.15
10	C	v	13	VII[f]	Globin	22.0	3.56	3.34	.12
11[e]	D	vi	14	VIII	Globin	22.0	3.32	5.69	

[a] Hemoglobin samples A, B and D consisted of carefully purified but uncrystallized carbonmonoxyhemoglobin. Hemoglobin sample C was twice crystallized carbonmonoxyhemoglobin described in this paper. [b] The weight of sample hydrolyzed varied from 100.0 to 117.5 mg.; the μmoles of DNP-valine isolated is calculated on the basis of exactly 100.0 mg. of sample in each determination. [c] DNP-ation i was carried out in alcohol–water solution; all other samples were dinitrophenylated in aqueous solution at 40° and *p*H 9.0. [d] Run no. 7 was hydrolyzed for 44 hr. Apparently the additional loss on hydrolysis due to the increased time is about balanced by the gain in DNP-valine due to complete hydrolysis of the protein. Hence the 3.44 μmoles of DNP-valine isolated does not deviate appreciably from the average. [e] Run no. 11 represents an independent determination by Dr. Anastasios Christomanos. [f] This sample is the same material as in sample VI but it has been further purified by additional washing with acetone and ether to remove occluded DNP-glutamic acid and di-DNP-lysine.

siderable variation in the number of end groups might be expected. Additional evidence for the completeness of dinitrophenylation is to be found in the molar equivalence of hemoglobin and DNP-globin in the various samples. Here we find identical results (76%) for DNP-protein sample III (dinitrophenylated for one hr.) and DNP-protein samples V and VII (dinitrophenylated 10 to 12 hr.). Again, the yield of DNP-globin actually obtained indicates that the dinitrophenylation as postulated is quantitative. For example, the dinitrophenylation of 0.910 g. of hemoglobin yielded 1.173 g. of air-dried DNP-globin. On standing *in vacuo* over sulfuric acid at room temperature for 88 hr. this material lost 4.7% in weight (assumed to be water) to yield 1.118 g. of purified DNP-globin. Assuming a molecular weight of 66,700 for hemoglobin, 2,600 for the loss of heme and 17,000 for the gain of 102 dinitrophenyl radicals we find a theoretical molecular weight of 81,100 and a theoretical yield of 1.106 g. of DNP-globin. Finally, the weight ratio of hemoglobin to DNP-globin 0.910/1.173 is 77.6%, as compared to 76% determined by comparison of the amino acid contents.

It is possible that some degradation of the hemoglobin does take place during dinitrophenylation at 40° and *p*H 9.0.[31] Other investigators have shown that bicarbonate solutions of DNFB may cause extensive degradation of insoluble proteins such as ichthylepidin,[32] while with soluble proteins such as "old yellow enzyme"[33] and carboxypeptidase[34] certain very labile peptide bonds undergo partial cleavage which results in slight degradation. On the other hand, Middlebrook[35] reported that even with insoluble keratin the dinitrophenylation was 97% complete and that no chain hydrolysis occurred at 40° and *p*H 7.0.

It seems clear from our results with hemoglobin that reaction for one to two hours at 40° and *p*H 9.0 in aqueous solution should be sufficient to prepare a completely dinitrophenylated product and that any side reactions that occur involve only a small fraction of the hemoglobin molecule. Extraneous products that may be formed during the dinitrophenylation are readily separated if the purification process is thoroughly done.

Loss of DNP-amino Acids during Acid Hydrolysis and Chromatography.—Porter and Sanger[2] hydrolyzed DNP-valine with 5.7 *N* hydrochloric acid and found losses of 20, 25 and 36% for 12, 16 and 24 hr., respectively. Masri and Singer[4] reported losses of 25 to 30% on refluxing DNP-valine for 14 to 20 hr. under the conditions existing in their laboratory. Havinga[3] used a correction of 25% for loss on hydrolysis and 10 to 15% for each chromatographic purification. Schroeder and LeGette[38] reported that the chromatographic procedure itself results in a loss of 7% per chromatogram for certain amino acids and peptides; DNP-valine itself was not studied. Since a correction of 20 to 40% for losses on hydrolysis and chromatography lies in the neighborhood of an entire end group for the hemoglobin molecule, the following definitive experiments were made in an attempt to evaluate these losses. The common procedure for evaluating these losses simply involves the determination of the destruction that results when the DNP-amino acid itself is "hydrolyzed" under the conditions employed. Actually, such an experiment measures the destruction of the DNP-amino acid after release from peptide linkage and in no way determines the destruction that may occur during the hydrolysis of the peptide bond. In the present experiments, not only DNP-valine but two peptides, DNP-val-gly and DNP-val-leu, have been hydrolyzed both in the

(31) By washing DNP-protein VI (Table I) with additional acetone and ether it was possible to remove small amounts of DNP-glutamic acid and di-DNP-lysine which presumably were occluded on the DNP-protein. These DNP-amino acids were not in sufficient quantity to effect the results (compare runs no. 8 and 9 with run no. 10). It was not established whether their presence resulted from partial degradation during the dinitrophenylation or slight decomposition of the hemoglobin solution on standing.

(32) R. W. Burley and C. Solomons, *Biochim. Biophys. Acta*, **18**, 637 (1955).

(33) F. Weygand and R. Junk, *Naturwiss.*, **38**, 433 (1951).

(34) E. O. Thompson, *Biochim. Biophys. Acta*, **10**, 633 (1953).

(35) W. R. Middlebrook, *ibid.*, **7**, 547 (1951).

presence and absence of DNP-globin in order better to evaluate the losses that occur; DNP-val-leu is derived from the actual N-terminal sequence of DNP-globin. The results are summarized in Table II.

TABLE II

STUDY OF LOSS OF N-TERMINAL DNP-VALINE DURING VARIOUS OPERATIONS

Run no.	Sample	μmoles hydrolyzed	μmoles recovered	% loss
Total operational loss (hydrolysis, extraction and chromatography)				
1	DNP-DL-Valine	5.20	4.56	12.3
2[a]	DNP-DL-Valine	5.20	4.51	13.3
3	DNP-L-Valine	3.83	3.37	12.0
4	DNP-L-Valine + DNP-Globin	3.83[c] 3.69	7.11	10.7
5[b]	DNP-DL-Valine	4.03	3.31	17.9
6	DNP-L-Val-gly	5.18	4.63	10.6
7	DNP-L-Val-gly	3.41	2.98	12.6
8	DNP-L-Val-gly + DNP-Globin	5.18[c] 3.39	8.22	(6.8)[d]
9	DNP-L-Val-gly + DNP-Globin	3.41[c] 3.53	6.97	(0.0)[d]
10	DNP-Val-leu[e]	3.15	2.50[f]	16.4
11	DNP-Val-leu + DNP-Globin	3.15[c] 3.58	6.08[f]	16.4
Chromatographic loss only (two chromatograms)				
12	DNP-DL-Valine	5.20	4.91	5.6
13	DNP-L-Valine	3.83	3.66	4.4
14	DNP-L-Valine	3.83	3.61	5.7

[a] Undistilled 6 *N* hydrochloric acid was used in this run to see whether traces of heavy metals present might affect the result. [b] This run was hydrolyzed 44 hr. If the value for loss on chromatography is subtracted from this run and from the 22-hr. run, one notes that the loss due to hydrolysis is essentially linear with time. [c] When the amino acid or peptide was hydrolyzed in the presence of DNP-globin, the top value in the bracket is the μmoles of amino acid or peptide used and the lower value in the bracket is the μmoles of DNP-valine to be expected from the sample of DNP-globin added. [d] We have no explanation for the apparent protective effect on the hydrolysis of DNP-val-gly in the presence of DNP-globin. [e] The DNP-val-leu used in these experiments was obtained from the partial hydrolysis of DNP-hemoglobin. [f] In these experiments, 0.16 μmole of unhydrolyzed DNP-val-leu was recovered from run no. 10 and 0.32 μmole from run no. 11.

The results of the hydrolysis of either DNP-DL-valine or DNP-L-valine, with or without DNP-globin, under conditions described in this paper indicate that the destruction and loss of the DNP-amino acid is about 12%, only one-third of previously reported values.[2-4] The results from the hydrolysis of DNP-val-gly are almost identical. In DNP-val-leu the bond is much more resistant, since 5% of the peptide remains after 22 hr. (see Table I). However, the destruction during the hydrolysis of DNP-val-leu, although a little higher than found with DNP-valine and DNP-val-gly, is certainly within the range expected for this type of correction. An average of all values[36] gives 13%, which seems to be a reasonable correction to apply in view of the good agreement obtained for the variety of material and conditions employed.

(36) Runs no. 8 and 9 were excluded from the results.

It should be emphasized that this loss of 13% represents all operational losses during the determination, that is, hydrolysis, extraction, chromatography and necessary transfers from vessel to vessel. Careful experimental technique will minimize any losses by extraction or transfer, and it was, therefore, of interest to determine the actual chromatographic loss and by difference the hydrolytic loss. The results of runs no. 12, 13 and 14 indicate that the chromatographic loss is about 5% and consequently that hydrolysis produces a destruction of only about 8%.

Our losses are greatly different from those previously reported (which presumably also really are total operational losses). It may be that the rather different chromatographic procedures are the cause. On the other hand, the concentration of the DNP-protein during the hydrolysis may have been vastly dissimilar. Thus, in the present experiments, 100 mg. of DNP-protein was hydrolyzed in 10 ml. of 6 *N* hydrochloric acid. None of the other authors present this information so comparison cannot be made.

The concordance of the results from DNP-valine, DNP-val-gly and DNP-val-leu shows that, in this example at least, destruction of the DNP-amino acid during the rupture of the peptide bond does not occur to any appreciable extent and that "hydrolysis" of the DNP-amino acid under the conditions employed gives a good estimate of the actual destruction.

Completeness of Hydrolysis of DNP-globin.—Hydrolysis of DNP-globin is most commonly carried out by refluxing 100 to 300 mg. of the air-dried material with approximately 6 *N* hydrochloric acid. Although the time of hydrolysis reported varies widely from 12 to 40 hr. little mention is made of the completeness of hydrolysis except in the original work of Porter and Sanger.[2] They reported that in the globins 10% of the DNP-valine was still in peptide form after refluxing for 16 hr. with 5.7 *N* hydrochloric acid and that DNP-valyl peptides could just be detected after hydrolysis for 40 hr. in 12 *N* hydrochloric acid at 100°.

Since the breakdown of DNP-amino acids on acid hydrolysis is also a function of time, any quantitative study of end group values requires the selection of a time for hydrolysis which gives a maximum for completeness of hydrolysis and a minimum for degradation of the N-terminal DNP-amino acids to be determined. A period of 22 hr. seems to meet these requirements adequately. In runs no. 6, 8, 9 and 10 (Table I) 0.15 μmole of DNP-val-leu was isolated with good accuracy. This was made possible by the observation that a mixture of DNP-valine, DNP-val-leu and dinitroaniline could be separated effectively by one chromatogram with 2F-8E-L as the developer. It should also be noted that hydrolysis is essentially complete after 44 hr. (run no. 7). In runs no. 10 and 11 of Table II we have the additional fact that purified DNP-val-leu (obtained from partial hydrolysis of DNP-hemoglobin) on hydrolysis for 22 hr.,

either alone or in the presence of DNP-globin, yields almost the same amount (0.16 μmole) of unchanged peptide. From these results we feel that the hydrolysis of DNP-globin in refluxing 6 *N* hydrochloric acid for 22 hr. is about 95% complete and that an average correction of 0.15 μmole of DNP-valine can be applied to the amount of DNP-valine actually isolated from the hydrolysis of 0.1-g. samples of DNP-globin.

The isolation of DNP-val-leu in quantity from the partial hydrolysis of DNP-hemoglobin indicates that the N-terminal amino acid sequence in at least some of the hemoglobin chains is valyl-leucine. Recently Ozawa and Satake[37] reported the same sequence for a variety of animal hemoglobins and Brown[6] obtained the same peptide from normal adult human hemoglobin. It is quite likely, then, that the complete hydrolysis of DNP-globin must involve the breaking of the bond between N-terminal valine and leucine. Although other investigators[38] have studied the kinetics of acid hydrolysis of dipeptides, information on valyl peptides is fragmentary and DNP-peptides have not been considered. Since DNP-val-leu was hydrolyzed for three different time intervals and the unchanged DNP-val-leu was recovered in each case, it is interesting to note that here too the hydrolysis follows the kinetics of a pseudo unimolecular reaction. Using the integrated form for a first order reaction, $\ln x_t/x_0 = -kt$, where x_0 is the initial μmoles of DNP-val-leu hydrolyzed and x_t the amount recovered in time t, a plot of $\ln x_t/x_0$ against time in minutes is a straight line. The slope gives the value 2.35×10^{-3} (min.$^{-1}$) for the rate constant for hydrolysis of DNP-val-leu in boiling 6 *N* hydrochloric acid.

Final Calculations of N-Terminal Residues per Molecule.—The data necessary for the calculation of the number of N-terminal residues in normal adult human hemoglobin are presented in Table III below. The standard deviation of 0.12 μmole (about 3.5%) seems well within the experimental error involved in this type of determination. The uncorrected average for DNP-valine (3.39 μmoles), on correction for loss on hydrolysis (8.0%), loss on chromatography (5.0%) and unhydrolyzed peptide (0.15 μmole), gives an average value of 4.04 μmoles of DNP-valine obtained from the free amino groups in 100 mg. of DNP-globin or DNP-hemoglobin. Since the ratio of DNP-globin to hemoglobin has been found to be 76% by comparison of the amino acid content and that for DNP-hemoglobin to hemoglobin to be 74% by calculation from this value, only the molecular weight of hemoglobin is needed to obtain the μmoles of sample hydrolyzed and hence the number of N-terminal valyl residues per molecule of hemoglobin.

A number of different molecular weights have been employed in work concerned with the nature of hemoglobin. Thus for calculating the free amino groups in normal adult human hemoglobin Sanger[2] used a molecular weight of 64,000 while Masri and Singer[4] used the value 66,000. Schroeder, Kay and Wells[26] based the number of amino acid residues per mole in both normal and sickle-cell-anemia hemoglobin on the value 66,700. The classical value 68,000 as determined by osmotic pressure measurements[39] and sedimentation and diffusion studies[40] is quite commonly used as a reference point, but it should be noted that Adair's figure was obtained from sheep and Svedberg's from horse rather than human hemoglobin. Chemical analysis for iron content as carried out by Butterfield[41] and more recently confirmed by Bernhart and Skeggs[42] gives an average value for iron of 0.335%, which corresponds to an equivalent weight of 16,700 and a molecular weight of 66,800 if four iron atoms per molecule are assumed to be present.

TABLE III

NUMBER OF N-TERMINAL VALYL RESIDUES IN NORMAL ADULT HEMOGLOBIN

Run no.	μmoles of DNP-valine: Isolated from 100 mg. (Table I)	Plus 13% operational loss	Plus 0.15 μmole DNP-val-leu isolated	No. of N-terminal residues: Mol. wt. 66,700	Mol. wt. 60,000
1	3.26	3.75	3.90	3.52[a]	3.17[a]
2	3.14	3.61	3.76	3.39[a]	3.06[a]
3	3.46	3.98	4.13	3.63	3.25
4	3.31	3.81	3.96	3.47	3.12
5	3.35	3.85	4.00	3.51	3.15
6	3.41	3.92	4.07	3.57	3.21
7	3.44	3.95	4.10	3.60	3.23
8	3.50	4.02	4.12	3.62	3.25
9	3.52	4.05	4.20	3.79	3.31
10	3.56	4.09	4.24	3.73	3.34
11	3.32	3.82	3.97	3.47	3.13
Av.	3.39	3.90	4.04	3.57	3.20

[a] Runs no. 1 and 2 were made on DNP-hemoglobin. From the value of 76% for the ratio of DNP-globin to hemoglobin found by comparison of the amino acid contents, a value of 74% was calculated for the ratio of DNP-hemoglobin to hemoglobin.

In recent years, much lower values for the molecular weight of normal adult human hemoglobin have been suggested. Thus Field and O'Brien[43] in 1955 reported a molecular weight of 59,400 based on newer data for sedimentation and diffusion constants. Srinivasan and Vinograd[44] have also reported a molecular weight of 60,000 ± 1100 (standard deviation) for normal adult human hemoglobin based on data obtained during the period of approach to sedimentation equilibrium. One of the several samples used by Srinivasan and Vinograd was the same hemoglobin solution on which six of the present determinations of N-terminal valyl residues were carried out (see Table I, hemoglobin sample C).

In Table III we have calculated the number of N-terminal valyl residues in normal adult human hemoglobin both on the basis of a "high" or classical value for the molecular weight and a "low" or relatively new value for the molecular weight. The

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former yields 3.6 N-terminal valyl residues and the latter 3.2 N-terminal valyl residues per molecule of hemoglobin.

Conclusions

Since our results differ from those of previous investigators, it seems important to summarize the points of similarity and difference. The following similarities should be noted: (1) the equivalence of DNP-globin to hemoglobin determined in these experiments from the amino acid content is in excellent agreement with Sanger's value determined from the amide nitrogen content, and (2) the nature of the DNP-protein hydrolyzed is the same as that of other investigators. The following differences are apparent: (1) our correction factor for the destruction of DNP-valine during acid hydrolysis is much less than that of other investigators, and (2) unhydrolyzed DNP-peptide that remains at the end of the hydrolysis has been quantitatively estimated in the present work.

The low destruction of DNP-valine that we have observed in our analyses has been discussed in a preceding section. Experiments to determine the cause of the difference cannot be devised because details about the conditions of hydrolysis are lacking in the other papers. Throughout the present investigation great stress has been laid upon the exact determination of the loss of DNP-valine during hydrolysis. To this end, not only DNP-valine but DNP-val-gly and DNP-val-leu have been hydrolyzed both in the presence and in the absence of DNP-globin. The consistent results reported in Table II lead us to conclude that confidence may be placed in the correction factor which has been determined. By use of this correction factor it has been shown that 0.1000 g. of DNP-globin, as prepared from purified hemoglobin and hydrolyzed according to the methods described in the present paper, contains 4.04 ± 0.15 μmole of DNP-valine.

If the above data and the commonly accepted value of approximately 67,000 for the molecular weight of human hemoglobin are then used to calculate the number of end groups, the non-integral value 3.6 ± 0.1 end groups is obtained. For the integral value of 4, a molecular weight of about 76,000 is required; for the integral value 3, about 57,000. The literature provides no evidence supporting a molecular weight of 76,000, but the work of Field and O'Brien[43] and of Srinivasan and Vinograd[44] suggests that the molecular weight may be about 60,000. On the other hand, if the non-integral value of 3.6 end groups per molecule is correct, it implies that preparations of hemoglobin that have been considered to be homogeneous are indeed heterogeneous. Although heterogeneities in normal adult human hemoglobin have been reported by Kunkel and Wallenius,[10] Morrison and Cook,[45] and Prins and Huisman,[46] it should be noted that uncrystallized hemoglobin was used in the experiments of these investigators.

Despite the immense amount of work that has been done on hemoglobin, it is apparent that uncertainties still exist about both the molecular weight and the homogeneity of this protein. Consequently, a final decision as to the exact number of N-terminal residues must be kept in abeyance until both the molecular weight of hemoglobin and the homogeneity of the preparations are established with more certainty.

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The Isolation and Characterization of a Melanocyte-stimulating Hormone (β-MSH) from Hog Pituitary Glands

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The isolation of a melanocyte-stimulating hormone (β-MSH) from porcine posterior pituitary powder has been described. The active material was extracted with warm glacial acetic acid and further purified by acetone and ether precipitations. It was then adsorbed on oxycellulose, eluted and further purified by zone electrophoresis on starch. Finally, it was submitted to countercurrent distribution. Amino acid analyses of the isolated product indicated the following composition: $Asp_2Glu_2Ser_1Gly_2Pro_3Met_1Phe_1Tyr_1Lys_2His_1Arg_1Try_1$. The molecular weight, 2177, calculated from these data, is shown to be the true molecular weight. The isoelectric point, determined in buffers of 0.1 ionic strength, has been found to be at *p*H 5.8. A comparison has been made between the isolated peptide and α- and β-MSH reported previously by other workers.

The presence of a melanocyte-stimulating hormone (MSH, intermedin) in the *pars intermedia* of the pituitary was first demonstrated by Zondek and Krohn in 1932.[1] Thereafter, few intensive investigations designed to isolate the pure hormone

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