Tiselius[25] reported on the separation of human serum albumin in three components using calcium phosphate. A possibly related observation has been made by Lapresle[26] who showed that, following enzymatic digestion, human serum albumin consisted of three immunochemically similar proteins. Furthermore, Luetscher[10] noted that the albumin of normal human plasma splits into two components upon electrophoretic analysis at *p*H 4.0. The same protein fraction derived from pathological plasma and investigated under the same conditions revealed also two components but their ratio varied depending on the type of disease. Thus, it would appear that the crystallized, "electrophoretically homogeneous" albumin may be identical with one of the three individual components of normal human serum albumin.

Acknowledgments.—The author wishes to express his appreciation for the gift of human mercaptalbumin from Dr. J. L. Oncley, Harvard Medical School. The technical assistance of Miss A. Polis, who carried out the electrophoretical and ultracentrifugal analyses, is kindly acknowledged.

BOSTON, MASS.

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(26) C. Lapresle, *Ann. Inst. Pasteur*, **89**, 654 (1955).

[CONTRIBUTION NO. 2199 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

A Quantitative Study of the Hydrolysis of Human Dinitrophenyl(DNP)globin: The Number and Kind of Polypeptide Chains in Normal Adult Human Hemoglobin

BY HERBERT S. RHINESMITH,[1] W. A. SCHROEDER AND LINUS PAULING

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A quantitative investigation of the partial hydrolysis of DNP-globin in refluxing 6 *N* hydrochloric acid has led to an explanation of our earlier conclusion that normal adult human hemoglobin contains a non-integral number, 3.6, of N-terminal valyl residues per molecule. It is now concluded that 4 N-terminal residues are present. Moreover, it has been found that the molecule contains two kinds of polypeptide chains, with respect to the N-termini. Under the above hydrolytic conditions the N-terminal valyl residues are released as DNP-val-leu almost quantitatively from two chains (A chains) within 15 min. On continued hydrolysis the other two chains (B chains) release DNP-valine. No N-terminal peptides originating from the B chains have been definitely identified.

Introduction

In a previous paper[2] we reported a non-integral value for the number of N-terminal valyl residues in normal adult human hemoglobin, and suggested that this finding raised questions about the molecular weight and homogeneity of hemoglobin. During that investigation DNP-val-leu was found to be the only peptide that could be isolated in significant amount from the partial hydrolyzates of DNP-globin. The isolation of DNP-val-leu accords qualitatively with the findings of Brown,[3] who reported a maximum quantity of DNP-val-leu corresponding to 2.5 chains per molecule of DNP-hemoglobin.

The original objective of the present investigation was the isolation and identification of N-terminal peptides other than DNP-val-leu. Although what may be other N-terminal peptides have been isolated, the most striking feature of the first experiments was the almost instantaneous release of much DNP-val-leu and the slow release of DNP-valine during hydrolysis in refluxing 6 *N* hydrochloric acid. It was evident that the maximum amount of DNP-val-leu is by no means equivalent to all of the DNP-valine that is ultimately released by continued hydrolysis. This fact suggested that at least two types of N-terminal sequences are present in human hemoglobin. The results of the quantitative study of the hydrolysis presented in this paper support this initial conclusion.

Experimental

Preparation of DNP-globin.—Carbonmonoxyhemoglobin, twice crystallized by the method of Drabkin,[4] was dinitrophenylated at 40° and *p*H 9.0 by the method of Levy and Li.[5] The heme was removed by the method of Anson and Mirsky[6] and the purified DNP-globin was equilibrated in the air to constant weight. Experimental details for preparing the DNP-globin used in this work were described.[2]

Partial Hydrolysis of DNP-globin.—Samples varying in weight from 50 to 500 mg. were employed for the hydrolyses. A 10-ml. portion of 6 *N* hydrochloric acid was used for each 100 mg. of sample. Foaming that occurred during the hydrolysis could be decreased materially if the DNP-globin was wetted thoroughly with acid by allowing it to stand at room temperature for one hr. with occasional swirling. The hydrolysis was carried out by refluxing on a hot plate except for the 15-min. runs, for which a pre-heated oil-bath was used. The time necessary to bring the solution to boiling was not counted as part of the time of hydrolysis. At the end of the period of hydrolysis the reaction was stopped by immersion in cold water and the hydrolyzate was extracted with 4 × 25 ml. of ether. The combined ether extracts were washed with 4 × 5 ml. of distilled water, each washing containing one drop of 6 *N* hydrochloric acid. The ether was evaporated, the residue was taken up in acetone, the acetone was evaporated, and the residue was reserved for chromatography.

Samples of DNP-globin were hydrolyzed for 0.25, 0.5, 1, 2, 4, 7, 12.5 and 22 hr., respectively; the 2-hr. and 4-hr. runs were in duplicate, and the 0.25-hr. run in triplicate. The result for 22 hr. of hydrolysis is the average of the eleven runs reported previously.[2]

In these hydrolyses the DNP-globin was not completely dissolved until about 4 hr. had elapsed. The insoluble portion in the 15-min. hydrolyzates was appreciable and could not be neglected in the quantitative evaluation of the data. The experiments described in the following section were accordingly made.

(1) Allegheny College, Meadville, Pennsylvania.

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

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

(4) D. L. Drabkin, *ibid.*, **21**, 224 (1949).

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

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

Investigation of DNP-globin Undissolved by Partial Hydrolysis.—In order to investigate the amount and nature of the material that did not dissolve during short periods of hydrolysis, 0.326 g. of air-dried DNP-globin was hydrolyzed for 15 min. with 30 ml. of 6 *N* hydrochloric acid. The reaction mixture was cooled and filtered through a weighed sintered glass funnel. The filtrate was extracted with ether and the ether extracts were analyzed for DNP-valine and DNP-val-leu as described below. The solid on the filter was bright yellow and indistinguishable in appearance from the original DNP-globin. After washing thoroughly with 6 *N* hydrochloric acid and distilled water, it was air dried to a constant weight of 0.083 g., 25% of the original sample.

The extracts of the filtrate contained far less DNP-val-leu than would have been anticipated from other experiments. When the solid was washed with 10 ml. of ether an additional small quantity of DNP-val-leu was removed. Much more DNP-val-leu together with unidentified materia :was removed by washing with 50 ml. of acetone. The final residue, which now weighed 0.071 g., was hydrolyzed for 22 hr. with 10 ml. of 6 *N* hydrochloric acid, in order to hydrolyze completely any unchanged DNP-globin. The ether extracts from this hydrolysis contained 0.57 μmole of DNP-valine. The quantitative amounts, in μmoles per 0.326 g. of DNP-globin, are summarized below.

Source	DNP-val	DNP-val-leu
Ether extracts, original filtrate	0.26	4.40
Ether extracts of solid residue		0.15
Acetone extracts of solid residue		2.06
Ether extracts, 22 hr. hydrolyzate	.57	
Totals	0.83	6.61

Partial Hydrolysis of DNP-val-leu.—In order to ascertain the rate of hydrolysis of DNP-val-leu, samples of this peptide were obtained from the partial hydrolysis of DNP-globin, purified by chromatography, and hydrolyzed in refluxing 6 *N* hydrochloric acid for 1, 2, 4, 8, 16 and 22 hr., respectively. The amounts of DNP-val and of unchanged DNP-val-leu were determined quantitatively as described below.

Chromatographic Procedure.—The method of Green and Kay[7] was used to separate and identify the DNP-compounds which were extracted from the hydrolyzates of DNP-globin. Three products were obtained readily from the ether extracts, namely, DNP-valine, DNP-val-leu and DNA (dinitroaniline); the latter is a by-product that has been reported to be formed by the decomposition of histidine.[8] As reported earlier,[2] these three compounds can be separated readily by a single chromatogram on silicic acid–Celite by using 2F-8E-L[7] as the developer. The DNP-peptide is more strongly adsorbed than DNA or the still faster moving DNP-valine. The quantities of DNP-amino acids and DNP-peptides were determined spectrophotometrically.[2]

N-Terminal Peptides Other than DNP-val-leu.—Several attempts have been made to isolate longer N-terminal peptides from 15-min. hydrolyzates. After the hydrolyzates were extracted with 4 × 25 ml. of ether to remove DNP-valine, DNP-val-leu and DNA, they were extracted exhaustively with ethyl acetate in order, if possible, to remove longer peptides unextractable by ether. This extraction is complicated by the fact that DNP-ε-lysyl peptides are also removed to some extent by ethyl acetate and tend to return to the aqueous phase when the extract is subsequently washed with acidified distilled water. Hence the course of the extraction is difficult to follow by change in color of the solutions.

Chromatographic procedures and developers, in general, followed the methods which are described by Schroeder and Honnen.[9] The residue obtained after evaporating the ethyl acetate could be dissolved partially in solvents such as 30AA-30A-B[7] for the purpose of transferring the sample to the column. Strongly adsorbed materials (probably ε-DNP-lysyl peptides) were present on the chromatograms and there were also other compounds that exhibited the chromatographic behavior to be anticipated for N-terminal peptides.

The hydrolysis of these supposed N-terminal peptides has yielded results that were unexpected from our earlier experience with DNP-valyl peptides. In order to ascertain the amino acid composition of these peptides, they were hydrolyzed in initial experiments for periods of 15 to 22 hr. When examination of the hydrolyzates yielded no ether-extractable DNP-amino acids we first assumed that the compounds were not N-terminal peptides. However, on hydrolysis of one of the zones for 8 hr. a small amount of DNP-valine was detected. When samples of three of these DNP-peptides were hydrolyzed for 4-hr. periods, N-terminal DNP-valine was found in quantities equal in each case to one-third to one-half of the moles of peptide hydrolyzed. The other amino acids in each of these three peptides were leucine and histidine in quantities of about one mole each per mole of peptide. These results are approximate due to the apparent decomposition of DNP-valine and the instability of histidine. Because these peptides had dissimilar chromatographic behavior, it was surprising to find apparently identical amino acid composition. Further investigation of these results is under way.

Control experiments during this study revealed the following pertinent information about di-DNP-histidine. (1) Di-DNP-histidine can be chromatographed nicely on silicic acid–Celite with 2AA-10A-B as the developer and 1:4 alcohol–ether as the eluent, but the loss is about 16% per chromatogram. (2) Di-DNP-histidine is destroyed on refluxing with 6 *N* hydrochloric acid; only 15% is recovered after 4 hr. (3) The presence of an equimolar amount of DNP-valine during refluxing in 6 *N* hydrochloric acid for 4 hr. increases the recovery to 46%. (4) That *im*-DNP-histidine is more stable than di-DNP-histidine was shown by the recovery of 80% of the expected amount when DNP-peptides that did not contain N-terminal histidine were hydrolyzed in refluxing 6 *N* hydrochloric acid for 4 hr.

Results and Discussion

The results are presented in Table I and Fig. 1. The solid circles and open circles represent the corrected values for the experimentally determined quantities of DNP-val-leu and DNP-valine, respectively, in the hydrolyzates calculated to the basis of 0.100 g. of air-dried DNP-globin.

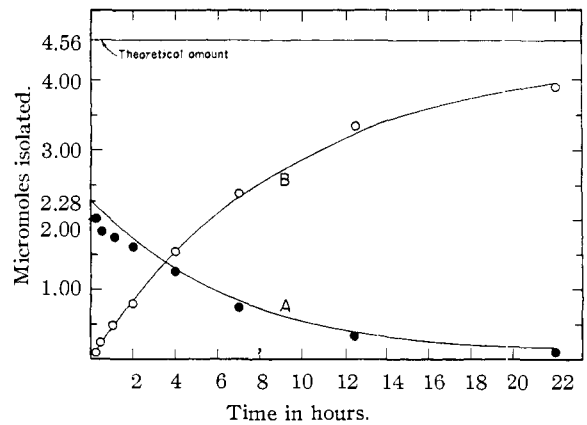

Fig. 1.—Course of the partial hydrolysis of DNP-globin in refluxing 6 *N* hydrochloric acid: O and ● are experimental points for DNP-valine and DNP-val-leu, respectively. The curves are calculated.

The data show that the release of DNP-val-leu is rapid, and essentially complete in 15 min. The release of DNP-valine is much slower. Because the increase in DNP-valine is more rapid than the de-

(7) F. C. Green and L. M. Kay, *Anal. Chem.*, **24**, 726 (1952). Abbreviations for solvents and developers follow Green and Kay, that is, AA for acetic acid, A for acetone, L for ligroin, F for formic acid and E for ethyl acetate. Thus, 2F-8E-L is 2 volume % F and 8 volume % E in L.

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

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

crease of DNP-val-leu, the latter cannot be the only source of DNP-valine.

The quantity 0.100 g. of air-dried DNP-globin is equal to 1.14 μmoles, on the basis of earlier work,[2] if the molecular weight of hemoglobin is assumed to be 66,700.[10] If we assume that 4 polypeptide chains exist in hemoglobin, it should be possible to isolate 4.56 μmoles of N-terminal DNP-valine on complete hydrolysis. Therefore, if all polypeptide chains rapidly released DNP-val-leu we might expect the isolated amount to approach 4.56 μmoles. The quantity of DNP-val-leu actually isolated is 2.03 μmoles, approximately half of the maximum amount, and it may be concluded that two chains only of the DNP-globin molecules release DNP-val-leu. In the following discussion it has been assumed that hemoglobin has four polypeptide chains and that two of them have the N-terminal sequence val-leu.

An exact kinetic interpretation of the data requires a knowledge of the rate of hydrolysis of DNP-val-leu in refluxing 6 *N* hydrochloric acid. In Fig. 2 the quantity $-\ln x_t/x_o$ is shown plotted against time, *t*, in hr.; x_o is the initial quantity of DNP-val-leu and x_t the amount after time *t*. The reaction is a pseudo unimolecular reaction and the constant for a first-order reaction is given by the slope of the best straight line through the points as 0.143 hr.$^{-1}$.

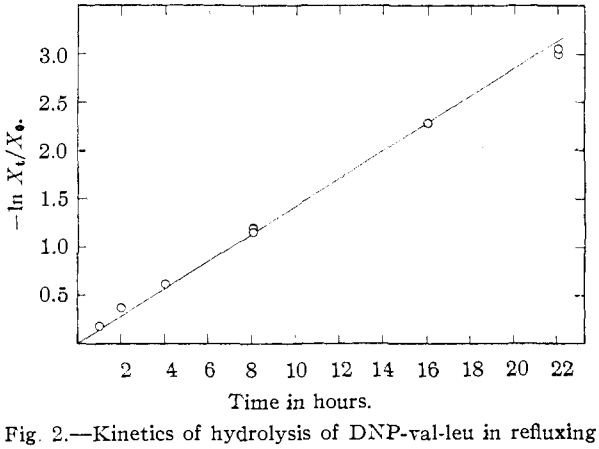

Fig. 2.—Kinetics of hydrolysis of DNP-val-leu in refluxing 6 *N* hydrochloric acid.

Let us assume that 0.100 g. of DNP-globin (1.14 μmoles) can theoretically release 2.28 μmoles of DNP-val-leu by refluxing in 6 *N* hydrochloric acid in a time (15 min.) that is short in relation to the period required for the complete hydrolysis of DNP-val-leu. Because the rate of hydrolysis of DNP-val-leu under these conditions is known, we may calculate the quantity of DNP-val-leu that will remain after various periods of hydrolysis if the initial quantity is 2.28 μmoles, and we may compare the calculated curve with the quantities of DNP-val-leu that have been isolated after hydrolysis of DNP-globin. In Table I the experimental data are presented in column 2 and calculated values in column 3. The close agreement between the calculated curve (A in Fig. 1) and the experimental points suggests that the assumptions are in the main correct. For short periods of hydrolysis the experimental points are about 10% below the curve. However, it is during short periods of hydrolysis that there is undissolved material which, if it contained unchanged DNP-globin, might be sufficient to represent the difference. This observation prompted the investigation of the undissolved material as described above. Although difficulties with adsorption were encountered that are not met when the hydrolyzate is extracted directly with ether, the total amount of DNP-val-leu that was finally isolated agreed well with other determinations (2.03 μmoles as compared with 1.95 and 2.10). The entire residue could not have been unchanged DNP-globin because it released only 0.6 μmole of DNP-valine on complete hydrolysis in contrast to the 3.2 μmoles that would be expected from 0.071 g. of unchanged DNP-globin. If this 0.6 μmole of DNP-valine originated in DNP-val-leu that was still adsorbed on the solid or in unchanged DNP-globin that was present, then this amount plus that which was actually isolated as DNP-val-leu is very close to the 2.20 μmoles per 0.100 g. of DNP-globin that one would anticipate after 15 min. of hydrolysis.

If we assume that 2.28 μmoles of DNP-val-leu was split from two chains (referred to as A chains) during the initial stages of hydrolysis, we may then calculate the quantities of DNP-valine that would result from its hydrolysis with use of the experimental value of the rate constant. These quantities are listed in column 4 of Table I. If now the other two chains of DNP-globin (referred to as B chains) are also releasing DNP-valine, amounts released from the B chains would be the differences between column 5 and column 4, as recorded in column 6. The first-order reaction rate constant for the hydrolysis of the B chains is found from these numbers

TABLE I

OBSERVED AND DERIVED AMOUNTS OF SUBSTANCES FROM THE PARTIAL HYDROLYSIS OF DNP-GLOBIN

1	2	3	4	5	6	7
Time, hr.	DNP-val-leu found[a] (cor.)[b]	DNP-val-leu calcd.	DNP-val from A chains calcd.	DNP-val found (cor.)[c]	DNP-val from B chains (col. 5 − col. 4)	DNP-val from B chains calcd.
0.00		2.28				
.25	2.03[d]	2.20	0.08	0.10[e]	0.02	0.04
.5	1.84	2.12	.16	.26	.10	.08
1	1.76	1.98	.30	.50	.20	.16
2	1.61[f]	1.71	.57	.80[g]	.23	.30
4	1.25[h]	1.29	.99	1.53[i]	.54	.56
7	0.75	0.84	1.44	2.42	.98	.88
12.5	.33	.38	1.90	3.35	1.45	1.33
22	.16	.10	2.18	3.90	1.72	1.79

[a] All numerical values in this table are expressed in μmoles obtained from the hydrolysis of 0.100-g. samples of DNP-globin. [b] Values for DNP-val-leu have been corrected for a loss of 4% per chromatogram. [c] Values for DNP-valine have been corrected for a loss of 2.5% per chromatogram and proportional loss on hydrolysis (8% for a 22-hr. period). [d] Average of values 2.10, 1.95 and 2.03 obtained in three experiments. [e] Average of 0.11 and 0.08. [f] Average of 1.56 and 1.65. [g] Average of 0.78 and 0.82. [h] Average of 1.18 and 1.31. [i] Average of 1.61 and 1.47.

(10) Recent experiments (J. Vinograd, private communication) show that a molecular weight close to 66,700 is a more probable value for hemoglobin than that of about 60,000 which was mentioned earlier.

to be 0.070 hr.$^{-1}$; the corresponding calculated values are given in column 7.

It is of interest to consider the significance of these calculations in terms of our earlier report that hemoglobin contained only 3.6 N-terminal valyl residues. Because DNP-val-leu was isolated in the earlier experiments, the value 3.6 includes a correction for the incomplete hydrolysis of this substance (from the A chains). However, the B chains produce no recognizable hydrolytic products other than DNP-valine, and the new experiments show that their hydrolysis is not complete in 22 hr. Indeed, the rate constant 0.070 hr.$^{-1}$ corresponds to 21.5% of B chains still unhydrolyzed after 22 hr. Because there are two B chains, about 0.43 N-terminal residues per molecule will be undetected. Increase of the earlier value of 3.57 N-terminal residues by 0.43 gives the number of N-terminal residues as 4.00. Similarly, if the data for 44 hr. of hydrolysis (Table I, run no. 7, ref. 2) are corrected for destruction (8% per 22 hr. of hydrolysis and 5% for loss by chromatography), the number of N-terminal residues is 3.81. To this must be added 0.09 residues because of incomplete hydrolysis giving 3.90.

Our earlier conclusion that 3.6 N-terminal valyl residues are present in hemoglobin resulted from the assumption that all chains are equivalent. The chains are not equivalent and when this fact is taken into consideration, it is necessary to conclude that the number of N-terminal residues is four. This result is still at variance with the five N-terminal residues per molecule that most investigators report[11]; Schramm, Schneider and Anderer have applied Edman's method to human hemoglobin and have reported[12] recently that 4 N-terminal valyl residues are present.

The destruction of DNP-amino-acids in the course of hydrolysis can occur before, during, or after release from the peptide bond. Earlier work[2] shows that little if any destruction of DNP-valine in the course of the hydrolysis of DNP-val-leu (and, hence, the A chains) occurs until after the release of DNP-valine. Destruction to the extent that it is found in the case of DNP-valine from the A chains will then be observed in any DNP-valine that is released in the course of hydrolysis. Throughout the above discussion, it has been assumed that the total destruction of DNP-valine from the B chains in the course of hydrolysis is no greater than that of DNP-valine from the A chains. If this assumption is invalid, the calculated rate constant for the hydrolysis of the B chains is an apparent one only. Now we may suppose that the penultimate amino acid residue differs in the A and B chains. As a result, there may be destruction of DNP-valine before or during release from the B chains. That the penultimate amino acid residue may influence the destruction of the DNP-N-terminal amino acid is known. Levy and Li[5] found that 80 to 90% of N-terminal DNP-serine was destroyed in the course of the hydrolysis of DNP-α-corticotropin. Yet when free DNP-serine was mixed with DNP-α-corticotropin before hydrolysis, the destruction of the added DNP-serine was only 7%. The main part of the destruction, therefore, must have occurred before or during the release. In other investigations in these laboratories[13,14] the destruction of DNP-serine in the course of the hydrolysis of many DNP-peptides has normally been of the order of 20 to 40% except that in the case of DNP-ser-cya[15] and di-DNP-ser-tyr it was 80 and 70%, respectively: it is of considerable interest that the N-terminal sequence of α-corticotropin begins ser-tyr-. The possibility exists, therefore, that the hydrolysis of the B chains of hemoglobin results in greater destruction of DNP-valine than that of the A chains. It may be, then, that a correction for added destruction of DNP-valine of the B chains rather than a correction for unhydrolyzed B chains is required.

A correlation of the kinetic interpretation of the results with the known facts of protein chemistry is not without difficulty. We have concluded that the B chains release DNP-valine at a rate that is very much less than that for the A chains. The A chains terminate in the sequence val-leu, which is known to be one of the most difficult sequences to hydrolyze completely.[16] The available evidence[16] suggests that the sequence val-val would be more resistant to hydrolysis than val-leu, but the results of Sanger's investigation of insulin[17,18] as well as studies of the completeness of protein hydrolysis[19] would lead one to believe that peptide bonds involving amino acids other than valine, leucine or isoleucine are not unusually difficult to hydrolyze. Yet, even if the B chains do terminate in such a sequence as val-val, detectable products of partial hydrolysis should result. The apparently unusual hydrolytic behavior that we have observed may be caused by some structure in which instead of four chains, two branched chains are present in the molecule. This possibility must be considered if it is correct, as Huisman and Dozy[20] find by means of carboxypeptidase, that one histidyl and one tyrosyl residue make up the C-terminal residues of human hemoglobin and if their result is not a consequence of the specificity requirements of the enzyme. Further evidence for this possibility lies in the work of Theodoropoulos and Craig,[21] who report that peptide bonds involving the ϵ-amino group of lysine have unusual stability. Perhaps, then, inasmuch as any normal peptide bond in which the carboxyl group of valine is concerned has heightened stability, a peptide bond between a carboxyl group of valine and an ϵ-amino group of lysine would have

(11) See ref. 2 for a discussion.

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

(13) L. M. Kay, W. A. Schroeder, N. Munger and N. Burt, THIS JOURNAL, **78**, 2430 (1956).

(14) W. A. Schroeder, L. M. Kay, N. Munger, N. Martin and J. Balog, *ibid.*, **79**, 2769 (1957).

(15) Cya denotes cysteic acid.

(16) The stability of peptide bonds is discussed thoroughly in a review by S. J. Leach, *Rev. Pure Applied Chem.*, **3**, 25 (1953).

(17) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

(18) F. Sanger and E. O. P. Thompson, *ibid.*, **53**, 353 (1953).

(19) See, for example, E. J. Harfenist (THIS JOURNAL, **75**, 5528 (1953)), E. L. Smith, A. Stockell and J. R. Kimmel (*J. Biol. Chem.*, **207**, 551 (1954)), and C. H. W. Hirs, W. H. Stein and S. Moore (*ibid.*, **211**, 941 (1954)).

(20) T. H. J. Huisman and A. Dozy, *Biochim. Biophys. Acta*, **20**, 400 (1956).

(21) D. Theodoropoulos and L. C. Craig, *J. Org. Chem.*, **21**, 1376 (1956).

the added stability required by the calculated rate constant for the B chains. The bond in the A chains between leucine and the third amino acid residue is certainly a labile one despite the fact that leucine is involved. Whether or not this bond is an unusually labile one cannot be decided because investigations of the kinetics of peptide hydrolysis usually are made with milder conditions than refluxing 6 *N* hydrochloric acid; however, a bond such as leu-ser or leu-thr probably would be rather labile, even though leucine is involved, because of the unusual lability of peptide bonds that involve the amino group of serine or threonine.[16]

Finally, then, the results of the present investigation lead to the following conclusions. Normal adult human hemoglobin contains 4 N-terminal valyl residues per molecule. Upon hydrolysis of DNP-globin in refluxing 6 *N* hydrochloric acid, two of these residues are released within 15 min. as DNP-val-leu. The other two residues have not been detected with certainty in any form other than DNP-valine. Whether hemoglobin contains branched or unbranched chains, our results and the fact that the C-terminal residues are unlike require that hemoglobin contain at least two kinds of polypeptide chains. On the basis of his examination of tryptic hydrolyzates of hemoglobin, Ingram[22] has concluded that hemoglobin contains identical half molecules, a conclusion that has also been drawn from the X-ray investigations of Perutz and his collaborators.[23] Ingram also concludes that four identical sub-units are not present.

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(22) V. M. Ingram, *Nature*, **178**, 792 (1956).

(23) M. F. Perutz, A. M. Liquori and F. Eirich, *ibid.*, **167**, 929 (1951).

PASADENA, CALIFORNIA

[CONTRIBUTION FROM THE STERLING-WINTHROP RESEARCH INSTITUTE]

New Amine-masking Groups for Peptide Synthesis

BY FRANK C. MCKAY AND NOEL F. ALBERTSON

RECEIVED MARCH 30, 1957

Acid-catalyzed cleavage of a urethan, R'NHCOOR, leads to the formation of an amine salt, carbon dioxide and a product derived from the carbonium ion, R^+. This paper reports the use in peptide synthesis of urethans derived from *t*-butyl alcohol, *p*-methoxybenzyl alcohol, cyclopentanol, cyclohexanol and diisopropylcarbinol. Some of these carboalkoxy protecting groups possess advantages over the well-known carbobenzoxy group.

It has been pointed out that the cleavage of a carbobenzoxy group from an amino acid or peptide by phosphonium iodide is an acid-catalyzed reaction,[1,2] and in recent years there have been a number of reports of the use of the more convenient anhydrous hydrogen halides in place of phosphonium iodide.[1-6]

The reaction may be written as $ROCONHR' + 2H^+ \rightarrow R^+ + CO_2 + H_3N^+R'$, where R is benzyl and R' is an amino acid or peptide residue. There are, however, sometimes objections to the use of benzyl (or allyl) groups. For example, benzyl iodide and benzyl bromide are lachrymators. In addition, the benzyl ion attacks the sulfur atom in methionine,[2] and peptides of tryptophan are frequently obtained in poor yield or no yield at all. It is also sometimes desirable to have a protecting group which would be stable to catalytic hydrogenation. Thus a benzyl ester could be unmasked while leaving the amine protected.

It already has been shown[2] that carboisopropoxyglycyl-DL-phenylalanine gives a diketopiperazine when treated with hydrogen bromide in nitromethane. Here the cleavage of the urethan is so slow that a competing reaction becomes of major importance. Thus, a carbonium ion more stable than the isopropyl is required. During 1952, we undertook the investigation of the use of other urethans as amine-masking groups; the results form the subject of the present paper.

An advantage of the carbo-*t*-butoxy group as a masking agent is that it is cleaved with extreme ease. Carbon dioxide evolution begins immediately when hydrogen chloride is bubbled into a solution of a carbo-*t*-butoxypeptide; the reaction is more rapid than the removal of a carbobenzoxy group with hydrogen bromide. It has been found that refluxing acetic acid will also liberate carbon dioxide from a carbo-*t*-butoxy tripeptide, whereas the carbobenzoxy group is stable under these conditions. However, the product is a diketopiperazine and not a peptide when acetic acid is used for the decomposition. It was suspected that the peptide was the first product and that this cyclized to a diketopiperazine and a free amino acid on further heating in acetic acid. The literature affords several examples of reactions of this type. Lichtenstein heated, di-, tri- and tetrapeptides in β-naphthol at 135–150° and obtained diketopiperazines (and a free amino acid in the case of tripeptides).[7] Emerson has pointed out that prolonged heating with hydrazine in the removal of the phthaloyl protecting group will lead to diketopipera-

(1) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

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