for the threonyl peptide to 5 hours for the sarcosyl peptide. The results of the degradations were determined by four methods: A, paper chromatography of the PTH's[50]; B, paper chromatography of the amino acids obtained by acid hydrolysis of the PTH's at 150°[51]; C, dinitrophenylation and hydrolysis of each successive peptide, followed by paper chromatography[31] of the resulting DNP-amino acids, and D, quantitative estimation of the amino acid content of each successive peptide after total hydrolysis and two dimensional paper chromatography.

Individual Steps of the Degradation. Hydroxypipecolic Acid.—The PTH (method A) had approximately the same R_f value in pyridine–heptane (3:7) as hydroxyproline-PTH and threonine-PTH. Methods B and C gave only decomposition products.

Threonine.—The PTH (method A) had the same R_f as authentic Thr-PTH, and the spot showed the characteristic pink center. Method B gave only decomposition products. DNP-Thr (method C) could be obtained when the DNP peptide was hydrolyzed for 6 hours instead of the usual 12–14 hours. Method D gave a ratio Leu:Thr of 10:1 showing the disappearance of Thr in this degradation step.

Leucine.—In this and the following steps method A gave no clear-cut results. Methods B and C clearly established Leu as the 3rd amino acid, and this was confirmed by method D, which gave a ratio Ala:DiMeLeu:Leu of 82:79:2.

Hydroxyproline.—The best evidence for the position of aHyPro is the disappearance of this amino acid in the 4th degradation step (method D). Hydrolysis of the PTH (method B) gave a small amount of HyPro, in spite of the vigorous conditions. The DNP-aHyPro was destroyed under the normal conditions used for hydrolysis of the DNP peptide (method C).

Sarcosine.—The only conclusive evidence was given by method D, which gave the ratio Ala:DiMeLeu:PheSar:Sar = 24:12:13:0.

Dimethylleucine.—Method D gave the ratio Ala:PheSar:DiMeLeu = 16:20:0.

Alanine.—Hydrolysis of the PTH produced Ala (method B), but this could not be regarded as conclusive because small amounts of Ala appeared in PTH hydrolysates of previous degradation steps.

The Hydrazinolysis[30] of Etamycin Acid.—A solution of 10 mg. of Etamycin acid in 0.3 ml. of anhydrous hydrazine (freshly distilled from barium oxide) was heated in a sealed tube at 105° for 16 hours. After evaporation of the hydrazine in a desiccator over sulfuric acid, the residue was dissolved in 1.0 ml. of water and shaken for 2 hours with 0.20 ml. of benzaldehyde. The aqueous layer was separated, extracted with 2–3 ml. portions of ether, and subjected to paper chromatography using butanol–acetic acid as the developing solvent. The only detectable amino acid spot was one due to α-phenylsarcosine, although a small spot with a very high R_f value also was observed (most likely a C-terminal peptide fragment).

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(50) J. Sjöquist, *Acta Chem. Scand.*, **7**, 447 (1951).

(51) A. L. Levy, *Biochim. et Biophys. Acta*, **15**, 589 (1954).

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Synthesis of the Pressor-Antidiuretic Hormone, Arginine-Vasopressin

BY VINCENT DU VIGNEAUD,[1] DUANE T. GISH, PANAYOTIS G. KATSOYANNIS AND GEORGE P. HESS

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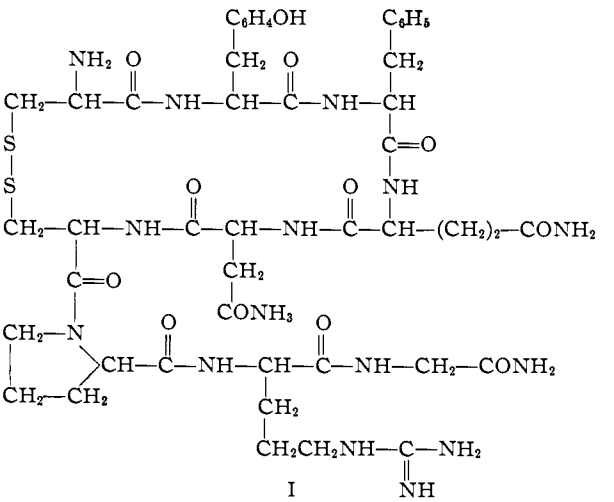
A product synthesized according to the structure proposed for arginine-vasopressin has been found to possess a potency approximately equivalent to that of the pressor-antidiuretic hormone isolated from the posterior pituitary glands of beef. The key intermediate in the synthesis of this hormone was the protected hexapeptide amide, carbobenzoxy-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide, which, after cleavage of the carbobenzoxy group with HBr–acetic acid, was coupled with the azide of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine. The resulting crude protected nonapeptide hydrobromide was treated with sodium in liquid ammonia to remove the protecting groups and then oxidized by aeration. The biologically active synthetic material thus obtained was purified by countercurrent distribution followed by electrophoresis and compared with natural arginine-vasopressin as to potency, partition coefficient, electrophoretic mobility, amino acid content and chromatography on the resin IRC-50. In all of these comparisons the synthetic material behaved the same as natural arginine-vasopressin.

Degradative studies[2–4] on highly purified preparations of arginine-vasopressin, the principal pressor and antidiuretic hormone of the posterior pituitary gland of beef, allowed structure I to be postulated for this hormone.[4,5]

Synthesis of the hormone according to this structure was undertaken[6] by a route which resembled the synthesis of oxytocin[7,8] in that each involved the preparation of a protected nonapeptide amide, in the case of vasopressin S-benzyl-N-carbobenzoxy - L - cysteinyl - L - tyrosyl - L - phenylalanyl - L - glutaminyl - L - asparaginyl-S - benzyl - L - cysteinyl - L - prolyl - L - arginyl-



I

(1) This work was supported in part by a grant (H-1675) from the National Heart Institute, Public Health Service, for which we wish to express our appreciation.

(2) E. A. Popenoe and V. du Vigneaud, *J. Biol. Chem.*, **205**, 133 (1953); **206**, 353 (1954).

(3) R. Acher, J. Chauvet and P. Fromageot, *Biochim. et Biophys. Acta*, **9**, 471 (1952); P. Fromageot, R. Acher, H. Clauser and H. Maier-Hüser, *ibid.*, **12**, 424 (1953).

(4) V. du Vigneaud, H. C. Lawler and E. A. Popenoe, THIS JOURNAL, **75**, 4880 (1953).

(5) R. Acher and J. Chauvet, *Biochim. et Biophys. Acta*, **12**, 487 (1953).

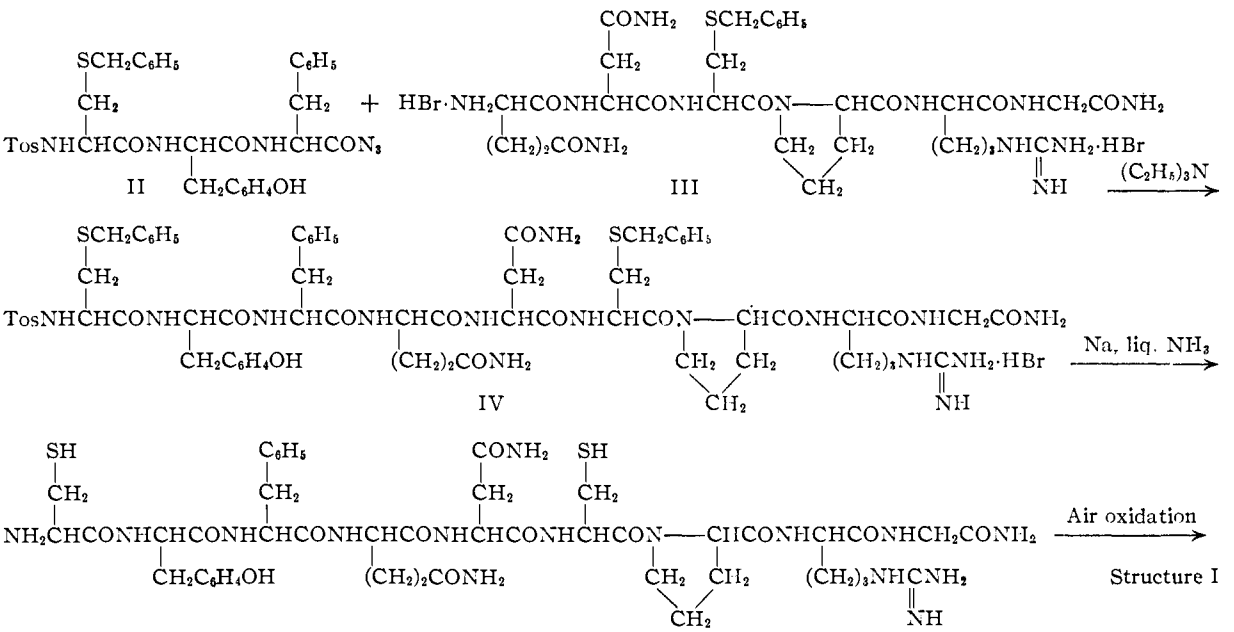
(6) V. du Vigneaud, D. T. Gish and P. G. Katsoyannis, THIS JOURNAL, **76**, 4751 (1954).

(7) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis and S. Gordon, *ibid.*, **75**, 4879 (1953).

(8) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts and P. G. Katsoyannis, *ibid.*, **76**, 3115 (1954).

glycinamide, removal of the protecting groups with sodium in liquid ammonia and oxidation of the resulting sulfhydryl nonapeptide to the active product. In the early synthetic studies,[6,9] the protected pentapeptide, S-benzyl-N-carbobenzoxy-L-cysteinyl - L - tyrosyl - L - phenylalanyl - L - glutaminyl-L-asparagine,[10] was coupled with the monohydrobromide of the tetrapeptide, S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide,[11] according to the tetraethyl pyrophosphite procedure[12] to give the protected nonapeptide amide. The more highly protected pentapeptide, S-benzyl-N-carbobenzoxy - L - cysteinyl - O - tosyl - L - tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparagine, was also used for coupling with the same tetrapeptide to give the corresponding O-tosyl nonapeptide amide,[9] tosyl being used herein as an abbreviation for the *p*-toluenesulfonyl grouping and expressed as Tos in the structural formulas. In both cases, treatment of the protected nonapeptide amides with sodium in liquid ammonia and oxidation of the resulting sulfhydryl compounds gave active products, which after purification by countercurrent distribution and electrophoresis possessed the biological properties associated with natural arginine-vasopressin. The specific activity of the synthetic preparations ranged in various experiments from 175 to 220 pressor units/mg. when assayed against the U. S. Pharmacopeia Posterior Pituitary Standard Powder.[9]

The specific activity of these synthetic products was considerably lower than the activity of the natural hormone. It was therefore suspected that the synthetic preparations might be contaminated with inactive components which possessed physical and chemical properties so similar to those of the active component as to render extremely difficult the separation of the compounds by countercurrent distribution, electrophoresis and other separation techniques employed. Since the chemical and optical purity of the respective pentapeptides and the tetrapeptide that were used for the preparation of the protected nonapeptides was considered satisfactory, the contamination was thought to be incurred during the last stage of the synthesis, namely, in the coupling of the pentapeptide with the tetrapeptide by the tetraethyl pyrophosphite method.[9] Other studies in this Laboratory have indicated that the formation of the peptide bond through the carboxyl group of asparagine with tetraethyl pyrophosphite may give rise not only to the expected peptide but also to an anhydro derivative of the peptide.[13,14] Since asparagine was the C-terminal amino acid in each of the pentapeptide intermediates used thus far for condensation with the tetrapeptide in attempts to synthesize arginine-vasopressin, the possibility occurred to us that the formation of an anhydro compound as a contaminating by-product in the coupling reaction to the nonapeptide intermediate might account for the difficulties encountered.



The present paper describes a new approach to the synthesis of arginine-vasopressin that has yielded a more highly active synthetic product with a potency similar to that of natural arginine-vasopressin. The key intermediate in this approach was the protected hexapeptide amide, carbobenzoxy - L - glutaminyl - L - asparaginyl-S - benzyl - L - cysteinyl - L - prolyl - L - arginylglycinamide monohydrobromide.[14] Treatment of this intermediate with HBr–acetic acid yielded L-glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl-L-prolyl-L-arginylglycinamide dihydrobromide (III), which was converted to the mono-

(9) P. G. Katsoyannis, D. T. Gish and V. du Vigneaud, THIS JOURNAL, *ibid.*, **79**, 4516 (1957).

(10) P. G. Katsoyannis and V. du Vigneaud, *ibid.*, **78**, 4482 (1956).

(11) D. T. Gish and V. du Vigneaud, *ibid.*, **79**, 3579 (1957).

(12) G. W. Anderson, J. Blodinger and A. D. Welcher, *ibid.*, **74**, 5309 (1952).

(13) D. T. Gish, P. G. Katsoyannis, G. P. Hess and R. J. Stedman, *ibid.*, **78**, 5954 (1956).

(14) P. G. Katsoyannis, D. T. Gish, G. P. Hess and V. du Vigneaud, *ibid.*, **80**, 2558 (1958).

hydrobromide with triethylamine and coupled with the azide of S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine[14] (II) to give the protected nonapeptide, S-benzyl-N-tosyl-L-cysteinyl-L-tyrosyl - L - phenylalanyl - L - glutaminyl - L - asparaginyl - S - benzyl - L - cysteinyl - L - prolyl - L-arginylglycinamide hydrobromide (IV).[15] The crude protected nonapeptide hydrobromide was treated with sodium in liquid ammonia to remove the protecting groups and the resulting sulfhydryl nonapeptide was oxidized by aeration in dilute aqueous solution at *p*H 6.7 and assayed for pressor activity by testing its ability to raise the blood pressure of the rat.[16] The treatment with sodium in liquid ammonia and subsequent oxidation of 1.42 g. of crude nonapeptide gave a solution which possessed approximately 130,000 pressor units. This solution upon concentration and lyophilization yielded a solid (4.15 g.) consisting of peptide material and inorganic salts. The specific activity of the material was approximately 20 pressor units/mg., indicating that approximately 83,000 pressor units were recovered after concentration and lyophilization.

For purification the crude active material was subjected to countercurrent distribution in 2-butanol–0.06 *M* *p*-toluenesulfonic acid. From the active peak 120 mg. of material was obtained, which on assay showed an activity of approximately 250 pressor units/mg. Further purification of this material by electrophoresis at *p*H 4.0 with cellulose as the supporting medium[17] yielded, as the most active fraction, 38 mg. of a product which possessed a specific activity of approximately 400 pressor units/mg.

The activity of this synthetic product, which varied from 360 to 440 units/mg. in a number of assays, compares favorably with that now found for highly purified preparations of natural arginine-vasopressin. The potency previously reported for natural arginine-vasopressin was approximately 600 pressor units/mg.[18] At the time these assays were performed, a value of 0.47 unit/mg. had been assigned to the U. S. Pharmacopeia Posterior Pituitary Standard Powder. A value of 0.40 unit/mg. has now been assigned to this standard. This would necessitate a change in the earlier figure to approximately 500 units/mg. However, it appears necessary to assign a lower value for the activity of natural arginine-vasopressin. The earlier assay value was determined in our Laboratory on dogs and cats, whereas more recently we have been using rats. Our most recent preparations have shown a pressor activity of 350 to 400 units/mg. for natural arginine-vasopressin,[19] which agrees quite well with the activity of our synthetic product within the limits of the assay.

The behavior of the synthetic product was the same as that of natural arginine-vasopressin on countercurrent distribution, paper electrophoresis at two different *p*H's and ion-exchange chromatography on IRC-50. The ratio of pressor to avian depressor activity in the synthetic material was identical with the ratio existing between these two activities in the natural hormone (1:0.15). Amino acid analysis of a hydrolysate of the synthetic compound showed the eight amino acids and ammonia to be present in the expected molar ratios to each other. Of particular significance was the complete absence of the unidentified ninhydrin-positive material, noted in our earlier synthetic preparations,[9] which emerged just following cystine.

These data warrant, in our estimation, the conclusion that the synthetic product is arginine-vasopressin and that structure I represents that of the hormone.

Experimental

Coupling of the Azide of S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine with L-Glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide Hydrobromide and Conversion of the Product to Active Material.—Carbobenzoxy-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide hydrobromide[14] (1.15 g., 1.18 mmoles) was treated with 30 ml. of acetic acid saturated with HBr for 30 minutes at 40–45° and then for 30 minutes at room temperature. The product was precipitated with ether, collected and washed with ether. For purification, the hexapeptide amide dihydrobromide (III) was precipitated three times from methanol (25 ml.) with ether (500 ml.); wt. 1.04 g.

S-Benzyl-N-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine hydrazide (0.84 g., 1.2 mmoles) was converted to the corresponding azide[14] (II) and then added to a solution of 0.8 g. (0.86 mmole) of the hexapeptide dihydrobromide in 12 ml. of dimethylformamide containing 0.16 ml. (1.15 mmoles) of triethylamine. The reaction mixture was stirred overnight at 5° and for 1 hour at room temperature. The product was precipitated with ethyl acetate, collected by filtration, washed successively with ethyl acetate, ethanol and ether and dried; wt. 0.92 g. This product was combined with a similar product (0.50 g.) from another run on a smaller scale. The material (1.42 g.) IV was divided into four portions and reduced with sodium in liquid ammonia according to the procedure employed for the synthesis of oxytocin.[7,8] The residue from each portion after evaporation of the ammonia was dissolved in about 600 ml. of 0.2% acetic acid, the *p*H was adjusted to 6.7 with NH_4OH and the solution was aerated with a slow stream of CO_2-free air for 1 hour. Solutions from these four runs were combined and upon assay were found to possess a total of 130,000 units of pressor activity.

The solution was concentrated in a rotary evaporator[20] and lyophilized to yield a mixture (4.15 g.) of peptide material and inorganic salt which possessed 83,000 pressor units, indicating some loss of activity during lyophilization.

Purification of Active Material.—The lyophilized product was placed in the first 31 tubes of the all-glass automatic countercurrent distribution apparatus[21] and distributed in 2-butanol–0.06 *M* *p*-toluenesulfonic acid. The progress of the purification was followed by determination of the Folin color according to the method of Lowry, *et al.*,[22] and the pressor activity of selected tubes. The distribution was carried through 1625 transfers and was interrupted four

(15) While this work was in progress a similar approach for a new synthesis of oxytocin was published independently from two laboratories [R. A. Boissonnas, St. Guttmann, P.-A. Jaquenoud and J.-P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955); J. Rudinger, J. Honzl and M. Zaoral, *Coll. Czechoslov. Chem. Communs.*, **21**, 202 (1956)].

(16) K. M. Lindquist and L. W. Rowe, *Drug Standards*, **23**, 153 (1955).

(17) H. G. Kunkel in "Methods in Biochemical Analysis," Vol. I, D. Glick, Ed., Interscience Publishers, Inc., New York, N. Y., p. 141.

(18) E. A. Popenoe, J. G. Pierce, V. du Vigneaud and H. B. van Dyke, *Proc. Soc. Exptl. Biol. Med.*, **81**, 506 (1952).

(19) The samples of natural arginine-vasopressin which were used for comparison of its pressor activity with that of the synthetic product were furnished by Dr. Albert Light and Dr. Rolf O. Studer of this Laboratory. The samples made available by them appeared to be homogeneous.

(20) L. C. Craig, J. C. Gregory and W. Hausmann, *Anal. Chem.*, **22**, 1462 (1950).

(21) L. C. Craig, W. Hausmann, E. H. Ahrens, Jr., and E. J. Harfenist, *ibid.*, **23**, 1236 (1951).

(22) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. S. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

times (after 250, 450, 650 and 1080 transfers) to replace with fresh solvent the solutions of the tubes containing material with no pressor activity. The solutions from 35 tubes containing the active material were pooled. The organic phase was extracted three times with water and the extracts were combined with the main aqueous phase. This solution was passed through a column of Amberlite IR-45 in the acetate form to remove the *p*-toluenesulfonic acid. The solution was then concentrated in a rotary evaporator and lyophilized to 120 mg. of a powder which had an activity of approximately 250 pressor units/mg.

This material was subjected to electrophoresis on a cellulose block[17,23] at *p*H 4.0 in a pyridine–acetate buffer for 42 hours at 5° with a potential of 400 volts. The most active fraction, weighing 38 mg., possessed 360 to 440 units/mg. of pressor activity on assay in the rat.[16]

Comparison of Synthetic and Natural Arginine Vasopressin.—The partition coefficients of the synthetic material as determined by countercurrent distribution in the systems 2-butanol–0.06 *M* *p*-toluenesulfonic acid and 2-butanol–0.1% acetic acid (*K*'s of 0.85 and 0.11, respectively) agreed within the deviations expected experimentally with the partition coefficients of natural arginine-vasopressin in the same solvent systems (*K*'s of 0.87 and 0.11,[24] respectively).

Samples of synthetic and natural arginine-vasopressin were placed side by side on a strip of Whatman No. 1 filter paper and subjected to paper electrophoresis[25] at *p*H 5.6 and at *p*H 4.0 in pyridine–acetate buffer. The synthetic product had a mobility identical with that of the natural product and migrated as a single component at both *p*H's.

A sample of the synthetic material was chromatographed[26] on an Amberlite IRC-50 column with an ammonium acetate buffer of *p*H 6.4. The synthetic product migrated on the column at the same rate as a sample of natural arginine-vasopressin chromatographed under identical conditions. No indication of the presence of more than one component was obtained under the experimental conditions used.

Amino acid analysis of the synthetic material after hydrolysis by the starch column chromatographic method[27] showed the expected amino acids and ammonia to be present in the following molar ratios, with the ratio for phenylalanine arbitrarily taken as 1: phenylalanine 1.00, tyrosine 0.88, proline 0.84, glutamic acid 1.06, aspartic acid 0.86, glycine 1.03, arginine 0.95, cystine 0.83 and ammonia 2.90.

Acknowledgments.—The authors are indebted to Mrs. Sylvia Kirsimagi White and Miss Gertrud Graubart for carrying out the assays for pressor and avian depressor activity and to Mrs. Lorraine S. Abrash for the amino acid analyses on the starch column.

(23) "Solka-Floc," a cellulose product of Brown Co. of New York, N. Y., was used.

(24) This value was determined by Dr. Peter G. Condliffe in this Laboratory.

(25) H. G. Kunkel and A. Tiselius, *J. Gen. Physiol.*, **35**, 89 (1951).

(26) The ion-exchange chromatography was performed by Dr. Albert Light of this Laboratory, to whom we wish to express our thanks.

(27) S. Moore and W. H. Stein, *J. Biol. Chem.*, **178**, 53 (1949).

NEW YORK, NEW YORK

[CONTRIBUTION NO. 2301 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The N-Terminal Sequence of the β Chains[1] of Normal Adult Human Hemoglobin

BY HERBERT S. RHINESMITH, W. A. SCHROEDER AND NANCY MARTIN

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The N-terminal sequence of the β chains of normal adult human hemoglobin is val-his-leu. Kinetic studies of the rate of hydrolysis of the val-his bond in the form of di-DNP-val-his-leu show that the hydrolysis is accompanied by a reaction of an unknown kind. The hydrolytic behavior at the N-termini is different for the α and the β chains of DNP-globin, but it has not been ascertained whether this difference is one of rate of hydrolysis or of destruction of the N-terminal amino acid in the course of the hydrolysis.

Introduction

Recent quantitative experiments[2] have led to the conclusion that normal adult human hemoglobin has four N-terminal valyl residues and that two kinds of N-terminal sequences are present. Although it was ascertained readily that two chains (termed the α chains[1]) release DNP-val-leu rapidly when DNP-globin is hydrolyzed, no N-terminal peptides that originated in the other chains (the β chains[1]) could be definitely identified at that time. This paper describes the isolation and identification of di-DNP-val-his-leu from the N-terminus of the β chains and a study of the kinetics of the hydrolysis of this peptide and of di-DNP-val-his.

Experimental

Preparation of DNP-globin.—Samples of DNP-globin were prepared by the methods previously described[2,3] except that in some preparations the *p*H was maintained at 8.15 instead of 9.0 during the dinitrophenylation. This change did not affect the final product.

Partial Hydrolysis of DNP-globin.—The procedure of partial hydrolysis was that previously described.[2] Samples ranged in size from 0.2 to 1.0 g., and the proportion of acid to weight of sample was also the same as previously used.

Extraction of the Partial Hydrolysates.—DNP-Valine and DNP-val-leu may be extracted easily with ether from a partial hydrolysate but the N-terminal peptides of the β chains are extracted with difficulty even with ethyl acetate. The following extractive procedure is the most satisfactory that has been devised in the course of these experiments.

The following quantities are adequate for the extraction of the hydrolysate of 0.2 g. of DNP-globin and should be increased proportionally for larger samples. After the hydrolysate had been transferred to a separatory funnel with 10 ml. of 6 *N* hydrochloric acid, the DNP-valine, DNP-val-leu and dinitroaniline (released from the α chains) were extracted with 5 × 25 ml. of ether. The procedure thus far is essentially that of ref. 2; if separation of the compounds of the ether extract is desired, the methods described in ref. 2 may be used. The hydrolysate was then extracted with 15 × 10 ml. of ethyl acetate in order to remove the N-terminal peptides from the β chains. Finally, the ethyl acetate extract was washed with 10 × 20 ml. of water to each portion of which 6 drops of 6 *N* hydrochloric acid was added. After evaporation of the ethyl acetate, the residue of peptides was ready for chromatography.

The extraction may be modified somewhat, depending on the purposes of the experiment. Because 65 to 75% of the

(1) Formerly called the B chains.[2] After discussion with Dr. Vernon Ingram, it was decided that if the polypeptide chains of normal adult human hemoglobin were designated α and β instead of A and B, there would be less likelihood of confusion with the names of the hemoglobins themselves, that is, hemoglobin A, S, C, etc.

(2) H. S. Rhinesmith, W. A. Schroeder and L. Pauling, THIS JOURNAL, **79**, 4682 (1957).

(3) H. S. Rhinesmith, W. A. Schroeder and L. Pauling, *ibid.*, **79**, 609 (1957).