was adjusted so that a flow of about 300 ml. per hour was obtained. The percolate (850 ml.) was discarded until the presence of base was indicated by picrate formation and basicity to moist litmus paper. The first 100 ml. of basic fraction was collected, concentrated to 10 ml., and the optical activity was determined. This process was repeated for the next six 100-ml. fractions. The first four basic fractions showed a slight positive rotation, while the remainder of the fractions had such small rotations that they could not be determined. A maximum positive polarimeter reading, $a = 0.13 \pm 0.01°$ (l 2 dm.), was obtained by combining and concentrating the first four basic fractions to 5 ml. The column was flushed with petroleum ether until the percolate contained no base. This required about 7 liters. All fractions of the basic percolate which did not demonstrate a positive rotation were combined, concentrated to 100 ml., filtered, and further concentrated to 5 ml. A polarimeter reading of $a = -0.06 \pm 0.01°$ (l 2 dm.) was determined for this concentrate.

Blank Determination.—A glass chromatographic cylinder (7.5 × 50 cm.) was packed with activated D-lactose hydrate to a depth of 40 cm. (about 1300 g.) and the column was wet with dry petroleum ether (b.p. 45–55°). Petroleum ether was added to the column, and the pressure at the top of the column was adjusted so that a flow of about 250 ml. per hour was obtained. Two liters of percolate was collected, concentrated to 5 ml., and a polarimeter reading of 0.00 ± 0.01° was determined. A Molisch test with α-naphthol and sulfuric acid failed to reveal the presence of carbohydrate in the percolate. Dry petroleum ether which had been shaken 24 hours with activated D-lactose hydrate and then filtered also failed to give a measurable optical rotation or a positive Molisch test.

Isomer B.—A glass chromatographic cylinder (3.7 × 60 cm.) was packed with activated D-lactose hydrate to a depth of 55 cm. (about 450 g.), and the column was wet with dry petroleum ether (b.p. 45–55°). A solution of 1.0 g. of hexahydrojulolidine, isomer B (from the reduction of julolidine and purification through the picrate) in 20 ml. of petroleum ether was added to the column, and the chromatogram was developed with additional quantities of petroleum ether. The pressure at the top of the column was adjusted so that a flow of about 100 ml. per hour was obtained. The percolate (325 ml.) was discarded until the presence of base was indicated by picrate formation and basicity to moist litmus paper. The first 25 ml. of basic fraction was collected, concentrated to 5 ml., and the optical activity was determined. The next 25-ml. portion was collected, added to the first, and the whole concentrated to 5 ml. and the optical activity again determined. The process of collection, combination, and concentration of the whole was continued until 1000 ml. of basic percolate had been collected and concentrated to 5 ml. After each concentration, the optical activity was determined. In each case, the polarimeter reading was $a = 0.00 \pm 0.01°$ (l 2 dm.).

Attempted Resolution of Octahydropyrrocoline.—A glass chromatographic cylinder (7.5 × 50 cm.) was packed with activated D-lactose hydrate to a depth of 45 cm. (about 1450 g.) and the column was wet with dry petroleum ether (b.p. 45–55°). A solution of 3.0 g. of octahydropyrrocoline[17] in 25 ml. of petroleum ether was added to the column and the chromatogram was developed with additional quantities of petroleum ether. The pressure at the top of the column was adjusted so that a flow of about 300 ml. per hour was obtained. The percolate (800 ml.) was discarded until the presence of base was indicated by picrate formation and basicity to moist litmus paper. The first 25 ml. of basic fraction was collected, concentrated to 5 ml., and the optical activity was determined. The next 50-ml. portion was collected, added to the first, and the whole concentrated to 5 ml. and the optical activity again determined. The process of collection, combination, and concentration of the whole was continued until 2 l. of basic percolate had been collected and concentrated to 5 ml. After each concentration, the optical activity was determined. In each case, the polarimeter reading was $a = 0.00 \pm 0.01°$ (l 2 dm.).

(17) Octahydropyrrocoline was prepared in the manner described by V. Boekelheide and S. Rothchild, THIS JOURNAL, **70**, 864 (1948).

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Sequence of Four Amino Acids at the Amino End of the Single Polypeptide Chain of Lysozyme

BY W. A. SCHROEDER

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Under certain conditions of partial hydrolysis, DNP-lysozyme produces a DNP-tetrapeptide and shorter DNP-peptides but longer peptides have not been isolated. The determination of the structure of the tetrapeptide as α,ε-di-DNP-lysyl-valylphenylalanylglycine thus fixes the sequence of the four amino acids at the amino end of the chain. On the basis of the results, it may be concluded that lysozyme consists of a single polypeptide chain which at the amino end terminates in the above sequence. A quantitative study of the composition of the mixture of end DNP-peptides which is produced by partial hydrolysis indicated the relative ease with which the various peptide bonds may be hydrolyzed: the valyl–phenylalanyl bond is stronger than the lysyl–valyl bond which in turn is stronger than the phenylalanyl–glycyl bond.

Sanger[1] in 1945 first described the use of 2,4-dinitrofluorobenzene (DNFB) for the determination of the free amino groups in proteins and at that time identified the free amino groups of insulin. Further application of the method by Porter and Sanger[2–4] has resulted in the identification of the terminal amino acids at the amino ends of the polypeptide chains of several proteins. Extension of the method by Sanger[5] lead to the isolation of dinitrophenyl-(DNP)-peptides from partial hydrolysates of two fractions of oxidized insulin and the determination of the sequence of the amino acids in these peptides. More recently, the entire sequence of amino acids in one of these fractions has been determined.[6]

In these laboratories, the application of Sanger's method to lysozyme showed that the terminal amino acid of this protein is lysine[7] but it was also found that a DNP-peptide (or peptides) could be isolated from certain hydrolysates. The fact that this DNP-peptide(s) yielded α,ε-di-DNP-lysine on complete hydrolysis lead to the conclusion that it is derived from the amino end of the lysozyme molecule. The present study was designed to

(1) F. Sanger, *Biochem. J.*, **39**, 507 (1945).
(2) R. R. Porter and F. Sanger, *ibid.*, **42**, 287 (1948).
(3) R. R. Porter, *Biochim. Biophys. Acta*, **2**, 105 (1948).
(4) F. Sanger, *Biochem. Soc. Symposia*, **3**, 21 (1949).
(5) F. Sanger, *Biochem. J.*, **45**, 563 (1949).
(6) F. Sanger and H. Tuppy, *ibid.*, **49**, 463, 481 (1951).
(7) F. C. Green and W. A. Schroeder, THIS JOURNAL, **73**, 1385 (1951).

determine the nature of this peptide material and to devise hydrolytic procedures which would produce long DNP-peptides from the DNP-lysozyme thus permitting the determination of as much as possible of the sequence of amino acids in this protein. A brief report of the results has been published previously.[8]

Experimental

Preparation and Properties of DNP-Lysozyme.—The lysozyme (Armour and Company, Lot 805L1) which was dinitrophenylated contained 95% of its activity in Peak A when chromatographed on a column of IRC-50 according to the procedure of Tallan and Stein[9]; that is, it behaved as unaltered lysozyme. The dinitrophenylation was made in essentially the way described by Sanger.[1] A 0.220-g. sample of lysozyme (about 10% moisture) was dissolved in 2 ml. of water, and 0.2 g. of sodium bicarbonate was added. A precipitate, probably of lysozyme carbonate, formed rapidly. A solution of 0.2 ml. of DNFB (prepared by dinitration of fluorobenzene) in 4 ml. of absolute alcohol was then added and the mixture was mechanically shaken for 3 hr. The yellow precipitate of DNP-lysozyme was centrifuged off, washed with 4 × 4 ml. of distilled water, 3 × 4 ml. of absolute alcohol, and 2 × 4 ml. of anhydrous ether, and finally air-dried to yield 0.190 g. of product.

Before the number of end groups in a protein molecule can be determined from the amounts of the DNP-amino acids in a complete hydrolysate, it is necessary to know or assume a molecular weight for the protein and to ascertain the equivalence between the protein and its DNP-derivative. Sanger,[1] for example, has determined the equivalence of insulin and DNP-insulin by comparing the content of amide nitrogen of the two materials. In the present work, the equivalence of lysozyme and DNP-lysozyme was found in the following manner. The content of certain amino acids in complete hydrolysates of both materials was analyzed for by starch[10] or ion exchange[11] chromatography and the quantities so estimated were compared. The equivalence as calculated individually from the content of about seven amino acids showed a spread of about 15% and consequently the average values given below probably are accurate only to ±5%. In this way it was found that 100 mg. of one preparation of DNP-lysozyme corresponded to 75 mg. of lysozyme whereas it corresponded to 80 mg. in another preparation. During these experiments, a zone which behaved like cysteic acid was found when a complete hydrolysate of DNP-lysozyme was chromatographed on Dowex-50.[11]

The yield of DNP-lysozyme when determined by this method was found to be rather poor (70–75%). The low yield may perhaps be caused by the precipitation of lysozyme carbonate in the reaction mixture. Investigation of the wash solvents from the preparation of DNP-lysozyme revealed the absence of any DNP-amino acids or DNP-peptides but it is possible that unreacted lysozyme was present in these wash solvents. However, the DNP-lysozyme itself contained no unreacted ε-lysyl groups: chromatography on starch[10] of a complete hydrolysate of DNP-lysozyme showed the absence of any free lysine.

Hydrolytic Procedures.—In all partial hydrolyses 15 ± 1 mg. of air-dried DNP-lysozyme and 10 ml. of hydrolyzing acid were used. Most hydrolyses were made by refluxing for various lengths of time in 6 *N* hydrochloric acid which had been doubly distilled in glass. Heating was done by means of a hot plate; since 10 minutes were required to reach refluxing temperature, the timing was begun when the solution started to boil. The hydrolysate was cooled quickly to room temperature at the end of the period of heating. For complete hydrolyses the time of refluxing in 6 *N* hydrochloric acid was 24 hr. DNP-Lysozyme is only slightly soluble in cold 6 *N* hydrochloric acid but solution is complete within a few minutes after refluxing begins.

Two other conditions of hydrolysis were also tried: refluxing *N* hydrochloric acid for 24 hr. and 6 *N* hydrochloric acid at 50° for 3 days. In the latter case, continuous mechanical agitation was necessary because of the insolubility of the DNP-lysozyme.

Extractive Procedures.—Sanger used ether[1] for the extraction of DNP-amino acids and ethyl acetate[5] for the extraction of DNP-peptides. In the present study both have been used: it was found that ether gives a cleaner extraction and removes all α,ε-di-DNP-lysine and dipeptide and most of the tri- and tetrapeptides from the hydrolysates whereas ethyl acetate in addition to removing all but traces of the remaining tri- and tetrapeptides also removes ε-lysyl material although because of its dipolar character one would not expect it to be extracted.

For the extraction, the hydrolysates were transferred to a separatory funnel and extracted first with 2 × 25 and 5 × 10 ml. of ether. The combined extracts were washed with the same volumes of water. The DNP-compounds are largely removed from the hydrolysate by the initial extraction. The washing with water does not remove any color from the ether but it should remove traces of undinitrophenylated amino acids and peptides.

After the extraction with ether, extraction was made with 5 × 10 ml. of ethyl acetate and the combined ethyl acetate extracts were washed with 5 × 10 ml. of water. Even the fifth extraction with ethyl acetate removes color from the hydrolysate. Most of the color is removed from the ethyl acetate by washing with water and has been found to be due largely to ε-DNP-lysyl material. However, some DNP-tripeptide and DNP-tetrapeptide which ether fails to remove remain in the ethyl acetate extract.

Further extraction in the same way with ethyl acetate was made after neutralizing about 85% of the acid with solid sodium carbonate. Extraction after partial neutralization was tried because it had been found in control experiments that DNP-pentaalanine is extracted by ethyl acetate from weakly acid solution but not from 6 *N* hydrochloric acid. Thus, if other high DNP-peptides behave similarly, it would not be possible to extract them directly from the hydrolysate. However, these extracts after partial neutralization contained no higher peptides but traces of the DNP-tripeptide and DNP-tetrapeptide as well as ε-lysyl material were present.

Chromatographic Procedures.—The chromatographic apparatus, the adsorbent, the packing and prewashing of the column, the types of developer, and the method of elution are identical with those used by Green and Kay[12] for the separation of DNP-amino acids by adsorption chromatography on silicic acid–Celite. The abbreviations used below are also those of Green and Kay.

The extracts of the hydrolysates were prepared for chromatography by evaporating the solvent from the wet extracts at reduced pressure and a bath temperature of 35°. The last traces of water which remained were removed by adding 2 ml. of acetone and re-evaporating. Since the residue must be dissolved in a small volume of solvent for chromatography, it should be concentrated on a small area of the flask by washing to the bottom with 2 ml. of acetone and then carefully removing the solvent under reduced pressure without splashing.

The most satisfactory solvent from which to place DNP-peptides on the chromatographic column is a 5 volume per cent. solution of glacial acetic acid in benzene (5AA–B). The peptide or peptide mixture should first be dissolved in the acetic acid and the solution then diluted with benzene. The volume of sample solvent should not be greater than 2 or 3 ml. for a no. 1 column (9 mm. diameter) and should be proportionately larger or smaller when other sizes are used. The sample solvents which were used when DNP-amino acids were chromatographed were those recommended by Green and Kay.[12]

Two chromatograms are required to separate the four compounds (α,ε-di-DNP-lysine, DNP-di-, -tri- and -tetrapeptides) which may be present in the extracts of a partial hydrolysate of DNP-lysozyme. If this mixture is developed with 5AA-B, the α,ε-di-DNP-lysine washes rapidly down the column and separates readily from the more slowly moving mixed zone of di- and tripeptides which in turn separates readily from the strongly adsorbed tetrapeptide. Thus on a 9 × 150-mm. column of 2:1 silicic acid–Celite the zones had the following positions in mm. from the top of the column after 42 ml. of 5AA–B had been used for development

(8) W. A. Schroeder, THIS JOURNAL, **74**, 281 (1952).
(9) H. H. Tallan and W. H. Stein, *ibid.*, **73**, 2976 (1951).
(10) S. Moore and W. H. Stein, *J. Biol. Chem.*, **178**, 53 (1949).
(11) S. Moore and W. H. Stein, *ibid.*, **192**, 663 (1951).
(12) F. C. Green and L. M. Kay, *Anal. Chem.*, **24**, 726 (1952).

DNP-Tetrapeptide	1–5
DNP-Di- and tripeptides	14–30
α,ε-Di-DNP-lysine	125-filtrate

Some 2,4-dinitroaniline which is formed by decomposition during hydrolysis washes through much more rapidly than α,ε-di-DNP-lysine.

Rechromatography of the mixture of the di- and tripeptides with 3% of glacial acetic acid and 15% of acetone in ligroin (60–70°)(3AA–15A–L) as developer results in the following positions on a 9 × 150-mm. column after 42 ml. have been used

DNP-Tripeptide 78–105
DNP-Dipeptide 113–137

At the completion of a chromatogram, the column was extruded from the chromatographic tube and the appropriate zones were cut out and eluted separately with 1:4 ethanol–ether (by volume). Eluted zones were prepared for rechromatographing in the same way as the extract of the hydrolysate (see above).

Development with 3AA–15A–L will separate the three peptides readily but will not separate the dipeptide from α,ε-di-DNP-lysine and hence, the two chromatograms described above are necessary when the four compounds are present in a mixture.

Analysis of the Isolated DNP-Peptides.—After the peptides had been isolated by the above procedures, it was necessary to determine their constituent amino acids. The analysis was done by hydrolyzing each peptide completely, extracting the DNP-amino acid thus released, dinitrophenylating the remainder in order to produce the DNP-amino acids of those amino acids which constituted the remainder of the peptide, and finally identifying the DNP-amino acids thus produced.

The hydrolyses were made by refluxing in 6 *N* hydrochloric acid for 24 hr. The cooled hydrolysate was extracted with ether as previously described in order to remove the end DNP-amino acid but the washing was reduced to 4 × 5 ml. These washings were combined with the extracted hydrolysate and this solution thus contained the free amino acids which constituted the remainder of the peptide. This solution was then evaporated to dryness at reduced pressure and a bath temperature of 60°, and 5 ml. of water was added and evaporated to remove traces of acid. The residue was taken up in one ml. of water and after the addition of 0.1 g. of sodium bicarbonate and a solution of 0.1 ml. of DNFB in 2 ml. of ethanol, the mixture was mechanically shaken for 3 hr. The reaction mixture was then transferred to a separatory funnel with 10 ml. of water, extracted while still basic with 4 × 25 ml. of ether to remove excess DNFB, acidified, and extracted with ether to remove the DNP-amino acids. This extract was washed and prepared for chromatography as described above.

The DNP-amino acids thus obtained from the extraction of the hydrolysate and from the dinitrophenylation were chromatographed according to the scheme of Green and Kay[12] and thus tentatively identified. This preliminary identification was confirmed by checking the chromatographic behavior of each of the isolated compounds with several different developers. After the qualitative work was complete, any quantitative determinations which were desired were made spectrophotometrically.

Spectrophotometric Determination.—Quantitative spectrophotometric determinations of the DNP-amino acids isolated were made with a Beckman quartz photoelectric spectrophotometer. Sanger[1,5] has used *N* hydrochloric acid or a 1% solution of sodium bicarbonate as the solvent for spectrophotometric determinations. In the present work, glacial acetic acid has been used because of its excellent solvent properties for DNP-amino acids and DNP-peptides. It has the additional advantage that most DNP-compounds can be recovered unchanged from the solution simply by evaporating the solvent: there is some evidence, however, that DNP-serine and DNP-threonine are altered by such a procedure. Although the absorption of acetic acid itself is so great that spectra cannot be taken at wave lengths shorter than about 250 mμ, this does not interfere with the determination of the DNP-amino acids at about 340 mμ. Spectrophotometric constants in acetic acid for DNP-amino acids which were isolated in the present work are given in Table I. The constants given in Table I are almost identical with those which Sanger reports and it is clear that the dinitrophenyl group determines to a large extent the spectra of this class of compounds. On this basis, the spectrophotometric constants of α,ε-di-DNP-lysine were used to calculate the quantities of the α,ε-di-DNP-lysyl peptides.

TABLE I

SPECTROPHOTOMETRIC CONSTANTS IN GLACIAL ACETIC ACID OF SOME DNP-AMINO ACIDS

Compound	Max., mμ	Min., mμ	F^a	$\epsilon^b \times 10^{-}$
α,ε-Di-DNP-lysine	343–344 260–261	287–288	3.10	3.22
DNP-Glycine	338–340 260–262	286	6.31	1.58
DNP-Phenylalanine	340–341 259	286–287	6.23	1.60
DNP-Valine	339–341 260	286–288	6.15	1.62

[a] This factor, (concn. in micromoles per 100 ml.)/(optical density for a 1-cm. length of solution), is convenient for calculation of quantity. [b] Molecular extinction coefficient.

Results

Qualitative Composition of the DNP-Peptides.—Complete hydrolysis and analysis of each DNP-peptide showed that its end amino acid was α,ε-di-DNP-lysine. In addition to the end group, the three peptides were found to contain the following amino acids

Most strongly adsorbed peptide: valine, phenylalanine, glycine
Intermediately adsorbed peptide: valine, phenylalanine
Least strongly adsorbed peptide: valine

These results immediately suggest that the sequence is lysylvalylphenylalanylglycine and further evidence confirms this conclusion. Partial hydrolysis (half an hr. in refluxing 6 *N* hydrochloric acid) of the most strongly adsorbed peptide, the tetrapeptide, yielded the other two peptides and α,ε-di-DNP-lysine in addition to a small amount of unchanged tetrapeptide. The identity of these peptides was established by their chromatographic behavior. Likewise, the intermediately adsorbed peptide on partial hydrolysis yielded unhydrolyzed peptide, the dipeptide and α,ε-di-DNP-lysine. Samples of dipeptide from partial hydrolysates of the tripeptide and from partial hydrolysates of DNP-lysozyme itself were analyzed and found to be identical. The identification of the constituent amino acids of the peptides is based upon the chromatographic behavior of their DNP-derivatives with several different developers and in the case of α,ε-di-DNP-lysine and DNP-glycine also on slight spectral differences. No attempt has been made to determine the configuration of the amino acids in these peptides.

Through the kindness of Dr. James R. Vaughan, Jr., it has been possible to compare synthetic L-lysyl-L-valyl-L-phenylalanylglycine with the tetrapeptide from lysozyme. In the form of the DNP-derivative, the chromatographic behaviors of the two are in good agreement under several conditions of development. More significantly, however, it was found that partial hydrolysis of each material yielded hydrolytic products in the same proportion.

During the identification of the constituent amino acids of the peptides, minor extraneous zones were

present on the chromatograms. These zones were found in the mixture which resulted from the dinitrophenylation of the extracted hydrolysate of each peptide. The chromatographic behavior of these zones, of which there are 3 or 4, resembles closely that of strongly adsorbed DNP-amino acids such as DNP-serine or DNP-aspartic acid but they can be shown to be artefacts and not constituents of the peptide because of the fact that approximately the same amount of each is produced when widely different amounts of the same peptide are hydrolyzed. When DNP-amino acids such as α,ϵ-di-DNP-lysine, DNP-glycine, DNP-valine, DNP-serine, and DNP-threonine are refluxed separately in 6 N hydrochloric acid and are extracted from the resulting "hydrolysates," these same artifact zones in approximately the same proportions are to be found after dinitrophenylation of the extracted "hydrolysates." They are produced independently of the fact that the DNP-amino acid may or may not have been chromatographed before refluxing in acid. These data show that the artifacts arise from the decomposition of the DNP-amino acids but it is surprising that different DNP-amino acids apparently produce the same detectable decomposition products.

Quantitative Composition of the Peptides.—The above qualitative identification of the amino acids in the peptides does not ascertain whether or not the ratio of the constituent amino acids to each other is unity. In order to resolve this question, the quantity of each of the DNP-amino acids which was isolated from a complete hydrolysate of a given peptide was determined spectrophotometrically. The bar graphs in Fig. 1 show the results of such determinations. These values have been corrected for chromatographic loss which in unpublished experiments has been found to be about 7% per chromatogram. It is clear that in these peptides the amino acids are present in equimolar quantity and that the end sequence in lysozyme is indeed lysylvalylphenylalanylglycyl---.

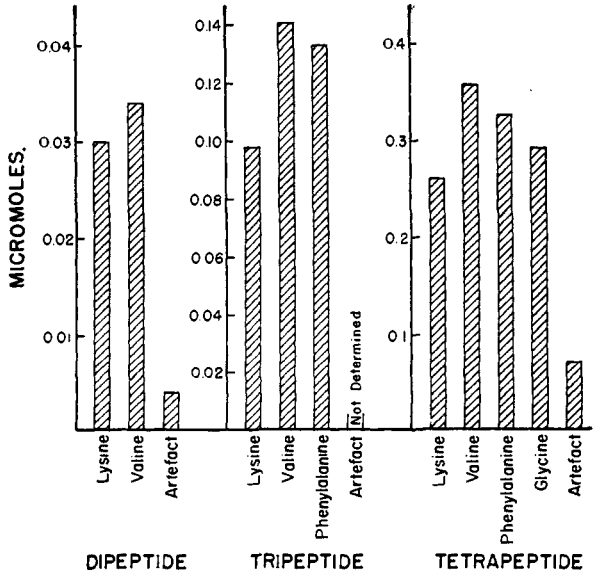


Fig. 1.—Results of quantitative determination of the constituent amino acids in the peptides.

The fact that in all of these peptides the quantity of lysine found is slightly less than that equivalent to the other amino acids is undoubtedly to be ascribed to the destruction of α,ϵ-di-DNP-lysine during hydrolysis. The extent of this destruction is about 30% in the tri- and tetrapeptide. The lesser destruction in the dipeptide is due to the fact that the period of hydrolysis here was only 4 hr.; in one experiment in which 24 hr. was used, the destruction of α,ϵ-di-DNP-lysine in the dipeptide was about 80%. The destruction of α,ϵ-di-DNP-lysine during hydrolysis appears to be somewhat variable even under apparently identical conditions of hydrolysis.

Incomplete experiments on the hydrolysis of α,ϵ-di-DNP-lysylalanine as a model substance indicate that the major destruction of the DNP-amino acid occurs during the rupture of the peptide bond and continues much more slowly after hydrolysis is complete. Thus, about 20% of the end group was destroyed during the first 2 hr. of hydrolysis in refluxing 6 N hydrochloric acid at which time hydrolysis is essentially complete; continued refluxing for 22 hr. destroyed only an equal amount.

Quantity of End Peptides in Various Types of Hydrolysate.—During hydrolysis in 6 N hydrochloric acid, it is observed that the initially insoluble DNP-lysozyme dissolves completely within 1 or 2 min. after refluxing begins. Then 5 min. after refluxing begins a small amount of insoluble material forms; all hydrolysates contain more or less insoluble material or show slight turbidity even after 24 hr. of hydrolysis.

Figure 2 shows the quantities of the several end DNP-peptides and of α,ϵ-di-DNP-lysine which can be isolated from samples of DNP-lysozyme after

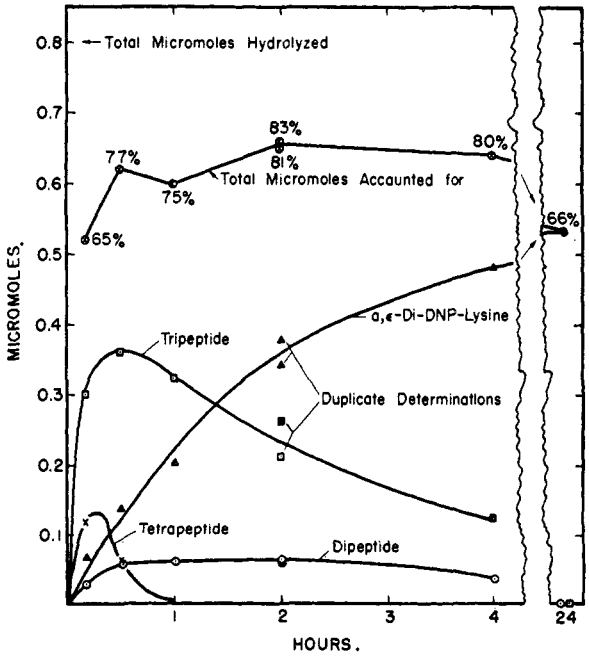
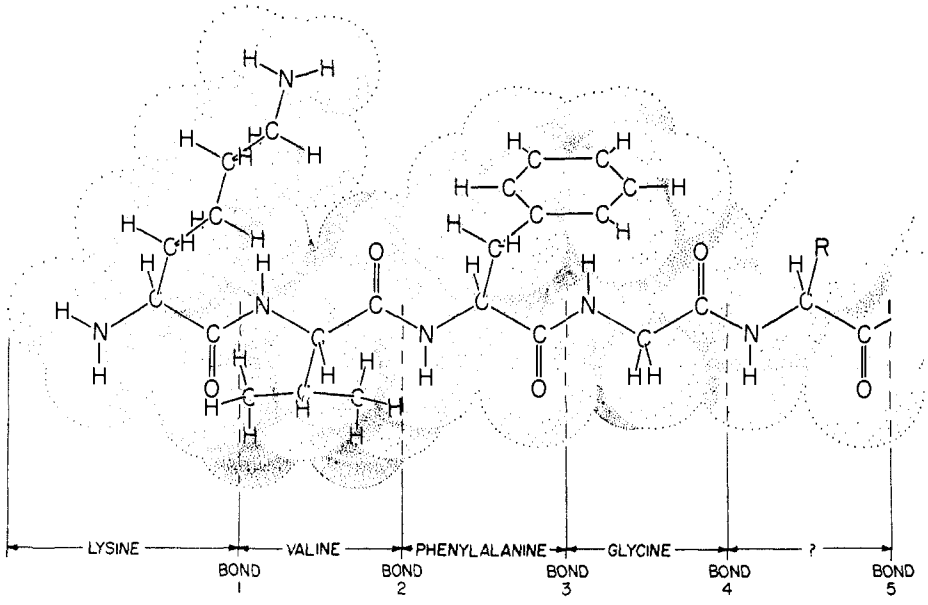


Fig. 2.—Quantity of α,ϵ-di-DNP-lysine, end DNP-peptides, and micromoles of starting material accounted for after hydrolysis of DNP-lysozyme in refluxing 6 N hydrochloric acid for various periods. The values are calculated on the basis of 15.0 mg. of starting material.



Relative Rate of Splitting of Bonds—3>4>1>2>5.

Fig. 3.—Sequence of four amino acids on the amino end of lysozyme.

refluxing in 6 *N* hydrochloric acid for varying lengths of time. Inspection of Fig. 2 indicates that this type of hydrolysis produces a rapid breakdown of the protein in which the formation of DNP-peptides from the intact protein has ceased after 15 to 30 min. and that thereafter the degradation of these peptides occurs.

No DNP-peptide longer than the tetrapeptide was found in any of the hydrolysates despite the fact that careful search was made for it. Moreover, had a pentapeptide or higher peptide been present in the extracts, it should have been easy to detect: the chromatographic properties of the DNP-peptides which were isolated, as well as those of synthetic DNP-peptides which were studied, lead one to believe that a pentapeptide should be more strongly adsorbed and readily separable from the tetrapeptide which was isolated. The possibility of course remains that small amounts of higher peptides may have been present but unextractable by the extracting solvents.

Figure 2 also shows the number of end groups per molecule of lysozyme which are accounted for by the sum of the end DNP-peptides and the α,ϵ-di-DNP-lysine. When considerable amounts of peptides are present and the time of hydrolysis is short, approximately 0.8 end group per 15,000 molecular weight is accounted for. A 24-hr. hydrolysis which completely hydrolyzes the peptides reduces this value to 0.66. In calculating these results, no correction has been made for chromatographic loss (about 7% per chromatogram) or for blanks which are of the order of 1 to 3%. If the corrections were applied, the end groups accounted for would be increased to the extent of 5 to 10%; a correction as high as 10% would result from the fact that in the isolation of the di- and tripeptides two chromatograms are necessary.

Because refluxing in 6 *N* hydrochloric acid failed to produce DNP-peptides longer than the tetrapeptide even when the time of hydrolysis was very short, other conditions of hydrolysis were investigated. When refluxing *N* hydrochloric acid was used, the DNP-lysozyme dissolved very slowly and little hydrolysis was evident after 4 hr.; after 24 hr. of hydrolysis the composition of the mixture was essentially the same as that produced by 10 min. of refluxing in 6 *N* hydrochloric acid and the tetrapeptide was the longest peptide which could be isolated.

In another experiment 6 *N* hydrochloric acid at 50° for 72 hr. was used for hydrolysis. Solution took place slowly and incompletely and constant mechanical agitation was necessary. The composition of the peptide mixture again showed much similarity to that which was obtained from 10 min. of refluxing in 6 *N* hydrochloric acid except that virtually no dipeptide was produced. No evidence of a pentapeptide was found.

Discussion

The portion of the polypeptide chain of lysozyme which has been determined in this study is pictured in Fig. 3. The results which are presented in Fig. 2 enable conclusions to be drawn as to the ease with which the various bonds may be split during hydrolysis. That the lability of the peptide bonds decreases in the following order is also substantiated by the results of the partial hydrolysis of individual peptides:

Bond 3 between phenylalanine and glycine—most labile
Bond 4 between glycine and fifth amino acid
Bond 1 between lysine and valine
Bond 2 between valine and phenylalanine
Bond 5 between the fifth and sixth amino acids—least labile (?)

The most stable peptide is the tripeptide and in it bond 2 is much more stable than bond 1 as is shown by the fact that during the period from 0.5 to 3.5 hr. of hydrolysis the rate of formation and hydrolysis of the dipeptide is essentially balanced

whereas the increase in α,ϵ-di-DNP-lysine is great and must result mostly from the tripeptide. Additional experiments showed that the dipeptide is completely hydrolyzed in 4 hr. (or less) whereas the tripeptide is not. The great stability of the valine–phenylalanine bond (Bond 2) is not surprising on the basis of results of Christensen[13,14] and Christensen and Hegsted[15] who found that the valyl peptides of gramicidin were very difficult to hydrolyze. Synge[16,17] also observed the same effects and made model experiments with such peptides as valylglycine, glycylvaline, leucylglycine and glycylleucine. When the larger group was on the carbonyl side of the peptide bond as in the valyl and leucyl peptides the rate of hydrolysis was greatly decreased especially in the valyl peptide. The glycyl peptides were split much more easily and one may thus expect that bond 4 would hydrolyze with some ease.

Information of the splitting of lysyl peptides and phenylalanyl peptides does not seem to be available in the literature but on the basis of the present work the phenylalanyl bond is rather easily hydrolyzed and the lysyl bond less so, although the latter may be affected by the DNP-groups which were present during this work.

In attempting to assess the reasons for the absence of the pentapeptide in the hydrolyzates, one must consider bonds 4 and 5. It may be that bond 5 is very stable, and hence prevents the formation of any pentapeptide during hydrolysis or it may be that the nature of the fifth amino acid really makes bond 4 very labile in comparison to bond 5. If the fifth amino acid were serine or threonine one would expect bond 4 to be unusually labile, since Desnuelle and Casal[18] have shown that peptide bonds involving the amino group of serine or threonine are very labile. The lability of bond 4 would also be enhanced by the presence of glycine as the fourth amino acid. The possibility also exists that the fifth amino acid is tryptophan, an amino acid which is present in appreciable amount in lysozyme and, in addition, is sensitive to destruction by acid. Either of these amino acids then might well be the cause of the failure of the protein to yield a penta or higher peptide.

Lysozyme is a protein which probably contains about 125 amino acid residues[19–21] of which about 20 are aspartyl, 11 arginyl, 11 glycyl, 10 alanyl and 10 seryl. It is interesting to note that of these five most prevalent amino acids only glycine appears in the tetrapeptide sequence whereas lysine, valine, and phenylalanine of which the content is only 6, 6 and 3 residues, respectively, are to be found near the end of the molecule.

Several authors have concluded that lysozyme contains one or at most two peptide chains. Thus, Lewis, *et al.*,[20] from microbiological and chemical analyses decided that lysozyme contained only one or two peptide chains. Likewise, Fraenkel-Conrat, *et al.*,[22] have suggested that lysozyme has only a single peptide chain which is internally cross-linked by di-sulfide bonds; they based this conclusion on the fact that reduced and alkylated lysozyme has a molecular weight which is the same as that of the original lysozyme. In previous work in these laboratories,[7] α,ϵ-di-DNP-lysine in amount equivalent to about 0.75 terminal group per molecule was isolated (based on a molecular weight of 13,900 and corrected for 7% chromatographic and 15% hydrolytic destruction); the conclusion was drawn that it is unlikely that more than one lysine residue occupies an amino terminal position in lysozyme. Recently, Thompson[23] has also found that lysine is the end amino acid of lysozyme. Her results indicate that tryptophan is responsible for the destruction of α,ϵ-di-DNP-lysine during hydrolysis but even after corrections are applied she is able to find sufficient α,ϵ-di-DNP-lysine to account for little more than half an end group per molecule.

The results of the analyses of the peptides which were isolated in the present study as well as those from the hydrolysis of α,ϵ-di-DNP-lysylalanine as a model substance indicate that the destruction of the end group during 24 hr. of refluxing in 6 *N* hydrochloric acid occurs to the extent of 25 to 40%. This correction is considerably larger than the 15% previously used.[7] If, for purposes of comparison, we consider the uncorrected amount of α,ϵ-di-DNP-lysine isolated in our previous work[7] and recalculated on the basis of a molecular weight of 15,000, the value obtained, 0.64 end groups per molecule, is in excellent agreement with 0.66 end groups per molecule from the present study (see Fig. 2). If chromatographic losses are assumed to be 7% and hydrolytic losses 25 to 40%, the end groups per molecule calculated from the present data will be 1.0 ± 0.1. Also to be considered is the end group accounted for by the sum of DNP-peptides and free DNP-amino acid in partial hydrolyzates (Fig. 2); without correction the value is about 0.8. On the basis of these results, it can be stated with considerable certainty that only one lysine occupies an amino terminal position.

There is the possibility, however, that in the acidic portion of the hydrolyzate of DNP-lysozyme which remains after extraction with ether and ethyl acetate there is a DNP-amino acid such as DNP-arginine which is unextractable by ether or by ethyl acetate and which might be the end amino acid of a second chain. However, Thompson[23] found no DNP-amino acid other than ϵ-DNP-lysine in the extracted acidic portion of a complete hydrolysate of DNP-lysozyme and the present work substantiates her results. Accordingly, we may conclude that lysozyme has a single polypeptide chain and that the terminal sequence is lysylvalylphenylalanylglycyl---.

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(22) H. Fraenkel-Conrat, A. Mohammed, E. D. Ducay and D. K. Mecham, THIS JOURNAL, **73**, 625 (1951).
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lysozyme which was used in these experiments in order to determine its purity and to acknowledge the work of Miss Lois M. Kay and Mr. Lewis Honnen in performing certain of the experiments reported here. The kindness of Dr. James R. Vaughan, Jr., in offering a sample of synthetic tetrapeptide is greatly appreciated. This work was aided by a grant from the National Foundation for Infantile Paralysis.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND CHEMICAL ENGINEERING, UNIVERSITY OF WASHINGTON]

The Synthesis of 5,6,7,7a,8,9,10,11-Octahydro-4H-benzo[ef]heptalene[1]

BY A. G. ANDERSON, JR., AND HELEN FRANCES GREEF

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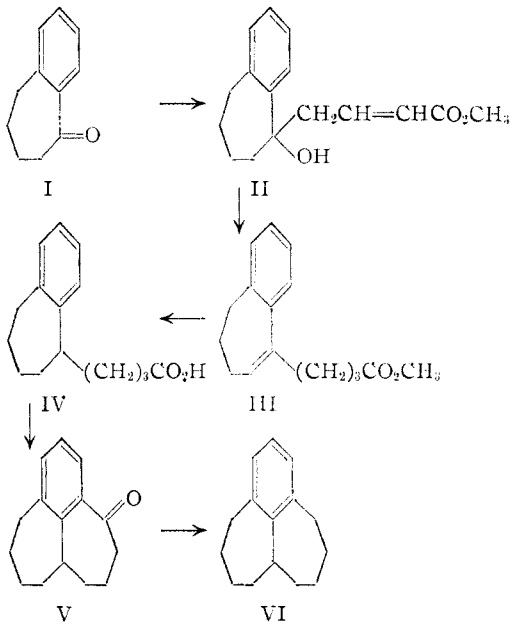
5,6,7,7a,8,9,10,11-Octahydro-4H-benzo[ef]heptalene, a hydrocarbon having a new tricyclic fused ring system, has been synthesized from 6,7,8,9-tetrahydro-5H-cycloheptabenzen-5-one.

In the course of investigations on the synthesis of hydrocarbons related to heptalene, pentalene or azulene which have new tricyclic fused rings systems, we have prepared 5,6,7,7a,8,9,10,11-octahydro-4H-benzo[ef]heptalene (VI). To our knowledge no compounds having this ring system have been reported previously. As the four other possible tricyclic systems containing five- and seven-membered rings fused to adjacent sides of a benzene ring[2] have been prepared previously,[3] the synthesis of VI completes this particular series of compounds.

A synthetic route to VI *via* 6,7,8,9-tetrahydro-5H-cycloheptabenzen-5-one (I) was selected and this intermediate was prepared in 89% yield by the cyclization of phenylvaleryl chloride in an inverse Friedel–Crafts reaction.



A Reformatsky reaction of I with methyl bromocrotonate gave a product (presumably II) which was unstable to heat and, accordingly, was subjected to catalytic hydrogenation without purification. The saturated product was also quite unstable and, similarly, was dehydrated without purification to give methyl 8,9-dihydro-7H-cycloheptabenzene-5-butyrate (III)[4] in 25% over-all yield from I. The observed instability of both the unsaturated and saturated hydroxy esters is in agreement with the results of Bachmann and co-workers[5] on similar compounds. The dehydration of the saturated hydroxy ester was unexpectedly difficult to accomplish. Heating the compound with anhydrous, fused potassium bisulfate at 150–160°[5] was the most satisfactory method found. The use of *p*-toluenesulfonic acid, β-naphthalenesulfonic acid, iodine or oxalic acid as catalysts gave over-all yields of 5% or less of III.

Saponification of III and catalytic hydrogenation of the acidic product, which was a viscous oil and unstable to heat, gave 6,7,8,9-tetrahydro-5H-cycloheptabenzene-5-butyric acid (IV) in 56% yield. A Friedel–Crafts cyclization of the acid chloride of IV afforded a 58% yield of 5,6,7,7a,8,9,10,11-octahydro-4H-benzo[ef]heptalen-4-one (V). A modified Wolff–Kishner reduction of V gave 5,6,7,7a,8,9,10,11-octahydro-4H-benzo[ef]heptalene in 38% yield. The structure of VI was shown by analysis and oxidation with potassium permanganate followed by esterification of the acidic product to give the known trimethyl hemimellitate.

Experimental[6]

Phenylvaleric Acid.—From 132 g. (1.0 mole) of cinnamaldehyde, 115 g. (1.1 moles) of malonic acid, 50 ml. of dry benzene and 13 ml. of 10% alcoholic potassium hydroxide was obtained, in a manner similar to that described by Welch,[7] 190 g. (83%) of cinnamylidenemalonic acid. The crude product melted at 198–202° and was used without further purification. A sample recrystallized from alcohol melted at 205–207° with decomposition.[8]

A solution of 109 g. (0.5 mole) of the crude cinnamylidenemalonic acid and 150 g. of sodium hydroxide in 1200 ml. of water was treated with Raney nickel catalyst powder[9] (100

(1) From the Ph.D. Thesis of Helen Frances Greef.

(2) The structure of this type having two five-membered rings fused to the benzene ring would not appear to be possible.

(3) J. v. Braun and J. Reutter, *Ber.*, **59**, 1922 (1926); J. v. Braun and E. Roth, *ibid.*, **60**, 1182 (1927).

(4) The position of the double bond was not determined and it may be exocyclic to the seven-membered ring.

(5) W. E. Bachmann and N. L. Wendler, THIS JOURNAL, **68**, 2580 (1946); W. E. Bachmann and A. S. Dreiding, *J. Org. Chem.*, **13**, 317 (1948).

(6) Melting points and boiling points are uncorrected.

(7) K. N. Welch, *J. Chem. Soc.*, 673 (1931).

(8) B. S. Bansal and K. C. Pandya, *J. Indian Chem. Soc.*, **24**, 443 (1947).

(9) D. Papa, E. Schwenk and B. Whitman, *J. Org. Chem.*, **7**, 587 (1942).