all), m.p. 156°. This substance gave a positive ninhydrin, but a negative biuret reaction. Chromatography gave a blue spot ($R_f$ value 0.17); *cf.* ref. 6.

*Anal.* Calcd. for $C_6H_{10}O_5N_2 + H_2O$: C, 34.6; H, 5.8; N, 13.5; N (Van Slyke), 6.7. Found: C, 34.9; H, 5.8; N, 13.2; N (Van Slyke), 6.4.

**N-Benzyl-β-*dl*-aspartyl-*dl*-alanine Ethyl Ester (XIV).** (A) From II in Dioxane Solution.—A cooled solution of II in dioxane was prepared from 9 g. of I in 200 ml. of dioxane; 10 g. of *dl*-alanine ethyl ester was added and the reaction mixture kept overnight at room temperature. No precipitate formed owing to the solubility of both *dl*-alanine ethyl ester hydrochloride and XIV in dioxane. The solution was evaporated to dryness *in vacuo;* the residue was recrystallized from a small quantity of ethanol and kept in an ice-box overnight. Recrystallization from ethanol yielded 4.1 g. (31.5%), m.p. 197°.

*Anal.* Calcd. for $C_{16}H_{22}O_5N_2$: N, 8.7. Found: N, 8.4.

(B) From Solid II.—A suspension of 13 g. of solid II in 200 ml. of dry toluene was shaken with 13 g. of *dl*-alanine ethyl ester in a 500-ml. glass-stoppered flask for 6 hours. The reaction mixture was filtered and the precipitate washed with ether and recrystallized from ethanol; 5.5 g. (37.5%) of XIV was obtained, which was identical with the substance prepared by method (A).

**β-*dl*-Aspartyl-*dl*-alanine Ethyl Ester (XV).**—XIV (3 g.) was dissolved in 50 ml. of glacial acetic acid, 0.2 g. of catalyst added and hydrogenolysis carried out for 4 hours. After filtration and evaporation of the solvent, the residue was recrystallized from ethanol. The substance, melting at 218°, was obtained in an almost quantitative yield. It gave a positive ninhydrin, but a negative biuret reaction.

*Anal.* Calcd. for $C_9H_{16}O_5N_2$: N, 12.0; N (Van Slyke), 6.0. Found: N, 11.9; N (Van Slyke), 6.1.

**β-*dl*-Aspartyl-*dl*-alanine (XVI).**—XIV (1 g.) was dissolved in 7 ml. of 1 *N* sodium hydroxide solution. After two hours, the solution was acidified with hydrochloric acid, but no precipitate formed. It was evaporated *in vacuo* to dryness and redissolved in 50 ml. of 50% acetic acid; after the addition of 0.2 g. of catalyst, hydrogenolysis was carried out for 4 hours. After filtration and evaporation of the solution *in vacuo* to dryness, the residue was recrystallized from water to yield 0.19 g. (30%) of short needles, m.p. 232°, which gave a positive ninhydrin, but a negative biuret reaction. Chromatography gave a blue spot ($R_f$ value 0.28).

*Anal.* Calcd. for $C_7H_{12}O_5N_2$: C, 41.1; H, 5.9; N, 13.7; N (Van Slyke), 6.8. Found: C, 41.1; H, 5.8; N, 13.7; N (Van Slyke), 6.8.

JERUSALEM, ISRAEL

---

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF IOWA STATE COLLEGE]

# Application of a Quantitative Method of Peptide Analysis to the N-Terminal Sequence of Lysozyme[1]

BY DOROTHY DE FONTAINE[2] AND SIDNEY W. FOX[3]

RECEIVED FEBRUARY 4, 1954

The microbiological phenylthiohydantoin subtractive method of peptide analysis has been applied to the terminal aminoid tripeptide residue of chicken egg lysozyme, without discernible alteration of the rest of the molecule. The sequence is shown to be L-lysyl-L-valyl-L-phenylalanyl-, which includes the configurational designations for the first time. Further evaluation of the quantitative method of peptide analysis is presented. Various assay media and microbes for the determination of lysine have been compared; one combination has given in this work significantly greater precision than two others.

A stepwise N-terminal method, representing a modification of the original Abderhalden–Brockmann[4] and Edman[5] methods has been applied with microbiologically quantitative results to synthetic peptides[6] and to ACTH preparations.[7] Some of these modifications have also been useful in qualitative structural studies of natural peptides.[8,9] In this paper are presented the results of study of applicability of the subtractive procedure to lysozyme, for which structural information is available, particularly from an investigation by Schroeder, with the DNP technique.[10] The molecular weight of lysozyme is sufficiently high (*ca.* 14,700)[11] to provide a severe test of the method.

## Materials and Methods

**Lysozyme.**—Lysozyme was a thrice recrystallized material[12] furnished by Dr. Joseph F. Foster. Electrophoretic analysis at *p*H 7.7 (phosphate–sodium chloride buffer $\gamma/2 = 0.20$) indicated this material was nearly homogeneous. There was observed in the electrophoretogram a trace (estimated 1–2%) component which moved slightly ahead of the main boundary. The descending boundary was somewhat asymmetric but showed no trace of the fast component. For some of the determinations of lysine, Armour lysozyme Lot 003 L1, was used.

**Aminoid Treatment.**—The procedure previously described[7] was followed with the following modifications: phenyl isothiocyanate (PTC) treatment was terminated after 4 hr. of incubation, selective fission of the treated product with dioxane–hydrogen chloride in a hydrogen chloride atmosphere was discontinued after 6 hr., and 6 *N* aqueous hydrochloric acid was used for total hydrolysis of protein.

**Assays.**—Standard curves covered the range of 0–50 γ of L-amino acid. The *p*H of the medium was adjusted in each case to 6.8 ± 0.1. Sixteen-hour inoculum cultures were used in the assays which were incubated for 72 hr. at 37° except for glycine assays which were incubated for 48 hr.

(1) Journal Paper No. J-2466 of the Iowa Agricultural Experiment Station, Project 863, supported by Grant No. G-3025(C) from the National Institutes of Health, Public Health Service.

(2) Taken in part from the M.S. Thesis of Dorothy De Fontaine, 1952.

(3) To whom inquiries should be directed.

(4) E. Abderhalden and H. Brockmann, *Biochem. Z.*, **225**, 386 (1930).

(5) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).

(6) S. W. Fox, T. L. Hurst and K. F. Itschner, THIS JOURNAL, **73**, 3573 (1951).

(7) S. W. Fox, T. L. Hurst and C. Warner, *ibid.*, **76**, 1154 (1954).

(8) W. Landmann, M. P. Drake and J. Dillaha, *ibid.*, **75**, 3638 (1953).

(9) W. Landmann, M. P. Drake and W. F. White, *ibid.*, **75**, 4370 (1953).

(10) W. A. Schroeder, *ibid.*, **74**, 281 (1952); **74**, 5118 (1952).

(11) C. Fromageot and M. B. de Garilhe, *Biochim. et Biophys. Acta*, **4**, 509 (1950).

(12) G. Alderton, W. H. Ward and H. L. Fevold, *J. Biol. Chem.*, **157**, 43 (1945); R. H. Forsythe, Ph.D. thesis, Iowa State College, 1949.

Titration was performed with 0.05 *N* sodium hydroxide solution except when *L. arabinosus* was used as the assay organism. In this case 0.10 *N* alkali was employed. Brom thymol blue was the indicator.

## Results and Discussion

In the course of analyses of lysine in the hydrolysate and after N-terminal and N-penultimate treatments of lysozyme it was apparent that one combination of assay organism and medium yielded more highly reproducible results than two others. Some comparative experiments, which were accordingly set up to test these hypotheses, confirmed the observations with this critical amino acid. In all, lysine was determined 72 times; all of the results are represented in Table I. Each row of figures represents a single simultaneously cultured assay, and each of the values, with few exceptions, is the average of four replicates at each of five different dilutions. The individual figures are presented only for the preferred combination, but average values, numbers of determinations and standard deviations are recorded for each of the organism–medium combinations. The results indicate that only in the case of the *mq* combination was the standard devation small enough to permit assigning 6 residues to the untreated and 5 each to the once-treated and twice-treated lysozyme. The corresponding figures for *fq* were 6–7, 3–5 and 4–5, whereas for *ms* they were 5–7, 4–5 and 4–5. No differences in lysine value between the Armour and Foster lysozymes were noted.

Repeated determinations of valine and phenylalanine did not exhibit the variability encountered with lysine.

In Table II are presented results of analyses of lysozyme from three laboratories, all figures being reduced to numbers of residues based upon an assumed molecular weight of 14,700. In this table, and in Table III, the values represent averages of duplicated tubes containing test sample at three dilutions.

TABLE I

LYSINE CONTENTS OF HYDROLYZED AND HYDROLYZED TREATED LYSOZYME WITH VARIOUS COMBINATIONS OF MEDIUM AND MICROBE, IN RESIDUES/14,700 g.

$AT_n$ = aminoid[12a] treatment *n* followed by hydrolysis. $AT_0$ thus indicates hydrolysate without such treatment.

| | $AT_0$ | $AT_1$ | $AT_2$ |
|---|---|---|---|
| | Combination *mq*[7] | | |
| Determination | 6.1 | 5.0 | |
| | 5.5 | 4.6 | 4.8 |
| | 6.2 | 4.9 | 4.9 |
| | 6.2 | 5.0 | 5.1 |
| | 6.2 | 5.0 | 5.1 |
| | 6.5 | 5.2 | |
| Mean no. of residues | 6.1 | 4.9 | 5.0 |
| Standard deviation | ±0.33 | ±0.21 | ±0.15 |
| | Combination *fq*[7] | | |
| No. of detns. | 8 | 8 | 3 |
| Mean no. of residues | 6.3 | 4.2 | 4.6 |
| Standard deviation | ±0.72 | ±0.95 | ±0.27 |
| | Combination *ms*[7] | | |
| No. of detns. | 15 | 15 | 7 |
| Mean no. of residues | 6.0 | 4.4 | 4.3 |
| Standard deviation | ±0.56 | ±0.48 | ±0.69 |

TABLE II

PARTIAL AMINO ACID COMPOSITION OF LYSOZYME

| Amino acid | No. of residues[a] per molecule of lysozyme: Present work | Fromageot and de Garilhe[11] | Lewis, *et al.*[13] |
|---|---|---|---|
| Alanine | 9.9(*cx*) | 10.0 | 9.6(c) |
| Arginine | 11.0(*fq*) | 12.5 | 10.8(m) |
| Glycine | 11.4(*bt*) | 11.0 | 11.2(m) |
| Histidine | 0.9(*mr*) | 1.0 | 0.9(m) |
| Isoleucine | 6.7(*aq*) | 7.0 | 5.8(a) |
| Leucine | 7.0(*aq*) | 7.9 | 7.7(a) |
| Lysine | 6.1(*mq*) | 6.0 | 5.7(m) |
| Methionine | 2.4(*fp*) | 2.3 | 2.1(a) |
| Phenylalanine | 3.1(*bt*) | 2.0 | 2.8(m) |
| Proline | 2.2(*bt*) | 1.7 | 1.8(m) |
| Serine | 9.4(*my*) | 10.1 | 9.4(m) |
| Threonine | 7.4(*fz*) | 6.6 | 6.6(f) |
| Tyrosine | 2.8(*bt*) | 3.1 | 2.9(m) |
| Valine | 5.5(*fq*) | 7.8 | 6.0(a) |

[a] Based on molecular weight of 14,700.[11]

The results illustrate substantial agreement from three different laboratories, although the experimental details available indicate a considerable divergence in methods by which the figures were garnered. The fact that crystalline lysozyme may consist of a family of molecules[14,15] must be borne in mind in evaluating such data. The data available are inadequate to permit explanation of many of the divergences between laboratories or from integral values, as for arginine, isoleucine, leucine, serine, threonine and valine. The largest discrepancy, percentagewise, is with respect to phenylalanine, 2 residues being found in one of the three studies. Uncertainty was expressed, however, for the figure of 2.37,[16] a maximum of three residues per molecule being suggested. The work of Lewis, *et al.*,[13] and the present figures, indicate that three residues per molecule of lysozyme is probably the correct figure.

Results for determination of sequence for the first three residues are presented in Table III. Those interpreted as signifying positional values are in boldface.

The applicability of this quantitative method of

(12a) The term *aminoid* is used to obviate confusion with the original terminus (referred to in this paper as N-terminus). It has proved to be desirable to be able to distinguish terminologically the original terminus from subsequently released termini, in studies involving either stepwise removal or progressive fission as in proteolytic studies. In addition to N-terminal *vs.* C-terminal and aminoid *vs.* carboxoid, the designations *amino* acid *vs.* amino *acid* have been employed [S. W. Fox, *Adv. Prot. Chem.*, **2**, 158 (1945); F. Wessely, *et al.*, *Monatsh. f. Chem.*, **83**, 1439 (1952), and earlier papers]. As pointed out by F. Sanger, *Adv. Prot. Chem.*, **7**, 1 (1952), the last type of denotation is difficult in oral communication.

(13) J. C. Lewis, N. S. Snell, D. J. Hirschmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **186**, 23 (1950).

(14) H. H. Tallan and W. H. Stein, *J. Biol. Chem.*, **200**, 507 (1953).

(15) Incidental to the immediate point of this discussion, but fundamental to the larger concept of amino acid composition of and residue arrangement in proteins, the Tallan and Stein data on lysozyme provide one further instance in support of the theory of molecular evolution which rests upon diversity in synthesis followed by Darwinian selection (see S. W. Fox, *Am. Naturalist.*, **87**, 253 (1953)).

(16) C. Fromageot and P. de Garilhe, *Biochim. et Biophys. Acta*, **3**, 82 (1949).

sequence determination may be partly evaluated in the results of Table III. Each value in boldface figures represents approximately 1.0 residue less than the value in the preceding treatment, and indicates the sequence to be lysylvalylphenylalanyl-. This conclusion is in agreement with the sequential arrangement first announced by Schroeder.[10] The actual decrements for lysine (1.2), valine (1.3) and phenylalanine (1.0) do not differ significantly from 1.0 in view of the size of the molecule analyzed.[16a]

From the results of this study it can now be stated that the sequence is L-lysyl-L-valyl-L-phenylalanyl, since each of the assay organisms used for these amino acids is known to be stereospecific in its requirement.

Although not all of the amino acids are represented in the analyses reported in Table III, the data strongly support the concept that lysozyme contains a single peptide chain,[17] particularly since an integral decrement is found only once in each of three positions. If simplifying assumptions are made, the chance that one of the amino acids not assayed occupies any one of the three positions may be calculated as $(4/18)^3$ or 1 in 91.

TABLE III

QUANTITATIVE RESIDUE SEQUENCE OF LYSOZYME

| Amino acid | $AT_0$ | $AT_1$ | $AT_2$ | $AT_3$ |
|---|---|---|---|---|
| Alanine(*cx*) | 9.9 | 9.7 | 9.9 | |
| Arginine(*fq*) | 11 | 11 | 11 | |
| Glycine(*bt*) | 11.3 | 11.5 | 11.3 | 11.3 |
| Histidine(*mr*) | 0.9 | 0.9 | 0.8 | 0.9 |
| Isoleucine(*aq*) | 6.7 | 6.7 | 6.7 | 7.2 |
| Leucine(*aq*) | 6.9 | 6.9 | 6.6 | 6.6 |
| Lysine(*mq*) | 6.1 | **4.9** | 5.0 | |
| Methionine(*fp*) | 2.4 | 2.2 | 2.1 | 2.1 |
| Phenylalanine(*bt*) | 3.1 | 3.0 | 3.1 | **2.1** |
| Proline(*bt*) | 2.2 | 1.9 | 1.9 | 1.8 |
| Serine(*my*) | 9.4 | 9.5 | 9.5 | 9.4 |
| Threonine(*fz*) | 7.4 | 7.2 | 7.0 | 7.3 |
| Tyrosine(*bt*) | 2.8 | 2.9 | 2.8 | 3.0 |
| Valine(*fq*) | 5.5 | 5.3 | **4.0** | 3.8 |

Some assessment of the limitations of the method may be gained from the following considerations. Arginine, for example, is present in high proportion in lysozyme. Assuming a ±10% experimental error in determination, one might be unable to assign the number of residues in a single determination any more closely than 10–12. Fortunately, an experienced microbiological analyst usually obtains precision, and probably accuracy, within limits that are closer than ±10%, particularly if statistical analysis be employed. Critical factors include size of the molecule, the number of residues of each type per molecule, and the accuracy of the method. In this and other studies in this Laboratory, the limitations of the method are believed usually to be the limitations of microbiological assay.

The analyses which have received most study in this work are those which concerned integral decrements. It is also of interest, however, to compare calculated molecular weights of the lysozyme preparation from the stoichiometry incidentally afforded by Table III. The essential results are presented in Table IV, on the basis of histidine = 1 or phenylalanine = 3. The result is of course subject to the earlier expressed reservations with regard to purity and to accuracy of assay. The figures may be compared with others in the literature as well as with one another, following successive treatments. Halwer, Nutting and Brice reviewed values of 14,900, 14,700, 13,900, 14,700, 17,500, 16,600 and propose a value of 14,800.[18] In addition, Fraenkel-Conrat, *et al.*,[17] report a value of 14,400 by osmotic pressure.

TABLE IV

CALCULATED MOLECULAR WEIGHT OF LYSOZYME

| | Aminoid treatment | | | |
|---|---|---|---|---|
| | $AT_0$ | $AT_1$ | $AT_2$ | $AT_3$ |
| No. of calcns. used | 14 | 13 | 12 | 9 |
| Average mol. wt. | 14,200 | 14,300 | 14,700 | 14,600 |
| Mean deviation from mean | ±710 | ±600 | ±920 | ±630 |
| Grand average mol. wt. | | | 14,450 ± 700 | |

Tables III and IV reveal that neither the individual amino acid values nor calculated molecular weights are significantly altered by repeated PTC and dioxane–HCl treatments. This should particularly be noted for serine and threonine linkages.[19] The amount of PTC used was a twenty-fold excess of that needed to combine not only with the terminal α-amino group but also with reactive sidechains of lysine, arginine, histidine, serine, threonine and tyrosine. With the exception of the terminal lysine, the contents of each of these amino acids was not significantly altered by the repeated treatments.

Lack of difficulty with reactive sidechains and applicability of this general type of method to quantitative studies of structure and biological metamorphosis are thus again indicated.[7] Discernible advantages and disadvantages of subtractive methodology of this type have been discussed.[7] In addition to the points previously summarized,[7] it should be repeated that the present kind of method permits configurational designations. The L-isomer may be determined directly as indicated; D-residues may be identified by comparison of microbiological and chromatographic results.[6,20]

**Acknowledgment.**—The gift of lysozyme and pertinent data from Dr. Joseph F. Foster are gratefully acknowledged.

AMES, IOWA

(16a) Progressively smaller decrements of amino acids through four steps of an Edman degradation, followed by total amino acid assay after each treatment, have been reported by V. du Vigneaud, C. Ressler and S. Trippett in *J. Biol. Chem.*, **205**, 949 (1953). The details of reaction differed from those employed in the present study.

(17) H. Fraenkel-Conrat, A. Mohammed, E. D. Ducay and D. K. Mecham, THIS JOURNAL, **73**, 625 (1951).

(18) M. Halwer, G. C. Nutting and B. A. Brice, *ibid.*, **73**, 2786 (1951).

(19) D. F. Elliott, *Biochem. J.*, **50**, 542 (1952).

(20) Data which have accumulated in this Laboratory suggest that acid hydrolysis decomposes PTH's partially to products other than amino acids. This relationship appears to hold also for a C-terminal method involving acid hydrolysis of peptides containing a C-terminal hydantoin (see E. Ware, *Chem. Revs.*, **46**, 444 (1950)). This type of decomposition reaction would seriously hamper extractive techniques, but would not vitiate a subtractive method.