THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 244, No. 8, Issue of April 25, pp. 1982-1987, 1969
Printed in U.S.A.

The Lysozyme of Bacteriophage λ

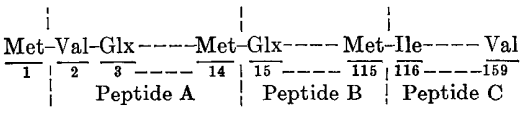
III. ORDERING THE CYANOGEN BROMIDE PEPTIDES*

(Received for publication, September 30, 1968)

LINDSAY W. BLACK‡ AND DAVID S. HOGNESS
From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

SUMMARY

The three cyanogen bromide peptides obtained from the single polypeptide chain of λ-lysozyme were isolated and ordered as the first step in a two-step program to determine the orientation of the structural gene for this enzyme. The results are summarized in the following diagram:



where the numbers indicate the residue position in λ-lysozyme.

In the preceding two papers of this series (1, 2), it was shown that λ-lysozyme consists of a single polypeptide chain of 159 residues bound by methionine at the amino terminus and by valine at the carboxyl terminus. The finding that there are only 2 internal methionine residues (2) suggests a solution to the problem of determining the orientation of *R*, the structural gene of λ-lysozyme located near the right end of the λ DNA (3). This solution is divided into two parts, the first of which is completed in this paper.

The problem of determining the orientation of a structural gene is that of determining the direction of its transcription, or its translation, or the direction from the codon for the amino-terminal residue to the codon for the carboxyl-terminal residue (the N to C direction), as these are all equivalent. Here we are interested in determining the N to C direction of R and, as the first step, wish to divide the polypeptide derived from this gene into two subpeptides or more which can be ordered relative to the complete polypeptide. Representing λ-lysozyme by

$$\underline{\text{Met}} - \underline{\text{aa}_i} \overset{\text{A}}{\text{----}} \underline{\text{Met}} - \underline{\text{aa}_j} \overset{\text{B}}{\text{----}} \underline{\text{Met}} - \underline{\text{aa}_k} \overset{\text{C}}{\text{----}} \underline{\text{Val}}$$

it is clear that reaction with cyanogen bromide (4) should yield homoserine,[1] and the cyanogen bromide Peptides A ($\underline{\text{aa}_i}$----Hser), B ($\underline{\text{aa}_j}$----Hser), and C ($\underline{\text{aa}_k}$----Val), where Hser represents homoserine. As the identification, and hence the ordering, of the three cyanogen bromide peptides should be a reasonably simple process, we chose them as the required set of ordered subpeptides.

The second, final part of the solution is, of course, the identification of which cyanogen bromide peptide contains the amino acid alteration caused by each of two mutations whose positions have been mapped on the λ DNA and which are sufficiently far apart so that they lie on opposite sides of at least one of the two internal methionine codons. In this article we present only the first part of the solution, *i.e.* the isolation and identification of the three cyanogen bromide peptides.

EXPERIMENTAL PROCEDURES

Materials

λ-Lysozyme was prepared according to the procedure described previously (1). Sephadex G-25 (coarse, bead form), G-50 (fine, 20 to 80 μ), and G-75 (40 to 120 μ) were from Pharmacia, and were suspended in 0.2 M acetic acid overnight and deaerated prior to use. *N*-Ethylmorpholine was redistilled before use, and redistilled pyridine, triethylamine, and anhydrous trifluoroacetic acid were gifts of G. R. Stark (Stanford University). The source of other chemicals used for electrophoresis in polyacrylamide gels and for end group and amino acid analysis has been described (1, 2).

Methods

End Group and Amino Acid Analysis—Amino-terminal residues were determined by the cyanate method of Stark and Smyth (5) as described for "small peptides." About 25 mμmoles of peptides were dissolved in 0.15 ml of the carbamylation solution (100 mg of KNCO, 0.6 ml of *N*-ethylmorpholine, 2 ml of H_2O) and incubated at 50° for 8 hours, and 0.16 ml of glacial acetic acid was added to end the reaction before removal of solvents by rotary evaporation. The Dowex 50-X2 column was used to

* This work was supported by grants from the National Institutes of Health and the National Science Foundation.

‡ Present address, Laboratoire de Biophysique, Universite de Genève, Geneva, Switzerland.

[1] In this paper, homoserine refers to the mixture of homoserine and homoserine lactone that results from the cyanogen bromide reaction or acid hydrolysis.

remove spurious glutamic acid end groups, and the amount of serotonin creatinine sulfate added was reduced to 2.5 mg because of the small amount of peptides analyzed.

The method for determining carboxyl-terminal residues by hydrazinolysis has been described (2), the procedure in which the benzaldehyde extraction is omitted being that used here.

Amino acid analyses were performed as described previously (2). The determination of homoserine in hydrolysates required a separate analysis on the medium column (57 cm) of the Beckman-Spinco analyzer at 30° (instead of 50°) to resolve it clearly from glutamic acid. Homoserine was assayed in the unhydrolyzed mixture after cyanogen bromide treatment by the use of the short column (12 cm) equilibrated and run with the pH 3.25 buffer. The decay of homoserine plus its lactone with time of acid hydrolysis was not measured, but was assumed to follow the kinetics described by Fruchter and Crestfield (6).

Edman Degradation—Most of the following modifications of the Edman degradation (7) were suggested by G. R. Stark. The dry λ-lysozyme (100 mμmoles) or Peptide A (46 mμmoles) was dissolved in 0.4 ml of H_2O, and then 0.9 ml of pyridine and 0.1 ml of triethylamine were added, in that order. After flushing the solution with N_2 (8), 0.05 ml of phenylisothiocyanate was added, and 1 hour later (room temperature) 1.0 ml of H_2O was added prior to extracting the solution five times with 5 ml of benzene. The final aqueous phase was evaporated to dryness, 1.0 ml of trifluoroacetic acid was added while flushing the tube with N_2 (8), and this solution was evaporated to dryness under reduced pressure over NaOH after 15 min at room temperature.

The Peptide A products were then dissolved in 50% acetic acid, but the λ-lysozyme product after the first round required prior solution in 0.3 ml of 99% formic acid before adding 3.0 ml of 50% acetic acid. These solutions were then applied to a column of Sephadex G-25 (2 × 20 cm) and 20-ml fractions were collected at a rate of one fraction per hour, eluting the column with 50% acetic acid and locating the position of peptide and the phenylthiohydantoin derivatives of the amino acids by their absorption at 265 mμ. Both products were evaporated to dryness.

To initiate the second round of degradation, the λ-lysozyme product had to be dissolved in less than 0.1 ml of 99% formic acid prior to adding, in order, 1 ml of H_2O, 2.8 ml of pyridine, 1.0 ml of triethylamine to bring the pH to 8.5, and finally 0.14 ml of phenylisothiocyanate. The remainder of the second round for λ-lysozyme and the entire second round for Peptide A followed the above procedure except that the polypeptides and phenylthiohydantoin acids were separated by ethylacetate extraction (9) rather than by Sephadex chromatography. The residue was dissolved in 5 ml of 1 M acetic acid and extracted four times with 15 ml of ethylacetate. The combined ethylacetate was extracted twice with 20 ml of H_2O, the water was added to the original aqueous phase, and both phases were then evaporated to dryness.

The phenylthiohydantoin acids were hydrolyzed in 0.1 M NaOH prior to analysis according to the procedure of Van Orden and Carpenter (10) except that the temperature of hydrolysis was 110° rather than 120°.

Assay of Peptides—The peptides were generally assayed by reaction with ninhydrin following alkaline hydrolysis (Figs. 1, 2, and 3). NaOH, 1.0 ml, 2.5 M, was rapidly mixed with samples of up to 3 ml in polypropylene tubes, which were then heated at 110° for 3 to 12 hours in a forced draft oven. Acetic acid, 2.0 ml, 30%, was added to the dry or nearly dry residues after cooling and, if necessary to ensure solution, the tubes were capped and heated for 10 min in a boiling water bath. The pH was checked to be roughly 5 with pH paper; 1.0 ml of ninhydrin reagent (11) was added and mixed rapidly. After heating the solution for 15 min in a boiling water bath, it was shaken vigorously to lower the background absorption through oxidation of unreacted ninhydrin. The absorbance at 570 mμ of the resulting solution was taken as the measure for the amount of peptide present.

RESULTS AND DISCUSSION

Isolation of Cyanogen Bromide Peptides

Reaction with Cyanogen Bromide—The reaction of λ-lysozyme with cyanogen bromide was carried out as described by Hofmann (12), with a 90- to 100-fold excess of cyanogen bromide (moles per mole) in 0.1 M HCl at 30°. The protein solution and the reagents (with the exception of the volatile cyanogen bromide) were thoroughly deaerated prior to starting the reaction in a tube which was immediately sealed. This deaeration procedure decreased the amount of unreacted methionine from 11% to 5% (reaction time, 30 hours).

Electrophoresis of 70 μg of peptides resulting from 20, 25, 30, or 42 hours of reaction with cyanogen bromide yielded band patterns which were equivalent. After removal of the reagents from the reaction mixture by rotary evaporation, electrophoresis in 15% small pore polyacrylamide gels at pH 4.5 (1) revealed only two bands, and no material in the position of λ-lysozyme. The yield of two rather than three bands is a puzzle to which we shall return; for present purposes, the above results indicate that the reaction was essentially complete by 20 hours.

This was also indicated by a barely detectable, probably insignificant change in the amount of unreacted methionine detected between 24 and 30 hours of reaction. After 30 hours, 2.72 residues of homoserine and 0.14 residue of unreacted methionine per molecule of λ-lysozyme were detected, accounting for 95% of the 3 methionine residues in the native enzyme. No significant change was observed in any of the other residue frequencies.

Separation of CNBr Peptides—The two peptide bands seen after electrophoresis at pH 4.5 in 15% polyacrylamide gels did not become three when the acrylamide concentration was varied between 8 and 20% or when the pH was changed to 3.8, 6.4, 8.5, and 10.5. Indeed, the best resolution of the two bands occurred under the original conditions described for the electrophoresis of λ-lysozyme in the first article of this series (1). We assume that the third peptide did not stain with Amido black sufficiently to be visible, as might be imagined were it small and therefore of quite limited mass and composition.

The three peptides were detected by chromatography on Sephadex G-50 columns in 0.2 M acetic acid as is shown in Fig. 1. A small peptide (A) is eluted from the column well after Peptides B and C which are only partially resolved by this procedure. The order of elution indicates that B is the largest and A the smallest of these peptides, which have been labeled, retrospectively, to fit the diagram given in the introduction. As can be seen from Fig. 1, Peptide A exhibits little, if any, absorbance at 280 mμ, indicating that it is deficient in tryptophan and tyrosine. The profile for the ninhydrin values in the region containing

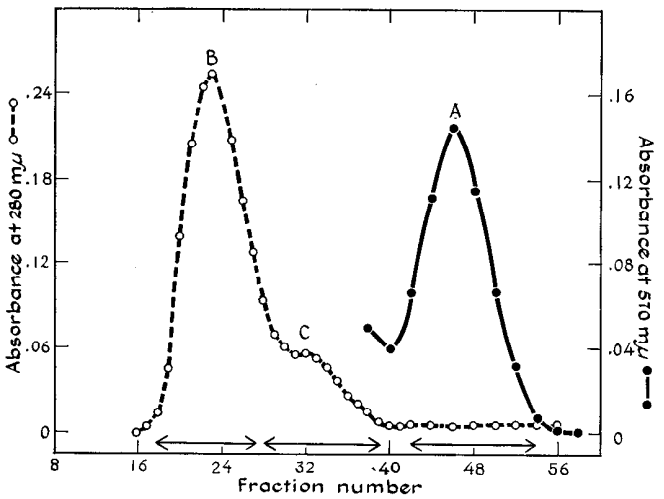


FIG. 1. Chromatography of the CNBr peptides on Sephadex G-50 columns. The peptide mixture (128 mμmoles of each peptide) dissolved in 0.2 M acetic acid was applied to a Sephadex G-50 column (0.9 × 159 cm) at 29° and eluted at a flow rate of 6.4 ml of this solvent per hour, 1.5-ml fractions being collected. Both the absorbance at 280 mμ and the amount of ninhydrin-reacting material present after alkaline hydrolysis ("Methods") were measured. The *double arrows* indicate the fractions pooled to yield Fractions A, B, and C of the respective peptides.

Peptides B and C parallels that for the absorbance at 280 mμ but is not shown for simplicity.

This chromatography formed the first step in the isolation of each peptide from 12.7 mg (710 mμmoles) of λ-lysozyme. After reaction for 30 hours with a 100-fold excess of cyanogen bromide in a final volume of 3.1 ml of 0.1 M HCl, the mixture was twice evaporated to dryness and the peptides were dissolved in 4 ml of 0.2 M acetic acid and applied to a Sephadex G-50 column as described in Fig. 1. Three fractions (A, B, and C), comparable to those indicated by the *arrows* in Fig. 1, were obtained.

Fraction A, after evaporation to dryness and dissolving in 3.5 ml of 0.2 M acetic acid, was applied to and eluted from a Sephadex G-25 column to remove any traces of Peptides B and C. The results and details of this step are given in Fig. 2. Fractions 11 and 12 were concentrated and used for subsequent analysis of Peptide A.

After concentrating the peptides in Fractions B and C as described above for Fraction A, they were subject to further chromotography on Sephadex G-75 and G-50 columns, respectively, as described in the legend of Fig. 3. In the case of Fraction B, the peptides were chromatographed twice under identical conditions, about 70% of the material eluted from the first column being applied to the second. The results of the second chromatography are shown in Fig. 3*b*, yielding an almost symmetrical peak from which Fractions 16 to 21 were combined and used for the analysis of Peptide B.

Since the pattern shown by Fraction C (Fig. 3*a*) indicates considerable amounts of Peptide B in the early fractions, only Fractions 17 to 19 in the terminal half of the peak were pooled for subsequent analysis of Peptide C.

The yields of Peptides A, B, and C were 65, 26, and 19%, although the yields of B and C could undoubtedly be increased by further chromatography of discarded fractions.

Homogeneity of Isolated Peptides—The molecules in the above preparation of Peptide C migrated as a single band during

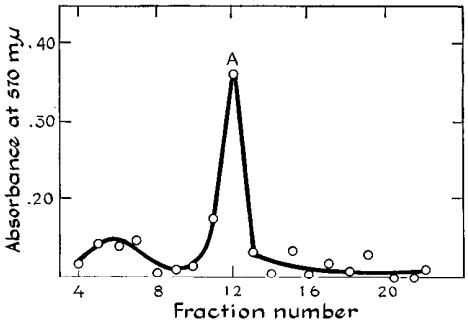


FIG. 2. The chromatography of Fraction A on Sephadex G-25 columns. Fraction A was dissolved in 3.5 ml of 0.2 M acetic acid, applied to a column of Sephadex G-25 (0.9 × 160 cm) at 25°, and eluted at a flow rate of 11 ml of this solvent per hour, 4-ml fractions being collected and analyzed with ninhydrin following alkaline hydrolysis ("Methods").

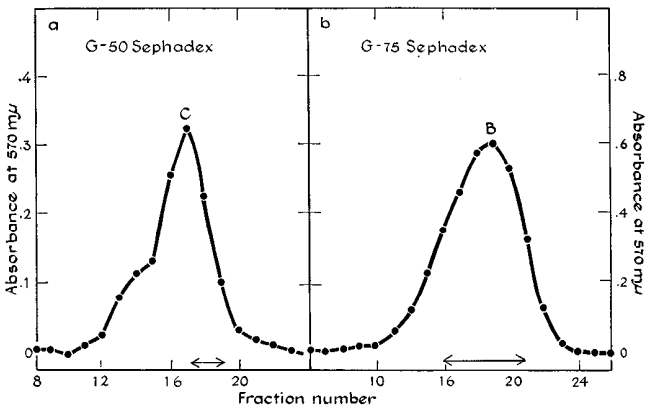


FIG. 3. Chromatography of Fractions B and C on Sephadex. Fractions C and B were eluted from columns of Sephadex G-50 (0.9 × 159 cm) and G-75 (0.9 × 103 cm) at 29° and 25°, respectively, with 0.2 M acetic acid. The flow rate and volume per fraction were 6.6 ml per hour and 3.3 ml for the Sephadex G-50 column and 6.9 ml per hour and 2.3 ml for the Sephadex G-75 column, these fractions being analyzed as described in Fig. 2. The *double arrows* indicate the fractions pooled to yield the purified preparations of Peptides B and C.

electrophoresis at pH 4.5 in the 15% polyacrylamide gel. The preparation of Peptide B yielded a nearly homogeneous pattern which, however, indicated a small amount of contaminating material leading the main band. Both Peptides B and C move faster than λ-lysozyme under these conditions, and occupy positions which indicate that they were the two peptides observed in the initial electrophoresis of the unfractionated cyanogen bromide reaction mixture. Peptide A is therefore the one which was not detected. The purified preparation of Peptide A was not further examined by gel electrophoresis because the fractionation given in Figs. 1 and 2 indicates it is free of B and C and because its amino acid composition is consistent with a single sequence (Table I).

Amino Acid Composition and Size of Peptides

The best fit of the number of residues per molecule to the amino acid frequencies was determined for each peptide by the method of Nyman and Lindskog (13) modified by us as described in the preceding paper (2). The results are shown in Fig. 4 which is identical with the plot given previously for λ-lysozyme

(Fig. 1 of Reference 2) except that the number of residues per molecule rather than molecular weight is given on the abscissa. The minimum, or best fit, for Peptide A is at 12.7 residues per molecule. The resulting values for the number of residues per amino acid in this peptide are given in Table I.

Similarly, the best fit for Peptide C occurs at 44 residues per molecule and its consequent composition is also given in Table I. The two smallest of the three peptides account for 57 of the 159 residues in λ-lysozyme, which, if we subtract the homoserine that should be formed from the amino-terminal methionine, leaves 100 or 101 residues expected per molecule of Peptide B depending on the location of the single cysteine residue which was not determined for any of the three peptides.

The fit of residue numbers to the amino acid frequencies found for Peptide B is anomalous since there are two essentially equivalent minima at 98 and 106 residues per molecule. We suppose that the peculiar behavior is due to contaminants in the preparation, creating two minima from one broad minimum by preferentially increasing the values of f at the true minimum. We have not analyzed the supposition further, but there being no reasons inherent to the amino acid analyses to choose one minimum over the other we take the mean of the two values, or 102 residues per molecule, to calculate the number of residues per amino acid given in Table II.

TABLE I

Amino acid composition of CNBr peptides

Amino acid[a]	CNBr peptides			ΣA, B, C[e]	λ-Lysozyme (2)
	A[b]	B[c]	C[d]		
	residues/molecule				
Cysteine				(1)	1
Histidine	0	2.0	0.93	3	3
Methionine + homoserine	1.01	0.93	0	2	3
Homoserine	1.01	0.93	0	2	0
Methionine	0	0	0	0	3
Tryptophan	0	2.1	1.06	3	3
Tyrosine	0	3.7	1.00	5	5
Phenylalanine	0.88	2.9	1.76	6	5
Proline	0	3.8	1.20	5	5
Threonine	0	4.6	1.11	6	6
Valine	0.94	4.3	1.92	7	7
Isoleucine	0.99	5.2	4.2	10	9
Serine	0	7.0	2.7	10	10
Lysine	1.06	7.7	2.9	12	12
Arginine	1.02	7.7	3.1	12	12
Alanine	1.04	8.3	4.8	14	13
Leucine	0.98	9.6	2.4	13	14
Glycine	0	10.3	5.0	15	15
Glutamic acid	2.05	10.4	4.7	17	17
Aspartic acid	2.72	11.6	5.2	20	19
Σ				161	159

[a] The amino acids are listed in the order of their frequencies in λ-lysozyme.

[b] Single amino acid analysis after 27 hours of acid hydrolysis.

[c] These values are the average of three analyses after 22, 37.5, and 48 hours of hydrolysis, except that (*a*) serine, threonine, and tyrosine values were extrapolated to zero time, (*b*) only the 37.5 and 48 hours of hydrolysis were used for valine, isoleucine, and leucine, and (*c*) homoserine and tryptophan values result from single determinations.

[d] These values are the average of three analyses after 20, 22, and 48 hours of hydrolysis with exceptions comparable to those given in Footnote *c* above.

[e] This sum is of the integers closest to the values given under A, B, and C.

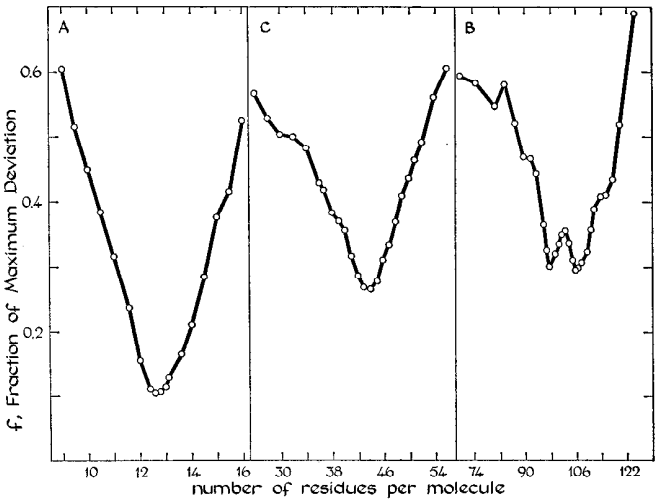


FIG. 4. The fit of number of residues per molecule to the amino acid frequencies of Peptides A, B, and C. See Fig. 1 of the preceding article (2) for the definition of and the procedure of computing f, the fraction of maximum deviation. Minimum values of f indicate the best fit.

The sum of the residues in Peptides A, B, and C for each amino acid is in good agreement with the corresponding number in λ-lysozyme (Table I), except for methionine + homoserine and for phenylalanine. The fact that there is one less homoserine among the peptides than there are methionines in the enzyme is expected since the conversion of the amino-terminal methionine should yield free homoserine which would be lost during purification of the peptides. We have no explanation for the higher value of phenylalanine among the peptides although the smaller discrepancies observed for isoleucine, alanine, and aspartic acid may be due to contaminants in Peptides B and C (see next section).

Ordering CNBr Peptides

Peptide C—Peptide C must be the carboxyl-terminal peptide of the set since it contains no homoserine (Table I). Its carboxyl-terminal residue should therefore be the same as that for λ-lysozyme, namely valine. Analysis of Peptide C by hydrazinolysis ("Methods") yielded 0.60 residue of valine per peptide molecule. This is to be compared with the 0.78 valine residue per molecule obtained from λ-lysozyme under the identical conditions (2). No other amino acid was formed in amounts approaching unity for the ratio of residues per molecule, although 0.25 residue of glycine per molecule of Peptide C was observed and some other amino acids were present at the level of 0.1 residue per molecule.

Analysis of the amino-terminal residue of Peptide C was carried out by the cyanate method of Stark and Smyth (5; see also "Methods"). Isoleucine was recovered in amounts equivalent to 0.63 residue per peptide molecule, no other amino acids being recovered in amounts approaching a unit ratio. However,

contaminating glycine was again formed at the level of 0.24 residue per molecule and, in addition, glutamate and aspartate were found at the appreciable levels of 0.16 and 0.27 residue per molecule. All other amino acids were recovered in only trace amounts.

Peptide A—Two rounds of Edman degradation ("Methods") of λ-lysozyme and amino acid analysis of the remaining polypeptide revealed the loss of 1 residue of methionine and 1 residue of valine (Table II). Since methionine has been identified as the amino-terminal residue to the enzyme (2), valine must be in the adjacent position. Analysis of the phenylthiohydantoin derivatives of the amino acids obtained after the second round of degradation yielded, upon regeneration of the free amino acids, 0.41 mole of valine per mole of polypeptide, supporting the identification of valine at position 2. Glycine was found in comparable amounts (0.39 mole per mole) but exhibited no significant decrease in the polypeptide (Table I). Other amino acids either were not present or were found in considerably smaller amounts (0.07, 0.10, and 0.16 mole per mole of glutamate, aspartate, and alanine, respectively).

The amino-terminal residue of either Peptide A or B should be valine, depending upon which is closer to the amino terminus in λ-lysozyme. Two rounds of Edman degradation of Peptide A (Table III) reveal that the sequence at its amino terminus is Val–Glx–, identifying it as the most amino-terminal peptide of the set since Peptide B contains no amino-terminal valine (see next section).

TABLE II

Amino acid composition of λ-lysozyme after two rounds of Edman degradation

Amino acid[a]	Residues per molecule	
	λ-Lysozyme	λ-Lysozyme after two rounds of degradation[b]
Cysteine	1	Not determined
Histidine	3	3.3
Methionine	3	1.9
Tryptophan	3	Not determined
Tyrosine	5	5.2
Phenylalanine	5	6.0
Proline	5	5.9
Threonine	6	6.2
Valine	7	6.0
Isoleucine	9	8.5
Serine	10	9.7
Lysine	12	11.5
Arginine	12	11.7
Alanine	13	13.2
Leucine	14	13.6
Glycine	15	14.7
Glutamic acid	17	17.2
Aspartic acid	19	18.8

[a] The amino acids are listed in order of their frequencies in λ-lysozyme.

[b] Calculated from the residue frequencies observed after 48 hours of acid hydrolysis assuming that the polypeptide contains 157 residues of which 1 is cysteine and 3 are tryptophan. Threonine, serine, and tyrosine were corrected according to the rates of disappearance with time of hydrolysis observed for the untreated enzyme (2). The increased values for phenylalanine and proline are not explained.

TABLE III

Amino acid composition of Peptide A after one and two rounds of Edman degradation

Amino acid[a]	Residues per molecule of peptide		
	Untreated	After the first round[b]	After the second round[b]
Homoserine	1	Not determined	1.22
Phenylalanine	1	1.02	1.00
Valine	1	0.40	0.19
Isoleucine	1	1.00	0.98
Lysine	1	0.43	0.33
Arginine	1	0.99	0.94
Alanine	1	1.15	1.07
Leucine	1	1.00	1.03
Glutamic acid	2	1.95	1.58
Aspartic acid	3	2.98	3.11

[a] The amino acids are listed in order of their frequencies in λ-lysozyme.

[b] Calculated from the residue frequencies after 44 hours (first round) or 36 hours (second round) of acid hydrolysis assuming that the sum of the isoleucine and leucine values is 2.

The fact that only 0.6 residue of valine was released after the first round of degradation is consistent with the subsequent loss of only 0.4 residue of glutamic acid in the second round, but it is surprising that the removal of the amino-terminal valine is so incomplete.[2] The loss of lysine in this Edman degradation ("Methods") is expected since reaction at the ε-amino group is extensive and free lysine is incompletely regenerated upon acid hydrolysis.

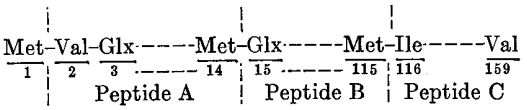
Peptide B—The only criteria necessary for the identification of Peptide B as the central peptide is the demonstration that it does not have valine at its amino terminus. Assay of residues with free α-amino groups in the Peptide B preparation by the cyanate method used previously yielded per molecule only 0.11 residue of valine as compared to 0.76 residue of glutamic acid, which appears to be the amino-terminal residue of this peptide, either as such or as glutamine. As with the end group analysis of Peptide C, contaminants (other than valine) were apparent in these analyses which use quite small amounts of peptides (25 mμmoles); glycine was detected at 0.2 residue per molecule of Peptide B, while aspartic acid, alanine, isoleucine, and leucine

[2] The low yield of valine may reflect a heterogeneity at the amino terminus of the molecules which results from aberrant reaction and cleavage of the amino-terminal methionine of λ-lysozyme during the cyanogen bromide treatment. Such an aberrance is suggested by the observation that free homoserine was recovered in very low yields (about 0.3 residue per enzyme molecule) when assayed after cyanogen bromide treatment and prior to hydrolysis of the peptides. While the observation of only 1 homoserine residue per molecule of Peptide A (Table I) indicates that no uncleaved homoserine residues remain at its amino terminus, the yield of 2.7 residues of homoserine from all 3 methionine residues in λ-lysozyme allows the supposition that about one-third of the amino-terminal methionines act aberrantly to produce an equivalent fraction of Peptide A molecules with masked valines.

were each present at 0.3 residue per molecule. We think that these contaminants arise from amino acids acquired during the extensive manipulations required for these determinations. Although they are deplorable and reduce the end group assays for the small amount of peptides that we used to the semiquantitative level, they do not appear large enough to alter the qualitative conclusions as to the terminal residues.

CONCLUDING REMARKS

The information that we have obtained in this and the preceding article (2) on the primary structure of λ-lysozyme can be summarized by the following diagram:



where the numbers represent the positions in the polypeptide chain.

We consider the determination of the number of residues in Peptide B (Fig. 4) to be less accurate than that for Peptides A and C and for λ-lysozyme itself (2). Hence we take the difference between the 159 residues in the enzyme and the sum of the 13 residues in A, and 44 residues in B, and the amino-terminal methionine, or 101 as the number of residues in B, assuming thereby that it contains the lone cysteine residue. Clearly, the residue positions in the diagram which are in the hundreds may by wrong by one or two.

With the isolation and ordering of the three cyanogen bromide peptides completed, the last part of the solution to the problem of determining the orientation of the *R* gene appears clear. J. Champoux, in our laboratory, has determined the position on the genetic map of vegetative λ of several different amber mutants of *R* by 2- and 3-factor crosses. Their spatial distribution appears to be sufficiently wide to span one of the two internal methionines. Hence the remainder of the problem consists in isolating the λ-lysozyme synthesized by the two most widely spaced of these nonsense mutants in an *E. coli* host cell which contains a strong amber suppressor such as *Su-1*$^+$ or *Su-3*$^+$ (14), and determining which cyanogen bromide peptide contains the requisite amino acid alteration. Alternatively, temperature-sensitive mutants of *R* (15) can be used. One of us (L. W. B.) is presently concerned with this final part of the problem.

Acknowledgments—We want to thank George R. Stark for his many valuable suggestions and instructions concerning the chemical analyses which constitute the greater part of this and the preceding article (2).

REFERENCES

1. Black, L. W., and Hogness, D. S., *J. Biol. Chem.*, **244,** 1968 (1969).
2. Black, L. W., and Hogness, D. S., *J. Biol. Chem.*, **244,** 1976 (1969).
3. Hogness, D. S., Doerfler, W., Egan, J. B., and Black, L. W., *Cold Spring Harbor Symp. Quant. Biol.*, **31,** 129 (1966).
4. Witkop, B., *Advan. Protein Chem.*, **16,** 268 (1961).
5. Stark, G. R., and Smyth, D. G., *J. Biol. Chem.*, **238,** 214 (1963).
6. Fruchter, R. G., and Crestfield, A. M., *J. Biol. Chem.*, **240,** 3875 (1965).
7. Edman, P., *Acta Chem. Scand.*, **4,** 277, 283 (1950).
8. Ilse, D., and Edman, P., *Aust. J. Chem.*, **16,** 411 (1963).
9. Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L., *Methods Biochem. Anal.*, **2,** 358 (1955).
10. Van Orden, H. O., and Carpenter, F. H., *Biochem. Biophys. Res. Commun.*, **14,** 399 (1964).
11. Moore, S., and Stein, W. H., *J. Biol. Chem.*, **211,** 907 (1954).
12. Hofmann, T., *Biochemistry*, **3,** 356 (1964).
13. Nyman, P.-O., and Lindskog, S., *Biochim. Biophys. Acta*, **85,** 141 (1964).
14. Weigert, M. G., Gallucci, E., Lanka, E., and Garen, A., *Cold Spring Harbor Symp. Quant. Biol.*, **31,** 145 (1966); Stretton, A. O. W., Kaplan, S., and Brenner, S., *Cold Spring Harbor Symp. Quant. Biol.*, **31,** 173 (1966).
15. Harris, A. W., Mount, D. W. A., Fuerst, C. R., and Siminovitch, L., *Virology*, **32,** 553 (1967).