Amino Acid Sequence Studies on the Tryptic Peptides of the Coat Protein of the Bacteriophage R17*

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ABSTRACT: The polypeptide chain of the bacteriophage R17 coat protein contains 129 amino acids. Tryptic digestion of the coat protein produced ten peptides and free lysine. Soluble peptides were separated on columns of Dowex 50-X4 and insoluble peptides were resolved by gel filtration in formic acid. The sum of the amino acids in the tryptic peptides accounted for all the residues in the protein. Amino acid sequences within the tryptic peptides were determined and compared with the amino acid sequence of the coat proteins from other ribonucleic acid phages. The peptides were found to be identical with the corresponding peptides of bacteriophage f_2 coat protein, except for position 88 (methionine in R17 coat protein, leucine in f_2 coat protein). This difference agrees with the amino acid analyses of the two coat proteins.

Since Loeb and Zinder (1961) described the small *Escherichia coli* bacteriophage f_2 containing only ribonucleic acid and a protein coat, several similar phages have been found, which are serologically related (Scott, 1965). Bacteriophage R17 isolated by Paranchych and Graham (1962) is one of these strains.

In a continuing study of coat proteins of these small RNA bacteriophages, the protein encapsulating R17 was examined. There were two principal reasons for selecting this strain for amino acid sequence analysis. One reason was that the serological data showed that the bacteriophage R17 was closely related but different from f_2 bacteriophage. It was therefore of interest to compare the amino acid sequences of these two coat proteins. The second reason was to provide the structure of the wild-type molecule so that amino acid substitutions in suppressed amber mutants could be localized (Tooze and Weber, 1967). A characterization of bacteriophage R17 coat protein by amino acid composition and by fingerprint studies has already been reported by Enger and Kaesberg (1965).

During this investigation an octapeptide was isolated that had the same composition but a different amino acid sequence from that of the corresponding peptide obtained from f_2 coat protein. A reinvestigation of this region from f_2 bacteriophage coat protein revealed an error that had been made in the amino acid sequence previously proposed for this protein (Weber *et al.*, 1966).

The structure shown in Figure 1 represents the amino acid sequence proposed for the R17 coat protein, which differs from that of f_2 bacteriophage coat protein (Weber and Konigsberg, 1967) by a single amino acid substitution of methionine for leucine at position 88.

Experimental Procedures

Materials. The R17 phage was prepared by the procedure of Gesteland and Boetker (1964). Iodoacetic acid was obtained from Mann Research Laboratories and ethylenimine was obtained from K & K Laboratories, Inc. Guanidine hydrochloride was prepared by the procedure of Anson (1941).

Trypsin (B grade, TPCK[1] treated, lot 53955) was obtained from Calbiochem. Chymotrypsin (twice recrystallized), pepsin (twice recrystallized), and leucine aminopeptidase (DFP treated) were obtained from Worthington. Carboxypeptidase A and carboxypeptidase B, both diisopropylphosphorofluoridate (DFP) treated, were purchased from Sigma. Crystalline "subtilisin" (*Bacillus subtilis*) was obtained from Mann Research Laboratories. Streptococcal proteinase was a gift from Drs. W. H. Stein and S. Moore. Bio-Gel P2 −400 mesh and Dowex 50-X4 (AG 50W-X4 20–35 μ) were obtained from Bio-Rad. All chemicals used were reagent grade. The pyridine was redistilled prior to use.

Phage Protein. The phage solution (5–10 mg/ml), buffered at pH 7, was made 2 M in KCl and kept for 5–10 min in a boiling water bath. The suspension was cooled immediately to 0° and centrifuged. The precipitate was washed twice with ice-cold 0.2 M KCl and then dissolved in 8 M guanidine hydrochloride containing 0.2 M Tris-HCl (pH 8.0). β-Mercaptoethanol (0.25 ml for about 100 mg of protein dissolved in 20

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[1] Abbreviations used: TPCK, L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone; LAP, leucine aminopeptidase; CPA, carboxypeptidase A; CPB, carboxypeptidase B; AECys, β-aminoethylcysteine; tryptic, chymotryptic, and peptic peptides are marked T, C, and P. Peptides derived from streptococcal proteinase or subtilisin digests are marked Sp and Sub.

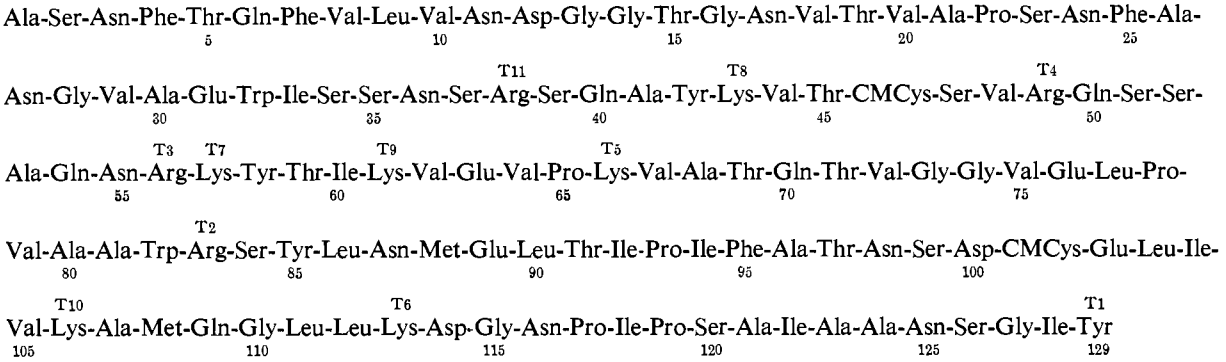

FIGURE 1: Proposed amino acid sequence of the coat protein of bacteriophage R17. The residue position is given above the line. The arrangement of the tryptic peptides was assumed to be the same as that found in f_2 coat protein (Konigsberg, 1966; see Discussion).

ml) was added and the solution was kept for 3–4 hr at 38°. Alkylation using a solution of sodium iodoacetate was carried out for 15 min at room temperature with the addition of enough 0.2 M NaOH to maintain a pH of 8 during the reaction. The protein solution was dialyzed against distilled water in the cold until it was salt free, resulting in a partial precipitation of the carboxymethylated protein. After dialysis the protein was lyophilized. Alkylation of the reduced protein was also carried out with ethylenimine. Soluble protein was prepared by the acetic acid method of Fraenkel-Conrat (1957).

Tryptic Digestion. The alkylated protein was suspended in cold aqueous ammonium hydroxide at pH 10.5 and sonically treated for several intervals of 30 sec to give a very fine colloidal suspension. The pH was then adjusted to 8.5 by the addition of powdered solid CO_2. The freshly prepared trypsin solution was added. A protein concentration of 5–10 mg/ml and an enzyme concentration of 0.01% was used. Digestion was carried out at 37° for 4 hr with the addition of an equal portion of enzyme after 2 hr. The digest was lyophilized until it was salt free and suspended in 0.2 M pyridine–acetate buffer (pH 3.1, 0.2 M in pyridine). The insoluble material was washed twice and the washings were combined, lyophilized, and dissolved in 2 ml of the pH 3.1 buffer. The peptides were then chromatographed on AG 50X-4.

Chymotrypsin digestion of the peptides T10 and T11 was carried out in NH_4HCO_3 solution in the same way as the tryptic digestion (25°, pH 7.5).

Pepsin digestion was performed in 10% formic acid using a peptide to enzyme ratio of 50 and a peptide concentration in the range of 0.05–0.5% at room temperature for 4 hr.

"Subtilisin" digestion was performed in 0.1 M NH_4HCO_3 for 6–12 hr at room temperature. The enzyme to substrate ratio was 1:50 and a peptide concentration of 0.2% was used.

Digestion with Streptococcal Proteinase. Activation of the enzyme was achieved by the procedure of Gerwin *et al.* (1966) Enzyme (4 μg) was used in 1.6 ml of solution with 1.5 mg of peptide T11C3. Digestion was performed at 35° for 150 min at pH 7.4 in 0.1 M NH_4HCO_3 solution containing 2×10^{-2} M β-mercaptoethanol.

Digestion with Leucine Aminopeptidase (LAP), Carboxypeptidases A and B (CPA and CPB), and Acid. LAP digestion was carried out at 40° in Tris buffer (pH 8.5) in the presence of 0.01 M $MgCl_2$. Extensive digestions were achieved after 4 hr. For amino-terminal sequence studies, digestion periods between 2 min and 3 hr were used. The results are expressed in number of residues per molecule.

CPA and CPB were used as reported by Guidotti *et al.* (1962). The results are expressed in number of residues per molecule. Partial acid hydrolysis was performed by the procedure of Tsung and Fraenkel-Conrat (1965) in 0.03 M HCl for 12 hr at 110°.

Hydrazinolysis was performed by the procedure of Akabori *et al.* (1952).

AG 50-X4 Chromatography. A 0.9 × 150 cm column of AG 50-X4 (20–35 μ) was used. The peptides were eluted with a linear gradient of increasing pH and ionic strength. The temperature was 60° and the column was eluted with a flow rate of 30 ml/hr. Fractions (2 ml) were taken. All further details have already been reported by Konigsberg *et al.* (1966). AG 50-X4 was also used for the separation of other peptides at 40°. The gradients are: (gradient I) 400 ml of pH 3.1 pyridine–acetate buffer (0.2 M in pyridine) and 400 ml of pH 5.6 pyridine–acetate (2.0 M in pyridine); (gradient II) 250 ml of 0.05 M pyridine–acetate buffer (pH 3.1); 250 ml of 0.2 M pyridine–acetate buffer (pH 3.1); and 250 ml of 2.0 M pyridine–acetate buffer (pH 5.6); and (gradient III) 250 ml of 0.1 M pyridine adjusted to pH 2.7 with formic acid and 250 ml of 0.5 M pyridine–acetate buffer (pH 5.6).

Gel filtration of the pH 3.1 insoluble peptides T10 and T11 was performed on Sephadex G-50 in 88% formic acid (Konigsberg *et al.*, 1966). Gel filtration on Bio-Gel P2 (−400 mesh) was carried out on a column 1.2 × 280 cm at a flow rate of 30 ml/hr in 0.2 M acetic acid.

TABLE I: Amino Acid Composition of R17 Coat Protein (expressed as mole ratios based on aspartic acid 14).[a]

Lysine	5.95
Arginine	3.90
Aspartic acid	14.00
Threonine	8.70
Serine	12.60
Glutamic acid	10.85
Proline	6.15
Half-cystine	1.90
Glycine	9.10
Alanine	14.20
Valine	13.70
Methionine	1.75
Isoleucine	7.80
Leucine	6.95
Tyrosine	3.70
Phenylalanine	3.90
Tryptophan	1.90

[a] The values are averaged from six different 22-hr hydrolysates on bacteriophage R17 coat protein. In the case of the amino acids valine and isoleucine the values were obtained from three different 72-hr hydrolysates. For serine and threonine the values reported were obtained after linear extrapolation of the number of residues calculated after 22 and 72 hr of hydrolysis. The number of micromoles was converted to residues per molecule and normalized to 14 aspartic acid residues. Half-cystine was determined as cysteic acid in oxidized protein (two 22-hr hydrolysates) and *S*-carboxymethylcysteine in carboxymethylated protein (two 22-hr hydrolysates) and the values were averaged. Tryptophan was determined by the method of Goodwin and Morton (1946).

Cyanogen Bromide Cleavage of T10. T10 was dissolved in anhydrous trifluoroacetic acid and incubated with a 50-fold molar excess of CNBr for 12 hr at room temperature. The acids were evaporated and the dry material was extracted with 0.2 M acetic acid. The suspension was centrifuged and the soluble part was purified by gel filtration on Bio-Gel P2.

Amino acid analyses were performed according to Konigsberg *et al.* (1966). End-group analyses were carried out as described by Konigsberg *et al.* (1966). The result of the subtractive Edman method was considered significant if only one amino acid decreased in the first step by at least 0.8 of a residue, in the second step by at least 0.75 residue, and in the third step by at least 0.6 residue.

Results

The amino acid compositions of R17 coat protein and the carboxymethylated derivative were determined according to Spackman *et al.* (1958). The results are given in Table I, expressed as mole ratios based on a value of 14 for aspartic acid.

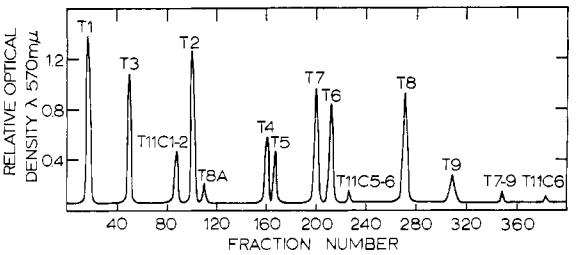

FIGURE 2: Chromatography of the tryptic peptides soluble in pyridine–acetate buffer (pH 3.1) on AG 50-X4 column (0.9 × 150 cm) at 60°. Fraction size, 2 ml. The solid line represents the pattern obtained by reaction of 10% of the effluent with ninhydrin after alkaline hydrolysis. The numbers for each peak represent peptides which are identified in Table II or in the text.

The protein contains no histidine. The values for isoleucine and valine after 22 hr of acid hydrolysis were about 0.5 residue lower than the next higher integer, 8 and 14, respectively. A 72-hour hydrolysate gave a fairly good approximation for these amino acids, 7.8 and 13.7, respectively. Since the two half-cystine residues can be reacted with iodoacetic acid in 8 M guanidine hydrochloride without prior incubation with mercaptoethanol the presence of two free SH groups was indicated. These data indicated a value of 129 amino acids per polypeptide chain, assuming 14 residues of aspartic acid.

End-Group Analyses on R17 Protein. CPA released about 0.9 residue of tyrosine assuming a molecular weight of 14,000 for the protein. Kinetic studies on the liberation of amino acids by CPA showed that isoleucine must be the penultimate residue in the chain (0.6 residue of isoleucine for 0.9 residue of tyrosine).

The amino-terminal residue of the polypeptide chain was already known to be alanine (G. Guidotti, unpublished data). About 0.9 residue of alanine was found by the procedure of Stark and Smyth (1963) after treating the protein with guanidine hydrochloride at 65° for 30 min.

Isolation of the Tryptic Peptides. The elution pattern for the pH 3.1 soluble peptides eluted from AG 50W-X4 at 60° using gradient I is shown in Figure 2. Table II shows the composition of the peptides T1–T9.

The peptides T2, T6, T8, T9, and T7–9 had less than 5% impurities. Peptides T4 and T5 were not always well separated. In a tryptic digest of aminoethylated coat protein peptide T4 is absent but two new peptides are found instead: T4A, eluted between T7 and T6; and T4B, eluted between T7–9 and T11C6. Trypsin splits T4 at the β-aminoethylated cysteine residue giving T4A and T4B in yields comparable to those of the other soluble tryptic peptides. T1 was eluted at column volume and required repurification using gradient III.

T2–T9 contained either lysine or arginine at the

TABLE II: Amino Acid Composition of the Tryptic Peptides from R17 Coat Protein.

Amino Acid	T1	T2	T3	T4	T4A	T4B	T5	T6	T7	T7–9	T8	T9	T10	T11	Total[a]
Lysine		0.05	0.05				1.05	1.05	1.00	1.95	1.03	1.05	1.06	0.10	6
Arginine		0.95	0.95	0.98		1.04							0.05	0.90	4
Aspartic acid	2.95	0.05	1.05		0.05								3.15	7.15	14
Threonine	0.05	1.90		0.95	0.98					1.03		0.95	2.03	3.10	9
Serine	1.87	0.05	1.90	1.00	0.05	0.97	0.05	0.05			0.98		1.90	5.15	13
Glutamic acid		2.00	2.00				1.00	1.00			1.00		2.05	1.90	11
Proline	2.00	0.95					1.03						1.05	1.10	6
Half-cystine[b]				0.85	0.98								0.85		2
Glycine	2.10	1.98	0.10				0.05	1.04					0.20	4.00	9
Alanine	3.00	3.05	1.05					1.00			1.02		1.15	4.05	14
Valine		3.90		2.05	1.00	1.03	1.95						0.80	4.80	14
Methionine								0.90					0.85		2
Isoleucine	2.94									0.95		1.00	2.80	0.95	8
Leucine		1.05						1.95					3.00	0.95	7
Tyrosine	0.80									0.94	0.95	0.95	0.90		4
Phenylalanine													0.95	2.94	4
Tryptophan		0.90												0.95	2
No. of residues	16	17	7	6	3	3	5	7	1	5	5	4	23	38	129

[a] The sum of each amino acid residue, rounded off to the next integer, in the tryptic peptides T1 to T11. The split peptides T4A and T4B and the overlapping peptide T7–9 are not included in this summation. [b] Determined as carboxymethylcysteine in peptides T4 and T10. Determined as β-aminoethylcysteine in peptide T4A.

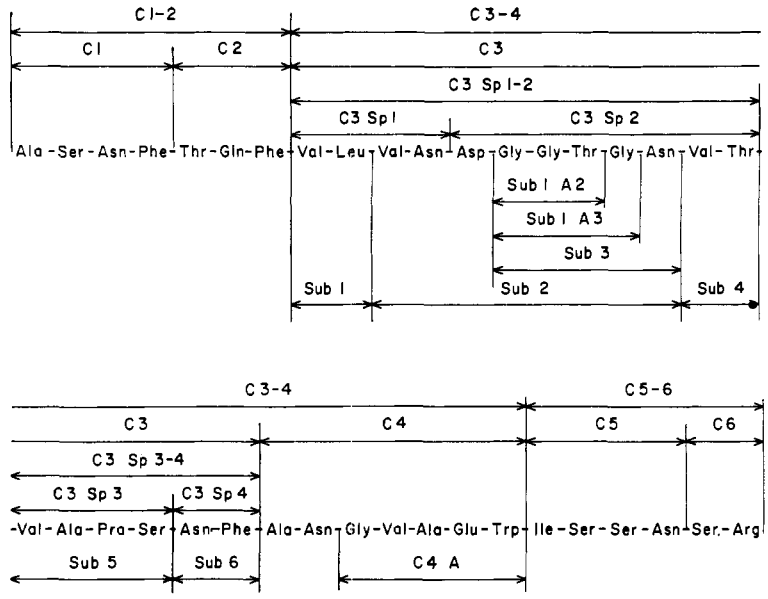

FIGURE 3: Fragmentation of the tryptic peptide T11 by chymotrypsin (peptides marked C) and further degradation of the chymotryptic peptide C3 by streptococcal proteinase (Sp) and "subtilisin" (Sub). The peptides marked A are derived from partial acid hydrolysis.

carboxyl terminus. Since T1 did not contain a basic amino acid and since it had the same carboxyl terminus as the intact protein, it was considered to be the carboxyl-terminal peptide of the coat protein. T7–9 was a peptide overlapping T7 and T9. In addition to these tryptic peptides, which were isolated in yields between 70 and 90%, the following peptides were also found which are not included in Table II: T11 Cl-2 (*Asx, Thr, Ser, Glx, Ala, Phe*$_2$), T8A (*Ser, Glx, Ala, Tyr*), T11C5-6 (*Asx, Ser*$_3$, *Ile, Arg*), and T11C6 (*Ser, Arg*).

The yields of these peptides are much lower compared to the yields of T1–T9 and varied in different digests. Even in very short tryptic digests T11C6 was found. In subsequent studies it was found that these peptides (except for T8A) are derived from T11. T8A is derived from T8. Since the elution pattern is not influenced by these peptides, no effort was made to minimize these chymotryptic-like splits. Shorter digestion time and lower temperatures resulted in poor yields of the tryptic peptides. Under limiting conditions of digestion, such as these, up to 50% of the coat protein remained undigested and precipitated together with the insoluble peptides, T10 and T11.

For the separation of T10 and T11 the procedure of Konigsberg *et al.* (1966) was used (gel filtration on Sephadex G-50 in 88% formic acid). The amino acid analyses of T10 and T11 are also included in Table II.

Amino Acid Sequence in the Tryptic Peptides. To simplify the discussion of the amino acid sequences in the tryptic peptides, the proposed amino acid sequence for the coat protein is given in Figure 1, assuming that the arrangement of the tryptic peptides is identical with that in the f_2 bacteriophage coat protein (Konigsberg, 1966; see also Discussion).

T11 (residues 1–38): Ala-Ser-Asn-Phe-Thr-Gln-Phe-Val-Leu-Val-Asn-Asp-Gly-Gly-Thr-Gly-Asn-Val-Thr-Val-Ala-Pro-Ser-Asn-Phe-Ala-Asn-Gly-Val-Ala-Glu-Trp-Ile-Ser-Ser-Asn-Ser-Arg. Alanine is the amino-terminal residue (DNP method). The carboxyl end was found by CPB and CPA treatment to be "Ser$_2$"-Arg ("Ser," amino acids eluted in serine position) since 1.8 residues of "Ser" were found/1.0 residue of arginine. Chymotryptic digestion of T11 and of both T10 and T11 yielded smaller fragments of T11, which were separated on AG 50-X4 using gradient II. The amino acid sequence in T11 is given in Figure 3.

T11C1: Ala-Ser-Asn-Phe. Ala-Ser was found by Edman degradation. LAP digestion showed the presence of asparagine. Phenylalanine was placed at the carboxyl terminus by the result of CPA digestion (0.90 Phe). The position of the asparagine residue is given by difference.

T11C2: Thr-Gln-Phe. Threonine is amino terminal (Edman degradation) and phenylalanine is at the carboxyl end (CPA, 0.95 Phe). LAP shows a glutamine residue, which was placed by difference.

T11C1–2: Ala-Ser-Asn-Phe-Thr-Gln-Phe. This peptide was isolated from some tryptic digests and allowed T11C1 to be placed proximal to T11C2, since alanine was amino terminal in T11C1–2.

T11C3: Val-Leu-Val-Asn-Asp-Gly-Gly-Thr-Gly-Asn-Val-Thr-Val-Ala-Pro-Ser-Asn-Phe. Four steps of Edman degradation gave the amino-terminal sequence Val-Leu-Val-Asx. Phenylalanine was found to be carboxyl terminal (CPA, 0.85 residue of Phe). From a streptococcal proteinase digest of T11C3, the follow-

ing peptides were obtained from AG 50-X4 using gradient II.

T11C3 Sp1: Val-Leu-Val-Asn. This was a neutral peptide and it must have been the amino-terminal part of T11C3, since it contained the sole leucine residue in T11C3.

T11C3 Sp2: Asx-(Asx,Thr,Gly_3,Val)-Thr. The amino-terminal residue was found by one step of Edman degradation. The carboxyl-terminal threonine was released by CPA digestion (0.85 residue). The peptide was acidic on paper electrophoresis at pH 4.5.

T11C3 Sp1–2: Val-Leu-Val-Asn-Asx-(Asx,Thr,Gly_3,-Val)-Thr. The four residues at the amino terminus were assigned by the use of LAP (see also T11C3). Threonine was found to be carboxyl terminal (CPA: 0.90 residue). T11C3 Spl-2 overlaps T11C3 Sp1 and T11C3 Sp2.

T11C3 Sp3: Val-Ala-Pro-Ser. See T11C3 Sp3–4.

T11C3 Sp4: Asn-Phe. The presence of asparagine was shown by LAP digestion. This peptide contained the only residue of phenylalanine in T11C3 and therefore can be placed at the carboxyl terminus of T11C3.

T11C3 Sp3–4. Val-Ala-Pro-Ser-Asn-Phe. The amino-terminal region was found by Edman degradation to be Val-Ala-Pro. Extensive digestion with LAP released only valine (0.95 residue). Since T11C3 Sp4 must be the carboxyl part of T11C3 Sp3–4 the serine residue can be placed by difference. This assignment was further supported by the isolation of the amino-terminal part of T11C3 Sp3–4 as a peptide denoted by T11C3 Sp3. The streptococcal proteinase peptides can be ordered as follows. Sp1-Sp2-Sp3-Sp4 and therefore a partial formula for T11C3 can be written *Val-Leu-Val-Asn-Asx(Asx,Thr,Gly_3,Val)-Thr-Val-Ala-Pro-Ser-Asn-Phe.* To get further fragments, T11C3 was digested with subtilisin and the peptides separated on AG 50-X4 using gradient II.

T11C3 Sub1: Val-Leu. This peptide can be assigned to the amino terminus of T11C3.

T11C3 Sub2: Val-Asn-(Asx,Thr,Gly_3)-Asn. This acidic peptide spans part of the unknown region in T11C3. Valine was found to be the amino-terminal residue by one step of Edman degradation. The asparagine residue in position 2 was released together with valine by LAP digestion (0.95 Val and 0.70 Asn residues). CPA liberated asparagine (0.5 residue).

T11C3 Sub3: Gly-(Thr-Gly_2)-Asn. Glycine is the amino-terminal residue as shown by Edman degradation. CPA released asparagine (0.60 residue).

T11C3 Sub4: Val-Thr. Edman degradation was used to prove the sequence of this dipeptide.

T11C3 Sub5: Val-Ala-Pro-Ser. This peptide has the same composition as T11C3 Sp3.

T11C3 Sub6: Asn-Phe. This peptide corresponds to T11C3 Sp4. The amino acid compositions of the subtilisin peptides Sub1, 2, 4, 5, and 6 sum up to the composition of T11C3. Sub2 has to be placed distal to Sub1, since the first four amino acids in T11C3 are known by Edman degradation. Sub5 and 6 have to span the carboxyl-terminal region, so that Sub4 can be placed by difference. The subtilisin peptides can be arranged as Sub1-2-4-5-6 and a partial formula can be written as *Val-Leu-Val-Asn-Asp-(Thr,Gly_3)-Asn-Val-Thr-Val-Ala-Pro-Ser-Asn-Phe.* The assignment of Asx as residue 5 in this formula is based on a comparison with the partial formula derived by use of the streptococcal proteinase peptides. Since Sub2 is an acidic peptide, residue 5 must be aspartic acid. T11C3 Sub3 accounts for all three glycine residues, and must be derived from the region of T11C3 which is shown in parentheses. The information on T11C3 Sub3 allows us to rewrite the sequence as *Val-Leu-Val-Asn-Asp-Gly-(Thr,Gly_2)-Asn* since the carboxyl-terminal asparagine residue must correspond to residue 10 in the formula given above. To arrive at a complete sequence, T11C3 Sub2 was subjected to partial acid hydrolysis and the peptides were separated on AG 50-X4 using gradient II. The free amino acids (aspartic acid, glycine, and valine) were found along with the following peptides.

T11C3 Sub2 A1: Gly-Gly. This dipeptide was identified by comparison with diglycine.

T11C3 Sub2 A2: Gly-Gly-Thr. Glycine was found to be amino terminal by Edman degradation. Threonine was placed at the carboxyl terminus by hydrazinolysis (0.5 residue).

T11C3 Sub2 A3: Gly-(Thr,Gly)-Gly. The glycine residues at the amino and carboxyl ends were placed by the information from Edman degradation and hydrazinolysis, respectively. Sub2 A3 spans the region in T11C3 still in parentheses. The partial structure of this peptide allows two possible formulas to be written as *Gly-Thr-Gly-Gly* and *Gly-Gly-Thr-Gly.* The isolation of diglycine (Sub2 A1) can be explained by both structures. Sub2 A2 can only come from *Gly-Gly-Thr-Gly.* Therefore the region still in parentheses in the partial formula can be written as *Gly-Thr-Gly.*

T11C4: Ala-Asn-Gly-Val-Ala-Glu-Trp. Edman degradation placed one residue of alanine at the amino terminus. CPA liberated only tryptophan (0.95 residue). Extensive digestion with LAP showed asparagine and glutamic acid. With LAP the following structure could be deduced Ala-Asn-Gly-(Ala,Val)-Glu-Trp (1.2 Ala, 0.8 Asn, 0.4 Gly, 0.2 Val, and 0.1 Glu). After an acid split by the procedure of Tsung and Fraenkel-Conrat (1965), a peptide C4A *Gly-Val-Ala-Glu-Trp* was isolated from AG 50-X4 using gradient II. Three steps of Edman degradation on this peptide established the amino-terminal sequence Gly-Val-Ala. The glutamic acid residue is placed by difference. With the results on peptide C4A and knowing the amino-terminal alanine in T11C4, the missing asparagine residue can be placed by difference.

T11C3–4: Val-(Asx_4,Thr_2,Ser,Pro,Gly_3,Ala,Val_3,Leu,-Phe)-Ala-Asn-Gly-Val-Ala-Glu-Trp was isolated from a short chymotryptic digest of T11 by paper chromatography using the system: 1-butanol–glacial acetic acid–pyridine–water (60:12:40:48) of Margoliash and Smith (1962). Since valine is the amino-terminal residue (Edman degradation) and tryptophan is the carboxyl-terminal residue (CPA), T11C3 can be placed proximal

to T11C4.

T11C5: Ile-Ser-Ser-Asn. Subtractive Edman degradation on this neutral peptide gave the sequence Ile-Ser. After one step of Edman degradation, the peptide Ser_2,Asn was digested with CPA. Asparagine and Ser-Ser were identified by comparison with standards.

T11C6: Ser-Arg. Arginine is placed by the result of CPB digestion on T11. This peptide must be at the carboxyl terminus of T11.

T11C5-6: Ile-Ser-Ser-Asn-Ser-Arg. Isoleucine is the amino-terminal residue (Edman degradation). From this result T11C5 is proximal to T11C6. The order of the chymotryptic peptides in T11 is C1–2-C3–4-C5–6 since only C1–2 has the same amino-terminal residue as T11 and C5–6 has the same carboxyl terminus as T11. The amino acid sequence of T11 itself is shown in Figure 3.

T8 (residues 39–43): Ser-Gln-Ala-Tyr-Lys. Digestion with LAP released glutamine. The sequence at the carboxyl-terminal end was found to be Ala-Tyr-Lys using CPB and CPA (CPB: 1.07 Lys; CPB and CPA: 0.35 Ala and 1.05 Tyr). One step of Edman degradation placed serine at the amino terminus. The position of the glutamine residue was determined by difference. It may be noted that the peptide T8A (Ser-Gln-Ala-Tyr) found in some tryptic digests was liberated by a chymotryptic-like split from T8.

T4 (residues 44–49): Val-Thr-CMCys-Ser-Val-Arg. T4 was isolated from tryptic digests of carboxymethylated protein. Valine is the amino-terminal amino acid (Edman degradation). From CPB and CPA treatment the carboxy-terminal sequence was shown to be Val-Arg (CPB: 0.95 Arg; CPB and CPA: 0.90 Val). Digestion of β-aminoethylated protein with trypsin gave two new peptides (T4A and T4B) instead of T4.

T4A: Val-Thr-AECys. Valine is the amino-terminal amino acid in this peptide as shown by Edman degradation. CPB liberated only one residue of AECys.

T4B: Ser-Val-Arg. Serine was shown to be at the amino terminus by Edman degradation.

T3 (residues 50–56): Gln-Ser-Ser-Ala-Gln-Asn-Arg. Paper electrophoresis of T3 at pH 4.5 showed a neutral spot with nearly no ninhydrin color in comparison to that obtained with the Sakaguchi reaction (Canfield and Anfinsen, 1963). The subtractive Edman failed and LAP did not release any amino acids. From these results and from the amino acid composition of T3, it may be concluded that a glutamine residue was at the amino terminus since glutamine cyclizes under the acid conditions used for chromatography to give pyrrolidonecarboxylic acid. Making use of this argument, the electrophoretic mobility of this peptide would show the presence of glutamine and asparagine rather than glutamic acid and/or aspartic acid. Arginine was found with CPB (CPB: 0.95 Arg). A special class of suppressible amber coat protein mutants of bacteriophage R17 has a serine residue in place of the glutamine residue at the amino-terminal position in T3 (Tooze and Weber, 1967). T3 obtained from these mutants was used for four steps of Edman degradation giving the amino-terminus Ser-Ser-Ser-Ala. After partial acid hydrolysis of normal T3 an acidic peptide *Ser-Ser-Ala-Glu* was isolated by gelfiltration on Bio-Gel P2. The amino-terminal region was shown to be Ser-Ser-Ala by Edman degradation. Deamidation during partial acid hydrolysis converted the glutamine residue to a glutamic acid residue. With these results the remaining asparagine residue can be placed by difference.

T7 (residue 57) is free lysine.

T9 (residues 58–61): Tyr-Thr-Ile-Lys. Tyrosine was amino terminal (Edman degradation). CPB and CPA treatment permitted the amino acid sequence Ile-Lys to be written (CPB: 1.05 Lys; CPB and CPA: 0.87 Ile). Threonine was placed by difference. The peptide T7–9 present in some digests overlapped T7 and T9. T7 was amino terminal, since CPA and CPB treatment liberated lysine and isoleucine in equal amounts.

T5 (residues 62–66): Val-Glu-Val-Pro-Lys. Edman degradation showed amino-terminal valine. CPB liberated lysine (0.7 residue). Extensive digestion with LAP gave only one residue of valine and glutamic acid. From the specificity of LAP the proline residue can be placed proximal to lysine and the second valine residue could be placed by difference. To confirm this amino acid sequence further, T5 was digested by LAP. A tripeptide T5L was isolated from AG 50-X4 using gradient I.

T5L Val-Pro-Lys. Two steps of Edman degradation gave the amino-terminal sequence Val-Pro.

T2 (residues 67–83): Val-Ala-Thr-Gln-Thr-Val-Gly-Gly-Val-Glu-Leu-Pro-Val-Ala-Ala-Trp-Arg. Three steps of Edman degradation gave the amino-terminal sequence Val-Ala-Thr. CPB showed arginine to be the carboxyl-terminal residue (CPB: 0.95 Arg). By CPB and CPA the carboxyl-terminal sequence Val-(Ala_2,-Trp)-Arg was deduced (CPB and CPA: 0.98 Trp; 1.70 Ala; 0.25 Val). Extensive digestion of T2 with LAP released equal amounts of glutamine and glutamic acid as well as the other amino acids proximal to the leucine residue in T2. T2 was digested with subtilisin and the following peptides were obtained from AG 50-X4 using gradient II.

T2 Sub1: Val-Ala-Thr-Gln. Three steps of Edman degradation were used to prove the amino acid sequence of this neutral tetrapeptide (electrophoresis, pH 4.5).

T2 Sub2: Thr-Val-Gly-(Glu,Pro,Gly,Val,Leu)-Val-Ala-Ala. Three steps of Edman degradation established the amino-terminal region Thr-Val-Gly. CPA released 0.3 residue of valine/1.9 residues of alanine. T2 Sub2 contains glutamic acid as shown by its electrophoretic mobility (pH 4.5) and LAP digestion.

T2 Sub3: Thr-Gln. The DNP method showed amino-terminal threonine. The presence of glutamine was shown by the electrophoretic mobility of the peptide.

T2 Sub4: Val-Ala. Valine is amino terminal (Edman degradation).

T2 Sub5: Thr-Val-Gly-Gly-Val. The amino-terminal sequence Thr-Val-Gly was found by Edman degradation. CPA released only valine and glycine (CPA: 0.50 Gly; 1.02 Val).

T2 Sub6: Glu-Leu-Pro-Val-Ala-Ala. Three steps of Edman degradation established the amino-terminal

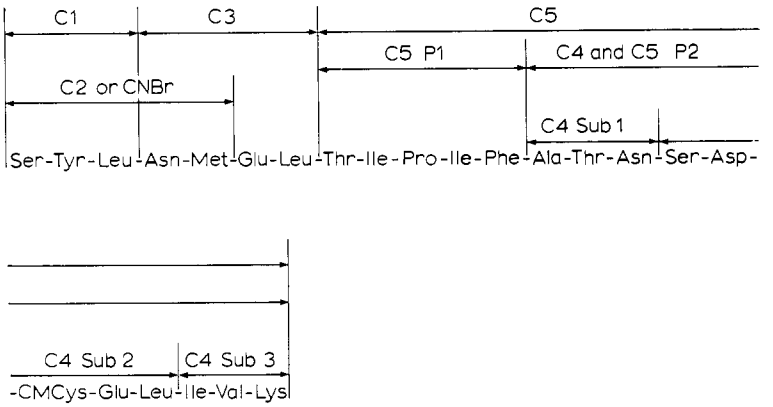

FIGURE 4: Fragmentation of the tryptic peptide T10 by chymotrypsin (peptides marked C) and CNBr. Further degradation of peptide C5 by pepsin (P) and of peptide C4 by subtilisin (Sub).

sequence Glu-Leu-Pro. The presence of a glutamic acid residue was shown by electrophoresis (pH 4.5) and by LAP digestion. CPA treatment liberated only alanine and valine (CPA: 1.95 Ala; 0.3 Val). T2 Sub1 and T2 Sub2 equal the composition of T2 minus one arginine and one tryptophan residue. Arginine was shown to be carboxyl terminal in T2 (CPB), so that the tryptophan residue can be placed penultimate to the arginine from the result of CPA and CPB digestion on T2. T2 Sub1 occupies the amino-terminal part of T2 as shown by the results of Edman degradation. T2 Sub3 and T2 Sub4 add up to T2 Sub1. The two peptides T2 Sub5 and T2 Sub6 equal the composition of T2 Sub2. The results of the Edman degradations prove that T2 Sub5 corresponds to the amino-terminal region of T2 Sub2.

T10 (residues 84–106): Ser-Tyr-Leu-Asn-Met-Glu-Leu-Thr-Ile-Pro-Ile-Phe-Ala-Thr-Asn-Ser-Asp-CMCys-Glu-Leu-Ile-Val-Lys. The DNP method showed amino-terminal serine. CPB and CPA treatment allowed the carboxyl-terminal sequence Leu-Ile-Val-Lys to be deduced (CPB and CPA: 1.03 Lys; 0.98 Val; 0.80 Ile; 0.50 Leu). From CNBr treatment of a mixture of T10 and T11, a fragment T10 CNBr was isolated by gelfiltration on Bio-Gel P2.

T10 CNBr: Ser-Tyr-Leu-Asn-Homoserine. One step of Edman degradation showed serine to be the amino-terminal residue. The presence of asparagine was established by LAP digestion. CPA and the specificity of CNBr were used to deduce the carboxyl-terminal sequence Leu-Asn-homoserine (CPA: 0.95 homoserine; 0.60 Asn; 0.45 Leu). The tyrosine residue was placed by difference. This peptide must arise from a CNBr split in T10, rather than in T11, since only T10 contains a methionine residue. The peptide has to be placed at the amino terminus of T10 since it contains a carboxyl-terminal homoserine residue. Chymotryptic digestion of T10 gave five peptides, which were isolated on AG 50-X4 using gradient II. Figure 4 shows the amino acid sequence of T10.

T10 C1: Ser-Tyr-Leu. This peptide was the amino-terminal part of T10 CNBr.

T10 C2: Ser-Tyr-Leu-Asn-Met. This neutral peptide corresponded to T10 CNBr. Ser-Tyr-Leu was found by three steps of Edman degradation. CPA treatment established the remaining part of the amino acid sequence (CPA: 0.98 Met; 0.65 Asn; 0.40 Leu).

T10 C3: Asn-Met-Glu-Leu. A short LAP digestion fixed the amino-terminal region as Asn-Met (LAP: 0.90 Asn; 0.60 Met; 0.35 Glu; 0.33 Leu). LAP digestion showed the presence of asparagine and glutamic acid. Leucine was placed at the carboxyl terminus by CPA digestion (CPA: 0.80 Leu). The position of the glutamic acid residue was assigned by difference. T10 C2 and T10 C3 have the sequence Asn-Met in common.

T10 C4: Ala-Thr-Asn-Ser-Asp-CMCys-Glu-Leu-Ile-Val-Lys. This peptide is the carboxyl-terminal region of T10. Alanine was the amino-terminal residue as shown by Edman degradation. CPA and CPB treatment allowed the carboxyl-terminal sequence to be written as Leu-Ile-Val-Lys (CPA and CPB: 1.05 Lys; 0.90 Val; 0.70 Ile; 0.50 Leu). T10 C4 was digested with subtilisin and the following peptides were isolated from AG 50-X4 using gradient II.

T10 C4 Sub1: Ala-Thr-Asn. Alanine was found to be the amino-terminal residue by Edman degradation. The peptide was neutral on paper electrophoresis at pH 4.5, showing the presence of asparagine rather than aspartic acid. CPA released only asparagine (CPA: 0.85 Asn). This peptide must be the amino-terminal part of T10 C4, since it contains the sole alanine residue of T10 C4 as its amino-terminal residue.

T10 C4 Sub2: Ser-Asp-CMCys-Glu-Leu. LAP digestion showed the presence of aspartic acid and glutamic acid. Two steps of Edman degradation proved the amino-terminal sequence Ser-Asp. CPA released only one residue of leucine. The assignment of the other two amino acids was possible from the result of LAP digestion (0.98 Ser; 0.80 Asp; 0.50 CMCys; 0.25 Glu; 0.25 Leu). Further evidence came from the isolation

of a dipeptide *Glu-Leu* by paper electrophoresis (pH 4.5) after an acid split of T10 C4 Sub2. The structure of this dipeptide was deduced by one step of Edman degradation.

T10 C4 Sub3: Ile-Val-Lys. Two steps of Edman degradation were used to prove the amino acid sequence of this tripeptide. T10 C4 Sub3 is the carboxyl terminus of T10 C4, since it contains the sole lysine residue of T10 C4. The subtilisin peptides can be ordered as Sub1-Sub2-Sub3.

T10 C5: (Thr,Pro,Ile$_2$,Phe)-Ala-Thr-Asn-Ser-Asp-CMCys-Glu-Leu-Ile-Val-Lys. Threonine is at the amino terminus (Edman degradation) and lysine is at the carboxyl terminus (CPB: 0.95 Lys). This peptide was digested with pepsin and the fragments isolated on AG 50-X4 using gradient II.

T10 C5 P1: Thr-Ile-Pro-Ile-Phe. LAP released only one residue of threonine, allowing the assignment of proline as the third residue. This result is consistent with the specificity of LAP. Three steps of Edman degradation giving the amino-terminal sequence Thr-Ile-Pro confirmed this conclusion. Phenylalanine was placed at the carboxyl terminus by CPA digestion (CPA: 0.85 Phe). The position of the second isoleucine residue was assigned by difference.

T10 C5 P2. This peptide had the same amino acid composition as T10 C4.

T6 (residues 107–113): Ala-Met-Gln-Gly-Leu-Leu-Lys. The amino-terminal part Ala-Met was deduced from Edman degradation. CPB and CPA released two residues of leucine for one residue of lysine. Glutamine was liberated by LAP digestion. Digestion with LAP allowed us to write the amino acid sequence as given (LAP: 1.0 Ala; 0.95 Met; 0.75 Gln; 0.15 Gly; 0.20 Leu).

T1 (residues 114–129): Asp-Gly-Asn-Pro-Ile-Pro-Ser-Ala-Ile-Ala-Ala-Asn-Ser-Gly-Ile-Tyr. Aspartic acid was at the amino-terminal end as shown by subtractive Edman degradation and paper electrophoresis before and after the first stage of Edman degradation. The peptide contained only one aspartic acid residue. CPA showed the carboxyl-terminal end to be Ile-Tyr (CPA: 0.95 Tyr; 0.65 Ile). Two digestions of T1 with subtilisin for 6 and 12 hr gave a set of peptides which were separated on AG 50-X4 using gradient II.

T1 Sub1: Asp-Gly-(Asn,Ser,Pro$_2$,Ile)-Ala. This peptide was acidic and therefore could be placed at the amino-terminal end of T1. The result of two steps of Edman degradation gave the sequence Asp-Gly. CPA liberated one residue of alanine.

T1 Sub2: Gly-(Asn,Ser,Pro$_2$,Ile)-Ala. This was a neutral peptide and had the same composition as T1 Sub1 but missing an aspartic acid residue. An acid split of T1 Sub2 gave three components (aspartic acid, glycine, and the peptide T1 Sub2A) which were separated on AG 50-X4 using gradient II.

T1 Sub2A: Pro-Ile-Pro-Ser-Ala. Three steps of Edman degradation resulted in the amino-terminal sequence Pro-Ile-Pro. Together with the result of CPA on T1 Sub1, serine can be placed by difference. These results establish the structure of T1 Sub1 as *Asp-Gly-Asn-Pro-Ile-Pro-Ser-Ala.* The asparagine residue was placed by difference, since the amino-terminal residues in T1 Sub1 and the sequence of T1 Sub2A were known.

T1 Sub3: Ser-Gly-Ile-Tyr. Serine was placed by Edman degradation and Ile-Tyr was assigned from the result of CPA digestion (CPA: 1.00 Tyr; 0.70 Ile). Glycine was placed by difference. T1 Sub3 was the carboxyl-terminal sequence of T1.

T1 Sub4: Asn-Ser-Gly-Ile-Tyr. This neutral peptide extends T1 Sub3 by one asparagine residue which was at the amino terminus of this peptide.

T1 Sub5: Ile-Ala-Ala. The amino acid sequence was shown by one step of Edman degradation. T1 Sub5 can be placed between T1 Sub1 and T1 Sub4.

Discussion

The coat protein of R17 bacteriophage contains 129 amino acids. This is based on the excellent agreement shown between the sum of the amino acids in all tryptic peptides and the over-all composition of the protein.

A comparison of the amino acid analyses of the coat proteins of the bacteriophages f_2 (Konigsberg *et al.*, 1966) and R17 indicates a minimum of one amino acid replacement in that bacteriophage R17 coat protein contains one more residue of methionine and one residue less of leucine per polypeptide chain.

This replacement was found in T10, since after CNBr treatment a peptide with the amino acid sequence *Ser-Tyr-Leu-Asn-homoserine* was isolated from R17 T10. This peptide spans the region of the first five amino acids in T10. The same region in T10 of bacteriophage f_2 coat protein has the amino acid sequence *Ser-Tyr-Leu-Asn-Leu* (Weber *et al.*, 1966). According to the genetic code, a change from leucine to methionine can be brought about by a single nucleotide replacement (Söll *et al.*, 1965). The remainder of T10 and the other tryptic peptides had the same amino acid composition as the corresponding peptides from bacteriophage f_2 coat protein (Konigsberg *et al.*, 1966).

The amino acid sequence studies of the short tryptic peptides T3, T4, T5, T6, T8, and T9 revealed the same amino acid sequence as that reported for bacteriophage f_2 coat protein (Weber *et al.*, 1966).

T3 could not be degraded by the Edman procedure, since it had an amino-terminal pyrrolidonecarboxylic acid residue after column chromatography. Here a mutant of bacteriophage R17 (Tooze and Weber, 1967) was of great advantage for the amino acid sequence study, since the amino-terminal residue of T3 obtained from this mutant was serine instead of glutamine which made a series of Edman degradations possible.

In the case of T4 a new point for tryptic cleavage was introduced by aminoethylation of the cysteine residues in the intact protein. As in the case of bacteriophage f_2 coat protein, no tryptic split was found in T10 after this treatment despite the presence of an aminoethylcysteine residue.

The chymotryptic peptides of T11, C1, C2, C4, C5, and C6 are identical with the corresponding peptides of T11 obtained from bacteriophage f_2 coat protein. The middle part of T11 is T11 C3, a peptide with 18 amino acids. The peptides from both bacteriophage coat proteins have the same amino acid composition. It has to be pointed out, however, that on T11 C3 Sp2, Edman degradation failed in the second step and CPA released only the carboxyl-terminal threonine residue and not the penultimate valine residue. The difficulties in determining the sequence of this peptide around the aspartic acid or asparagine residues will be reported elsewhere (Weber and Konigsberg, 1967). These difficulties stem from the possibility of formation of the imide and β form of these residues under the conditions used in chromatography. The assignment of *Gly-Gly-Thr-Gly* as part of T11 C3 Sub2 to residues 13–16 was based mainly on T11 C3 Sp2, which allowed us to place an aspartic acid residue in position 12. Within these limitations, T11 seems to have the same amino acid sequence as the corresponding peptide from bacteriophage f_2 coat protein.

The similarity of the two coat proteins is so striking that no attempt was made to order the tryptic peptides of bacteriophage R17 coat protein, since it was expected that the arrangement would be the same as that found for bacteriophage f_2 coat protein T11-T8-T4-T3-T7-T9-T5-T2-T10-T1 (Konigsberg, 1966). The proposed amino acid sequence for the bacteriophage R17 coat protein as shown in Figure 1 is based on this assumption.

There is one main difference between our results and the previous study of Enger and Kaesberg (1965) on bacteriophage R17 coat protein. Enger and Kaesberg found seven lysines and ten glutamic acid residues. We have found only six lysines and all of them are accounted for in our amino acid sequence. In addition all the eleven glutamic or glutamine residues that we have found can also be placed unequivocally in the amino acid sequence. This apparent discrepancy could easily be resolved if the phages are different despite the same nomenclature and the same original source (Paranchych and Graham, 1962). In support of this, Enger and Kaesberg have described a tryptic peptide 15 (Val, Ile, Leu, Lys) which has never been found in our studies. The composition of this peptide is unique, therefore it is probably derived from one region in the amino sequence. Since the carboxyl-terminal region of T10 had the sequence *Glu-Leu-Ile-Val-Lys* in our study it is possible that glutamic acid (residue 102) is replaced by lysine in this case.

The amino acid composition of bacteriophage MS2 coat protein (M. Wallis, private communication; Lin *et al.*, 1967) and of bacteriophage M12 coat protein (Enger and Kaesberg, 1965) when normalized to 14 residues of aspartic acid, was the same as for bacteriophage R17 coat protein. As far as the amino acid sequence of bacteriophage MS2 coat protein is known (M. Wallis, private communication; Lin *et al.*, 1967), no difference is apparent when it is compared to bacteriophage R17 coat protein. Recently a nearly complete sequence for the coat protein of f_r bacteriophage was published by Wittmann-Liebold (1966). Comparing the amino acid sequences of the coat proteins of bacteriophages R17 and f_r one can account for the exchange of about 20 amino acids. The length of the polypeptide chains however is the same. The replacements are not randomly distributed. The region between residues 20 and 54, which corresponds to one-quarter of the amino acid sequences is identical for both proteins. Nearly 80% of the amino acid exchanges can be explained assuming single nucleotide replacements in the corresponding codons (Söll *et al.*, 1965).

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Purification and Properties of Tuna Myosin*

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ABSTRACT: Myosin was extracted from the white muscle of skipjack tuna (*Euthynnus pelamis*) and purified by the chromatographic method of Richards *et al.* (Richards, E. G., Chung, C.-S., Menzel, D. B., and Olcott, H. S. (1967), *Biochemistry 6*, 528). The product showed a hypersharp boundary in the ultracentrifuge, $s^0_{20,w} = 6.22$ S, and possessed adenosine triphosphatase (ATPase) activity and amino acid composition similar to that of rabbit myosin. Molecular weight by high-speed sedimentation equilibrium was approximately 530,000, compared to 510,000 obtained for rabbit myosin under the same conditions. The pH–activity curve showed two maxima similar to that of the unpurified preparation.

The purified tuna myosin tended to aggregate during storage; this was not prevented by dimethyl sulfoxide, 1,3-butanediol, *N*-ethylmaleimide, or 2-mercaptoethanol.

Disagreements in the reported molecular weight of myosin and in details of its subunit structure are thought to be due to the different methods of extraction and purification used (Tonomura *et al.*, 1966; Dreizen *et al.*, 1966). Among these may be mentioned the conventional precipitation–resolution method (Szent-Györgyi, 1943) and the ammonium sulfate fractionation procedure (Szent-Györgyi, 1951; Tsao, 1953; Kielley and Bradley, 1956; Small *et al.*, 1961). Column chromatographic methods have been applied to the purification of myosin (Brahms, 1959; Perry, 1960; Asai, 1963; Smoller and Fineberg, 1964; Baril *et al.*, 1964, 1966). Recently we described a modified chromatographic procedure with DEAE-Sephadex A-50 and KCl gradient in pyrophosphate or phosphate buffer, which has provided preparations of highly purified myosin from several sources (Richards *et al.*, 1967).

The present investigation describes (1) the preparation and purification of skipjack tuna myosin by the modified chromatographic procedure, (2) determination of its molecular weight by the high-speed sedimentation equilibrium method of Yphantis (1964), (3) study of some of its enzymatic properties, and (4) its amino acid composition. These properties are compared with those of highly purified rabbit myosin.

Experimental Procedure

Materials and Methods. Methods and reagents used for the extraction of myosin, the chromatographic procedures, and methods for the analysis for protein and ATPase[1] activity and identification of RNA were those previously described (Richards *et al.*, 1967). DMSO (Stepan Chemical Co., Chicago) and 1,3-butanediol (Eastman) were used as obtained.

Live skipjack tuna (*Euthynnus pelamis*), about 45 cm in length (obtained at the Research Laboratory of the Fish and Wildlife Service in Honolulu, Hawaii), were denervated by cutting the spinal cord and brought to the Biomedical Research Center of the University of Hawaii for further handling. The time between denervation and extraction of myosin was about 1 hr.

The preliminary processing of the myosin, including the first high-speed centrifugation, was carried out in Hawaii. At this stage an equal volume of glycerol was added to the myosin solutions and the resulting solutions were held at −20°. When needed, the myosin was precipitated from the 50% glycerol solution by the addition of nine volumes of water; the precipitate

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[1] Abbreviations used: ATPase, adenosine triphosphatase; DMSO, dimethyl sulfoxide; NEM, *N*-ethylmaleimide.