Peptide Mapping Study of R17 Coat Protein Synthesized *in Vitro**

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ABSTRACT: Translation of R17 ribonucleic acid (RNA) by *Escherichia coli* cell-free extracts was examined by studying lysine incorporation into soluble tryptic peptides from the *in vitro* coat protein. A sensitive thin-layer peptide mapping technique showed that several minor peptides containing lysine were present in tryptic digests both of protein coat synthesized *in vitro* and of natural coat protein. Radioactive double labeling of these minor peptides showed that they arose from irregularities of tryptic cleavage during protein digestion rather than from errors in translation. Five unique lysyl tryptic peptide sequences were shown to contain essentially equivalent amounts of radioactivity when the lysine content of the minor peptides was taken into account in a quantitative estimation of total lysine incorporation *in vitro*. This result demonstrates a high fidelity of *in vitro* translation of R17 RNA in the *E. coli* cell-free system.

One of the three known cistrons of bacteriophage R17 RNA codes for viral coat protein. There is a preferential translation of the coat protein cistron when R17 RNA is used to direct protein synthesis in cell-free extracts of *Escherichia coli* (for review, see Bergquist and Burns, 1969). This phenomenon parallels the situation found during bacteriophage replication in the bacterial host. Several investigators have used peptide mapping techniques to compare protein synthesized *in vitro* with the natural viral coat protein (Nathans *et al.*, 1962; Nathans, 1965; Capecchi, 1966; Yamazaki and Kaesberg, 1966). In most cases, close similarities between peptide maps from tryptic digests of the two types of protein have been reported. These similarities are indicative of a general correspondence in the respective amino acid sequences. A known difference in sequence arising because the initiating *N*-formylmethionyl residue is not cleaved from the *in vitro* protein (Adams and Capecchi, 1966; Webster *et al.*, 1966) cannot be detected by normal fingerprinting techniques because the amino-terminal tryptic peptide is insoluble in these systems.

It is known that the fidelity of translation of synthetic copolymers of nucleic acid bases may be upset by such factors as temperature, magnesium ion concentration, polyamines, and certain amino glycoside antibiotics (summarized by Weinstein *et al.*, 1966). While the effects of these agents on peptide synthesis *in vitro* is well documented, there is little evidence to show that natural messenger RNA is translated without some miscoding or ambiguity in the cell-free system. The fact that protein synthesized *in vitro* fingerprints in a manner apparently identical with digests of the analogous protein synthesized *in vivo* does not prove absolute accuracy of translation of all appropriate code words in the mRNA. Quantitative information on the incorporation of specific amino acids into specific peptide sequences is a necessary first step in the establishment of the level of translational fidelity in the cell-free system. A brief examination of this kind was made by Lin and Fraenkel-Conrat (1967) who followed the incorporation of alanine into the major soluble tryptic peptides of coat protein synthesized by *E. coli* extracts under the direction of phage f2 RNA. These workers concluded that either *in vitro* translation was incomplete or that the carboxy-terminal end of the coat protein molecule was particularly susceptible to proteases in the cell-free system.

In this paper we report an examination of lysine incorporation into the soluble tryptic peptides of R17 coat protein synthesized by *E. coli* cell-free extracts. We observed a number of minor peptides on fingerprints of the protein synthesized *in vitro*, which suggested to us that miscoding could be occurring at a low level during translation of R17 RNA. When we found the minor peptides to be present in fingerprints of R17 coat protein produced in the natural host, we examined the possibility that the minor peptides arose either by nontryptic cleavage or by inhibition of the action of trypsin at certain sites in the protein sequence. In order to make a quantitative estimation of incorporation, we investigated the compositions of several minor peptides containing lysine which were common to digests of *in vitro* and *in vivo* protein. The amount of lysine in each of the five soluble standard[1] tryptic peptide sequences was very similar when the lysine content of the minor peptides was taken into account. This correspondence in lysine content plus the absence of peptides unique to the fingerprints of protein synthesized *in vitro* was interpreted to indicate that a high level of translational fidelity operates in the *in vitro* system.

Methods

E. coli Cell-Free Extracts. Cell-free extracts were prepared from *E. coli* S26 according to the method of Capecchi (1966)

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[1] We define standard tryptic peptide sequences as those resulting from tryptic cleavage at lysyl and arginyl residues in a polypeptide chain.

except that the S-30 fraction was dialyzed against 0.01 M Tris-acetate (pH 7.8)–0.01 M magnesium acetate–0.03 M ammonium chloride–0.006 M 2-mercaptoethanol.

In Vitro Protein Synthesis. The *in vitro* amino acid incorporating system directed by R17 RNA was essentially that described by Capecchi (1966), except that the contribution of the preincubation and incubation mixtures toward the magnesium ion concentration in the final reaction mixture was 0.0095 M. *E. coli* tRNA which had been treated to remove amino acids was used to supplement the system. The kinetics of incorporation have been described previously (Bergquist *et al.*, 1968).

Preparation of tRNA. The *E. coli* S26R1e tRNA was obtained from cells in midlogarithmic phase of growth as described previously (Bergquist *et al.*, 1968).

R17 RNA and Coat Protein. Bacteriophage R17 was grown in broth and purified according to the procedure of Gesteland and Boedtker (1964). Phage stocks were grown and assayed on *E. coli* Hfr. R17 RNA was prepared according to Bergquist *et al.* (1968) and R17 coat protein by acetic acid extraction of a bacteriophage suspension (Capecchi, 1966).

For the preparation of coat protein labeled *in vivo* with radioactive amino acids, the phage was grown in a glycerol–amino acids medium containing minimal amounts of the amino acids to be used as the radioactive labeling species (Bergquist *et al.*, 1968). The addition of between 80 and 320 μCi per l. of each radioactive amino acid and 2–4 mg/l. of each unlabeled carrier amino acid was adjusted to give an approximate tenfold excess of amino acid molecules to the sites available in the coat protein subunits estimated to be synthesized. Before extraction of coat protein the labeled phage suspension was further purified by centrifugation to equilibrium on a cesium chloride gradient (Argetsinger and Gussin, 1966).

Sucrose Density Gradient Centrifugation. Reaction mixtures from the *in vitro* system were treated with ribonuclease and centrifuged on 25-ml preparative sucrose density gradients to free the coat protein fraction from the contaminating 20S synthetase (Capecchi, 1966). The linear gradients were 5–20% sucrose containing 0.08 M KCl–0.01 M Tris-acetate–0.01 M magnesium acetate (pH 7.8). The gradients were centrifuged, fractionated, and assayed as described previously (Bergquist *et al.*, 1968).

Peptide Mapping. Sucrose gradient fractions of coat protein synthesized *in vitro* were pooled, 0.5–1.0 mg of unlabeled carrier coat protein was added, and the total protein was then precipitated and washed with 7% trichloroacetic acid followed by ether (Capecchi, 1966). Residual ether was removed and the protein was carboxymethylated, digested with TPCK[2]-treated trypsin for 5 hr at 37°, lyophilized, and mapped on cellulose thin layers (Burns and Turner, 1967). ^{14}C-Labeled peptides were detected by dry film autoradiography using Kodak Royal Blue Medical X-Ray film. For determination of radioactivity, the peptide positions were marked and the layer was spread with a cellulose acetate mixture (Bieleski and Turner, 1966). The radioactive peptide areas were then cut out and transferred to vials for scintillation counting.

Chemicals. Radioactive amino acids were obtained from New England Nuclear Corp., Boston, Mass.; the Radiochemical Centre, Amersham, U. K., and Schwarz BioResearch, Orangeburg, N. Y. The specific activities of [^{14}C]amino acids and [^{3}H]lysine used in the final *in vitro* reaction mixtures ranged from 2.1×10^4 to 2.5×10^5 cpm per mμmole. Pyruvic kinase, ATP, CTP, TPCK, and phosphoenolpyruvate were obtained from Calbiochem, Los Angeles, Calif.; ribonuclease, deoxyribonuclease (ribonuclease free), and trypsin (twice recrystallized) from Worthington Biochemical Corp., Freehold, N. J.; sucrose and [^{12}C]amino acids were obtained from Mann Laboratories, New York, N. Y.

T1 Asp-Gly-Asn-Pro-Ile-Pro-Ser-Ala-Ile-Ala-Ala-Asn-Ser-Gly-Ile-Tyr
T2 Val-Ala-Thr-Gln-Thr-Val-Gly-Gly-Val-Glu-Leu-Pro-Val-Ala-Ala-Trp-Arg
T3 Gln-Ser-Ser-Ala-Gln-Asn-Arg
T4 Val-Thr-CMCys-Ser-Val-Arg
T5 Val-Glu-Val-Pro-Lys
T6 Ala-Met-Gln-Gly-Leu-Leu-Lys
T7 Lys
T8 Ser-Gln-Ala-Tyr-Lys
T9 Tyr-Thr-Ile-Lys
T7-9 Lys-Tyr-Thr-Ile-Lys

FIGURE 1: The amino acid sequences of the major soluble tryptic peptides from R17 coat protein (from Weber, 1967).

Results and Discussion

Preliminary experiments were directed toward examining the degree of correspondence between peptide maps of coat protein synthesized *in vivo* and *in vitro*. Natural coat protein and *in vitro* protein samples which were labeled with [^{14}C]-arginine and [^{14}C]lysine were prepared. The fingerprint autoradiographs of tryptic digests of the two preparations showed a close correspondence between the locations of the major radioactive peptides. Furthermore, the mapping positions of the radioactive *in vitro* peptides coincided with ninhydrin-positive peptides from the unlabeled natural coat protein used as carrier protein during tryptic digestion. In addition a number of minor radioactive peptides common to digests of both natural and *in vitro* protein were found on the autoradiographs. We concluded from the agreement in mapping patterns that reasonably accurate translation occurs in the cell-free system.

The total number of fingerprint peptides detected by the thin-layer mapping procedure exceeded the number of known standard tryptic peptides of the R17 coat protein sequence (see below). Previous work had shown similar results for other known protein sequences (Burns and Turner, 1967). The additional R17 coat protein peptides were assumed to result either from cleavage at sites other than lysyl and arginyl residues or from partial inhibition of tryptic cleavage at these susceptible residues in the polypeptide chain. Since we intended to examine the level of translational fidelity in the cell-free system by making a quantitative study of amino acid incorporation, it was apparent that the composition of both standard and nonstandard peptides would have to be determined in order to carry out this study.

The Mapping Position of the Major Tryptic Peptides. The sequence of R17 coat protein has been determined (Weber, 1967). The single polypeptide chain consists of 129 amino acid residues, and on tryptic digestion yields 11 standard tryptic peptides (T1–T11) only nine of which are readily soluble

[2] The abbreviation used is: TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

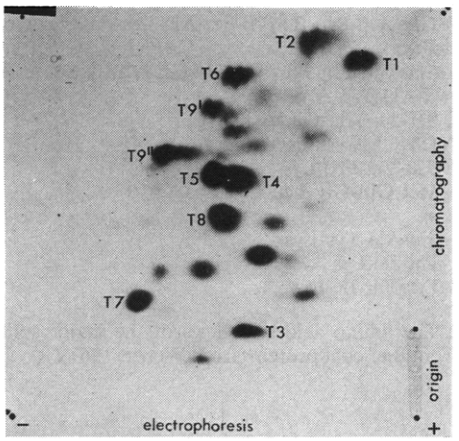

FIGURE 2: The mapping positions of the major soluble tryptic peptides. The peptides which could be identified from preliminary *in vitro* radioactive labeling experiments are marked on the peptide map of a 160-μg sample of a tryptic digest of natural R17 coat protein. The separation was carried out by electrophoresis at pH 2, followed by ascending chromatography in 1-butanol–acetic acid–pyridine–water (15:3:12:12). The band origin is in the lower right-hand corner of the map. The direction of electrophoresis was horizontal and that of chromatography was vertical. This orientation of peptide maps has been followed in the remaining figures. The peptides were stained with a ninhydrin reagent (Heilmann *et al.*, 1957). The amino acid sequences of the peptides are given in Figure 1. Peptides $T9^{I}$ and and $T9^{II}$ were subsequently shown to correspond to peptides T7 and T7-9, respectively. All the unidentified peptides were consistently present on ninhydrin-stained maps. Several of these peptides were studied subsequently (see text).

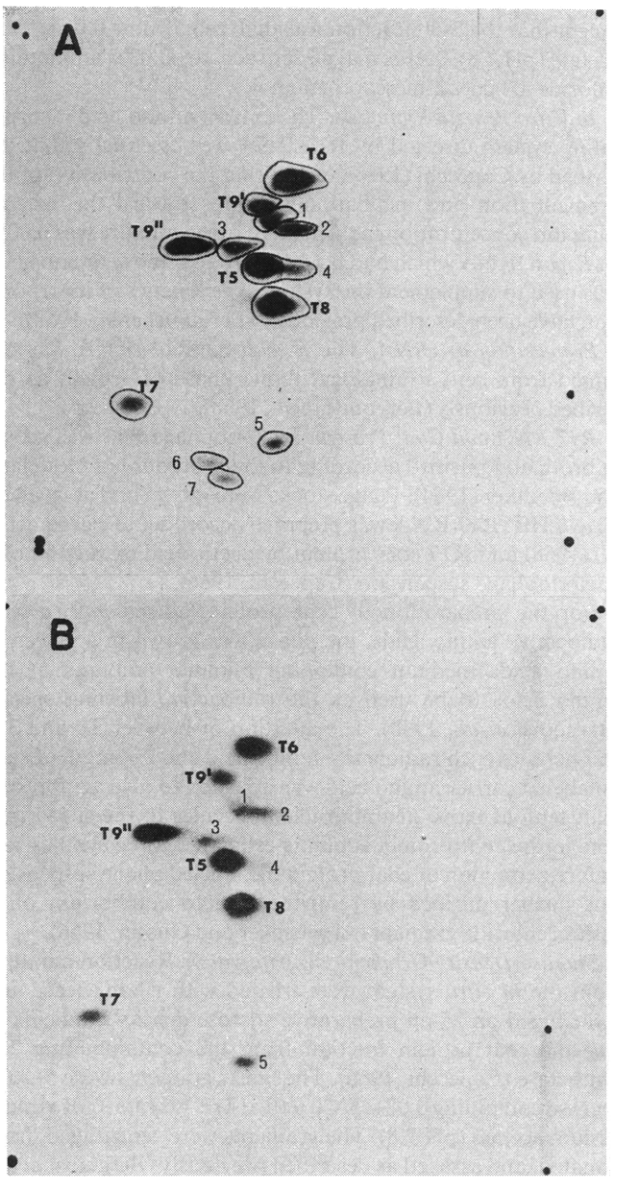

FIGURE 3: Autoradiographs showing peptides labeled with [^{14}C]-lysine from R17 coat protein synthesized *in vivo* and *in vitro:* (A) *in vivo* coat protein; (B) *in vitro* coat protein. The radioactivity data and the preparation of the two coat proteins are given in Table I. The samples were mapped as described for Figure 2 and the peptides were detected by autoradiography. The major peptides correspond to those in Figure 2; the minor peptides are labeled 1–7.

(see Figure 1). In addition, a nonstandard peptide is produced in large yield. This peptide (T7–9) results from partial inhibition of cleavage between two standard tryptic peptide sequences, T7 and T9.

The mapping positions of the major soluble tryptic peptides (Figure 2) were determined by comparing peptide maps of different *in vitro* coat protein preparations, each of which had been labeled with a single radioactive amino acid. The peptides were identified by correlating their observed labeling properties with those expected for the peptides found by Weber (1967). It was not possible to determine peptide compositions directly by elution and hydrolysis of natural coat protein peptides from thin-layer plates because the cellulose adsorbent was found to contain material which eluted with the peptides and caused extensive interference with subsequent amino acid estimations (D. J. W. Burns, 1968, unpublished work). The peptides T9 and T7–9 could not be distinguished because they were labeled with the same radioactive amino acids (see Figure 1). The two peptides were designated $T9^{I}$ and $T9^{II}$ until further identification.

The Peptides Containing Lysine. Since six of the ten major soluble peptides contain lysine (see Figure 1), the lysine content of these peptides was selected as a suitable basis for making quantitative estimations of yield. Two coat protein preparations which were labeled with [^{14}C]lysine were prepared and mapped. One preparation was from intact bacteriophage while the other was *in vitro* protein. There was good qualitative agreement between the fingerprint autoradiographs, although the map of the *in vivo* protein showed two additional spots (peptides 6 and 7, see Figure 3). The radioactivity content of the peptides was determined (Table I). A comparison showed considerable differences in the relative lysine content of some major peptides from the two preparations. These quantitative differences might have arisen either as a result of translational errors *in vitro* or because of variation in rates of tryptic hydrolysis. The latter explanation could also apply to the nonequivalence of lysine content for the peptides T5 and T8 compared with T6, in the *in vivo* protein. The origins of the unidentified peptides were studied in order to differentiate between these possibilities.

Identification of T7–9. The peptides T9 and T7–9 differ by the presence of an additional lysyl residue in T7–9 (see Figure 1). Hence, the proportion of lysine in T7–9 compared with T9 will always be double that of the relative yields of the two

TABLE I: Incorporation of [^{14}C]Lysine into Tryptic Peptides of R17 Coat Protein Synthesized *in Vivo* and *in Vitro*.

Peptide[a]	*In Vivo* Synthesis[b] cpm	Ratio[c]	*In Vitro* Synthesis[d] cpm	Ratio[c]	Ratio (cpm) *in Vivo*/ *in Vitro*
T6	2270	1.00	825	1.00	2.75
T5	3190	1.41	1060	1.29	3.01
T8	3075	1.35	930	1.13	3.31
$T9^{II}$	2350	1.04	1336	1.62	1.76
$T9^{I}$	662	0.29	317	0.38	2.09
T7	605	0.27	315	0.38	1.92
1	434	0.19	166	0.20	2.61
2	744	0.33	202	0.24	3.68
3	350	0.15	199	0.24	1.76
4	539	0.24	80	0.10	6.74
5	436	0.19	243	0.29	1.79
6	405	0.18	Not present		
7	244	0.11	Not present		

[a] The numbering of the peptides is given in Figure 3. [b] R17 was grown in a labeling medium containing 80 μCi/l. of [^{14}C]lysine (see Methods). The bacteriophage preparation was harvested and purified by centrifugation on a cesium chloride density gradient. Coat protein was prepared by acetic acid extraction of the preparation, and analyzed by peptide mapping. The radioactivity in the peptides was determined by scintillation counting. [c] T6 was used as a standard for comparing radioactivity in the peptides. [d] The *in vitro* reaction mixture contained: 250 μl of preincubation mixture, 300 μl of incubation mixture, 1.2 mg of R17 RNA, 1.0 mg of stripped *E. coli* S26R1e tRNA: [^{14}C]lysine (249 cpm/ $\mu\mu$mole) and 19 [^{12}C]amino acids in a total volume of 1.0 ml. The reaction mixture was incubated for 30 min at 37° and fractionated on 25-ml sucrose density gradients. The gradients were dripped out, the fractions were assayed, and those containing coat protein were pooled, carboxymethylated, digested, and mapped.

peptides based on content of another amino acid. This approach was used to identify the peptides $T9^{I}$ and $T9^{II}$. Two separate *in vitro* coat protein preparations were labeled with [^{3}H]lysine and either [^{14}C]threonine or [^{14}C]isoleucine and mapped.

[^{14}C]Threonine and [^{3}H]Lysine. From the peptide sequence data [^{14}C]threonine was expected to label T2 (2 threonyl residues), T4 (1 threonyl residue), and T7–9 and T9 (with the latter two peptides having a combined radioactivity content equivalent to 1 threonyl residue). The peptide map autoradiograph showed that T2, T4, $T9^{I}$, and $T9^{II}$ were the major radioactive peptides (see Figure 4). The radioactivity content in each peptide was determined, and the data showed that the total [^{14}C]threonine radioactivity for the T9 sequence ($T9^{I}$ and $T9^{II}$) was almost exactly one-half that of T2 (Table II). The ratio of ^{14}C radioactivities for $T9^{I}$:$T9^{II}$ was 1:2 whereas the corresponding ratio of ^{3}H radioactivities was close to one-half of this value (1:3.66). This result indicated

FIGURE 4: Autoradiograph showing peptides labeled with [^{14}C]-threonine from R17 coat protein synthesized *in vitro*. The radioactivity data and the preparation of the sample are given in Table II. The numbering of the peptides corresponds to that given in Figures 2 and 3.

that $T9^{II}$ was probably the T7–9 peptide of Weber (1967).

[^{14}C]Isoleucine and [^{3}H]Lysine. Besides T9 and T7–9, only T1 (3 isoleucyl residues) was expected to be labeled with [^{14}C]-isoleucine *in vitro*. The autoradiograph demonstrated that these three peptides were the major radioactive peptides from the coat protein digest (Figure 5). The data showed that the combined [^{14}C]isoleucine radioactivity in $T9^{I}$ and $T9^{II}$ was slightly less than one-third of that found in T1 (Table III). The ratio of the ^{14}C radioactivities for $T9^{I}$:$T9^{I}$ was again 1:2, and that of the ^{3}H radioactivities was 1:3.53. From the results above, it was concluded that $T9^{II}$ corresponded to the T7–9, and $T9^{I}$ to the T9 peptides of Weber (1967).

Noncleavage between T3 and T7. All autoradiographs displaying the peptides containing lysine showed one minor spot mapping in the position of T3 (see peptide 5, Figure 3a,b). The detection of a labeled peptide in this region was unexpected, since T3 has an amino-terminal arginyl residue. However, since the peptide sequences T3, T7, and T9 occur sequentially in the coat protein (Weber, 1967) it appeared possible that inhibition of cleavage between T7 and T9 might also extend to the bond between T3 and T7, giving rise to a peptide with the T3 sequence plus T7 as an amino-terminal lysyl residue. Further, it seemed likely that if T3 and the postulated T3–7 peptide were mapping together, an extended period of electrophoresis might achieve separation because of the additional charge imparted to T3–7 by the lysyl residue.

An *in vitro* coat protein preparation was labeled with [^{14}C]serine and [^{3}H]lysine and mapped under conditions of prolonged electrophoresis in the first dimension. It was anticipated that [^{14}C]serine would label peptides T1 and T3 (2 seryl residues each) as well as T4 and T8 (1 seryl residue

TABLE II: Incorporation of [^{14}C]Threonine and [^{3}H]Lysine into Tryptic Peptides of R17 Coat Protein Synthesized *in Vitro*.[a]

Peptide[b]	^{14}C (cpm)	Ratio[c]	Theoretical Ratio[d]	Relative % Yield of $T9^{I}$ and $T9^{II}$[e]	^{3}H (cpm)	Ratio[c]	Calculated Ratio[f]
T2	3177	2.06	2.00	*g*	*g*	*g*	*g*
$T9^{I}$	516			33.4	188	1.00	1.00
		1.00	1.00				
$T9^{II}$	1025			66.6	687	3.66	3.98
T4	562	0.36	1.00	*g*	*g*	*g*	*g*

[a] The *in vitro* reaction mixture was similar to that described in the legend for Table I, but contained [^{14}C]threonine and [^{3}H]-lysine. [b] The peptides are shown in Figure 4. [c] The combined ^{14}C radioactivity in $T9^{I}$ and $T9^{II}$ was taken as a standard figure for comparing the radioactivity content of the peptides. $T9^{I}$ was taken as a standard for comparing the ^{3}H radioactivity in $T9^{I}$ and $T9^{II}$. [d] Based on amino acid sequence data (see Figure 1). [e] Based on relative content of ^{14}C radioactivity. [f] Based on relative yields of $T9^{I}$ and $T9^{II}$ with the assumption that $T9^{I}$ had one lysyl residue and $T9^{II}$ had two lysyl residues. [g] Not applicable.

TABLE III: Incorporation of [^{14}C]Isoleucine and [^{3}H]Lysine into Tryptic Peptides of R17 Coat Protein Synthesized *in Vitro*.[a]

Peptide[b]	^{14}C (cpm)	Ratio[c]	Theoretical Ratio[d]	Relative % Yield of $T9^{I}$ and $T9^{II}$[e]	^{3}H (cpm)	Ratio[c]	Calculated Ratio[f]
T1	2595	3.69	3.00	*g*	*g*	*g*	*g*
$T9^{I}$	238			33.8	219	1.00	1.00
		1.00	1.00				
$T9^{II}$	465			66.2	773	3.53	3.92

[a] The *in vitro* reaction mixture was similar to that described in the legend to Table I but contained [^{14}C]isoleucine and [^{3}H]-lysine. [b] The peptides are shown in Figure 5. [c–g] As for Table II.

TABLE IV: Incorporation of [^{14}C]Serine and [^{3}H]Lysine into Tryptic Peptides of R17 Coat Protein Synthesized *in Vitro*.[a]

Peptide[b]	^{14}C (cpm)	Ratio[c]	Theoretical Ratio[d]	Relative Yield of $T3^{I}$ and $T3^{II}$[e] (%)	^{3}H (cpm)	Ratio[c]	Theoretical Ratio[d]	Yield of $T3^{II}$[f] (%)
T1	2645	2.14	1.00	*g*	*g*	*g*	*g*	*g*
$T3^{II}$	581			23.5	155	0.27	*g*	26.7
		2.00	2.00					
$T3^{I}$	1893			76.5	72	0.12	*g*	12.4
T8	1295	1.05	1.00	*g*	580	1.00	1.00	*g*
T4	494	0.40	1.00	*g*	120	0.21	*g*	*g*

[a] The *in vitro* reaction mixture was similar to that described in the legend to Table II but contained [^{14}C]serine and [^{3}H]lysine. [b] The peptides are shown in Figure 6. [c] The combined ^{14}C radioactivity in $T3^{I}$ and $T3^{II}$ was taken as a standard figure for comparing the ^{14}C radioactivity content of the tryptic peptides. T8 was used as a standard for comparing the ^{3}H radioactivity content. [d,e] As for Table II. [f] Based on [^{3}H]lysine content of T8 as a standard. [g] Not applicable.

each). The autoradiograph of the peptide map showed that the major areas of radioactivity corresponded to T1, T3, T8, and T4. There were two incompletely resolved spots in the region of T3 which were designated $T3^{I}$ and $T3^{II}$, respectively (Figure 6). The radioactivity content of the peptides (Table IV) showed that the total ^{14}C radioactivity in $T3^{I}$ and $T3^{II}$ was very similar to that in T1 and double that in T8. The yield of $T3^{II}$, based on the total ^{14}C radioactivity in

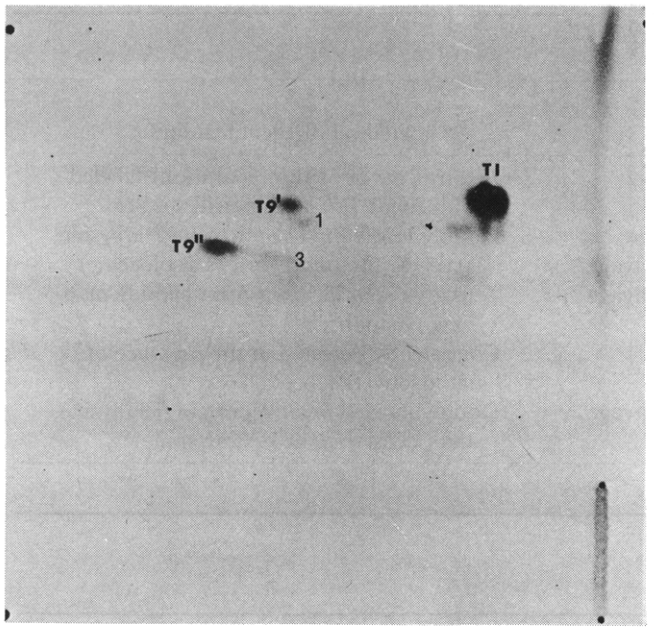

FIGURE 5: Autoradiograph showing peptides labeled with [^{14}C]-isoleucine from R17 coat protein synthesized *in vitro*. The radioactivity data and the preparation of the sample are given in Table III. The numbering of the peptides corresponds to that given in Figures 2 and 3.

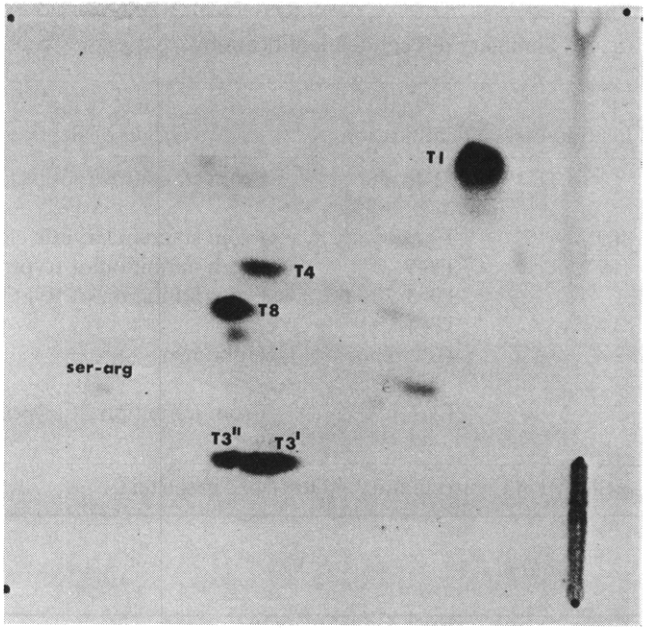

FIGURE 6: Autoradiograph showing peptides labeled with [^{14}C]serine from R17 coat protein synthesized *in vitro*. The radioactivity data and the preparation of the sample are given in Table IV. Electrophoresis was carried out for 30 min instead of the usual 25-min period. The numbering of the peptides corresponds to that given in Figures 2 and 3. However, the area of peptide T3 is partially resolved into two spots, the faster moving spot being designated T3^{II} and the slower moving spot being designated T3^{I}. The dipeptide Ser-Arg corresponds to that found by Konigsberg *et al.* (1966), and results from nontryptic cleavage in the insoluble T11 sequence.

both T3 peptides, was 23.5%. If T3^{II} contained a single lysyl residue then the same percentage of lysine (compared with the lysine content of T8, as a standard) should be found in the peptide. The calculated lysine content of T3^{II} on this basis was 26.7%. Some lysine radioactivity was also found in T3^{I}, possibly because of incomplete separation from T3^{II}. The presence of residual T3^{II} in T3^{I} would be expected to raise both the ^{14}C radioactivity and ^{3}H radioactivity of T3^{I}, resulting in a slightly lowered estimation of the yield of T3^{II}. The lysine content of T3^{I} indicated a 12.4% level of contamination with T3^{II}, raising the yield of T3^{II} based on lysine content to a maximum of 39.1%.

These results demonstrated the existence of two peptides, T3^{I} and T3^{II}, with the properties anticipated for T3 and T3–7, respectively, mapping in the region of T3. The possibility still remained that T3^{II} might arise from a mistranslation of the T3 arginine codon for lysine (this substitution would be necessary in order to maintain tryptic cleavage). An *in vitro* preparation labeled with [^{14}C]arginine and [^{3}H]lysine was used to examine this possibility. We found that the T3 region contained an equivalent amount of arginine to T2, another major soluble peptide containing arginine. Thus the T3 arginine codon is being translated correctly.

Other Minor Peptides. Other minor peptides containing substantial amounts of lysine were peptides 1–4 (Figure 3).

Peptides 1 and 3. A consideration of several autoradiographs showed that peptides 1 and 3 appeared whenever T9 or T5 were radioactively labeled. A tentative identification of the peptides was made on the assumption that cleavage between T9 and T5 was measurably inhibited. Two additional peptides would be expected to result from such inhibition. The peptides should correspond to sequences T9-5 and T7-9-5 containing 9 and 10 amino acids, respectively. T7-9-5 would probably map between T7–9 and T5, thus coinciding with the location of peptide 3. Similarly, T9-5 would probably map between T9 and T5 coinciding with the location of peptide 1. A peptide with the composition of T7-9-5 contains three lysyl residues, whereas one with the composition of T9-5 contains two lysyl residues. Hence in theory it is possible to examine peptides 1 and 3 further in terms of their content of lysine compared to another amino acid. A preliminary examination of the two peptides was carried out. The amount of lysine relative to threonine or isoleucine found in peptide 3 compared with peptide 1 was found to be approximately that anticipated for their presumed identities. However, the mapping areas of the peptides could not be established exactly because the low level of ^{14}C radioactivity in the peptides resulted in diffuse spots on the autoradiograph. Hence the radioactivity data, and consequently the identification of peptides 1 and 3 as T7–9 and T7-9-5, respectively, could only be regarded as tentative.

Peptides 2 and 4. These two peptides were found to be labeled only when T6 was also labeled (for example, with [^{14}C]-methionine). It was assumed that these two peptides arose from the T6 sequence in some manner. No simple explanation for this phenomenon was apparent. Neither peptide arose from partial reaction of the T6 methionyl residue during carboxymethylation, nor did either peptide correspond to an unusual peptide found by Konigsberg *et al.* (1966) in digests of the almost identical f_2 coat protein. The latter peptide had a composition differing from that of T6 by valine replacement of methionine.

TABLE V: Summary of Peptide Identifications.

Peptide[a]	Final Identification	Origin of Peptide	Method of Identification[b]
T1 to T8	T1 to T8	From standard tryptic cleavage	*In vitro* coat protein preparations labeled with single [^{14}C]amino acid species
T9^{I}	T9	From standard tryptic cleavage	Doubly labeled *in vitro* protein ([^{3}H]lysine, and [^{14}C]threonine, or [^{14}C]isoleucine)
T9^{II}	T7–9	From inhibition of tryptic cleavage	
1	T9–5	From inhibition of tryptic cleavage	Tentative identification from peptide mapping positions
3	T7-9-5		
2,4	T6 "minor peptides"	Unknown	Consistently detected in the presence of radioactively labeled T6
5	T3-7	From inhibition of tryptic cleavage	Doubly labeled *in vitro* protein ([^{3}H]lysine, and [^{14}C]serine, or [^{14}C]arginine)

[a] Shown in Figures 2 and 3. [b] Described in the text.

TABLE VI: Incorporation of [^{14}C]Lysine into the Standard Tryptic Peptides of R17 Coat Protein Synthesized *in Vivo* and *in Vitro*.

	In Vivo Synthesis		*In Vitro* Synthesis			Ratio (cpm) *in Vivo*/*in Vitro*
Peptide[b]	cpm	Ratio[c]	cpm	Ratio[c]	Expected Ratio[d]	
T6	3553	1.00	1107	1.00	1.00	3.21
T5	3524	0.99	1210	1.09	1.00	2.91
T7	2333	0.66	1292	1.17	1.00	1.81
T8	3075	0.87	930	0.84	1.00	3.31
T9	2171	0.61	1135	1.03	1.00	1.91

[a] The raw data and descriptions of the different preparations are given in Table I. [b] The calculations of the lysine radioactivity in each of the standard tryptic peptides are based on the proposed spot identifications described in the text (also see Table V). T9^{II} was taken as being equivalent to (=) T7-9; T9^{I} = T9; 5 = T3-7; 2 and 4 = T6 minor peptides; 1 = T9-5; 3 = T7-9-5. Hence:

Peptide from Table VI		Peptides from Table I
T6	=	T6 + 2 + 4
T5	=	T5 + (0.5 × (1)) + (0.33 × (3))
T7	=	T7 + (5) + (0.5 × T9^{II}) + (0.33 + (3))
T8	=	T8
T9	=	T9^{I} + (0.5 × T9^{II}) + (0.5 × (1)) + (0.33 × (3))

[c] T6 was used as a standard to compare the radioactivity in the peptides. [d] Based on amino acid sequence data (see Figure 1).

A summary of peptide identifications is given in Table V.

Lysine Incorporation into the Standard Tryptic Peptides. The data for the coat protein preparations labeled with [^{14}C]-lysine (Table I) was reassessed in terms of total lysine content in each of the five standard tryptic peptides containing lysine. The proposed origins of minor peptide sequences were taken into account in estimation of lysine content (Table VI).

The *in vitro* preparation showed approximately equivalent incorporation into the five peptide sequences when T6 was used as an arbitrary standard. This result, plus the ability to account for the minor peptides as products of incomplete tryptic digestion, was interpreted as good evidence for a high level of translational fidelity in the cell-free system. The yields of the peptides T7 and T9 were somewhat less than anticipated for the natural coat protein sample. Subsequent experiments showed that the release of internal peptide sequences during tryptic digestion of milligram amounts of natural coat protein was slower than that of the carboxy-terminal peptides (D. J. W. Burns and P. L. Bergquist, 1968, unpublished data). Although all the peptides containing lysine are internal, the rate of release of T7 and T9 may be further reduced because tryptic cleavage in this region is usually inhibited. A prolonged time of digestion (20 hr) increases the yield of these two peptides.

The Nonstandard Peptides. The existence of the minor peptides found on the peptide maps cannot be accounted for by assuming a prematurely curtailed time of digestion instead of inhibition of complete tryptic cleavage. After 30-hr tryptic digestion all minor peptides were still present and there was an increase in the yields of some peptides known to result from chymotryptic-like cleavage (for example, the Ser-Arg dipeptide shown in Figure 6). The isolation of

several nonstandard tryptic peptides has been reported during amino acid sequence studies on R17 coat protein (Weber and Konigsberg, 1967). Most of these peptides resulted from chymotryptic-like cleavage in the large (38 residue) amino-terminal T11 peptide fragment. With the exception of T7–9, no peptides which arose from inhibition of tryptic cleavage at lysyl or arginyl residues were reported by these workers. Our results indicate that further inhibition of cleavage occurs in the same general region of the coat protein sequence as T7–9 giving rise to peptide T3–7, and possibly peptides T9-5, and T7-9-5. A number of other minor peptides were also detected both on natural coat protein fingerprints stained with ninhydrin, and on autoradiographs of radioactively labeled natural or *in vitro* protein. Presumably, some of these peptides correspond to the nonstandard tryptic peptides reported in the sequence studies. We suggest that the remainder are also nonstandard tryptic peptides which are detectable because of the high resolution of the mapping procedure.

The T4 Peptide. Although the peptides containing arginine have not been examined in detail in this paper, one anomaly is apparent from the data presented. The yield of T4 synthesized *in vitro* was always much lower (about 40%) than anticipated, whereas the yield of T4 was normal for the natural coat protein. There is some evidence that a proportion of the bacteriophage coat protein cistrons are incompletely translated *in vitro* (Nathans, 1965; Lin and Fraenkel-Conrat, 1967). During previous work we compared the *in vitro* incorporation of alanine and glycine into two internal peptide sequences and the carboxyl-terminal T1 sequence. We found that the proportion of incomplete *in vitro* coat protein chains (that is, less than 127 of 129 residues) is less than 10% (Bergquist *et al.*, 1968). Therefore, we believe that the low yield of T4 does not reflect the synthesis of incomplete coat protein subunits in the *E. coli* cell-free system. However, we have no alternative explanation for the reduced T4 yield. Nathans (1965), using autoradiography to detect peptides on paper chromatograms, found that one tryptic peptide from an *in vitro* system directed by MS-2 RNA was present in variable yields in different preparations. It is possible that the MS-2 peptide may have corresponded to T4 as MS-2 and R17 coat proteins have the T4 sequence in common (Lin *et al.*, 1967; Weber, 1967).

Conclusion

An examination of lysine incorporation into R17 coat protein synthesized in a cell-free system showed that the fidelity of *in vitro* translation is high when the radioactivity content of the minor peptides found on fingerprints is taken into account. All minor peptides which consistently appeared on autoradiographs of [^{14}C]lysine-labeled preparations were common to natural and *in vitro* protein. These minor peptides could be largely accounted for by assuming that tryptic cleavage was inhibited at certain sites in the protein chain. This is in agreement with previous conclusions as to irregularities of cleavage during tryptic digestion (Burns and Turner, 1967).

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