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Demonstration of Further Differences between *in Vitro* and *in Vivo* Synthesized MS2 Coat Protein*

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ABSTRACT: When MS2 ribonucleic acid (RNA) is used as messenger in the *Escherichia coli* cell-free system, most of the proteinaceous material synthesized is less acidic than the virus coat protein. When the small tryptic peptides of MS2 coat protein obtained from such material are analyzed for their content of *in vitro* produced components, the C-terminal peptide is found present in much lesser amounts than the other peptides.

The ability of viral RNA to stimulate [^{14}C]amino acid incorporation in extracts of *Escherichia coli* has been well documented (Nirenberg and Matthaei, 1961; Tsugita *et al.*, 1962; Nathans *et al.*, 1962). However, the definitive identification of the resulting identification of the resulting proteinaceous products has been more problematical. When TMV–RNA was used, little if any of the product resembled native or denatured TMV coat protein (Aach *et al.*, 1964; Tsung and Fraenkel-Conrat, 1965a). Using the RNA of several closely related coliphages, much more of the ^{14}C-labeled product remained associated with the added coat protein during various fractionation procedures (Nathans *et al.*, 1962; Tsung and Fraenkel-Conrat, 1965a; Nathans, 1965; Yamazaki and Kaesberg, 1966; Ohtaka and Spiegelman, 1963; Viñuela *et al.*, 1967). However, the realization that all products are believed to carry N-terminal formylmethionyl groups (Adams and Capecchi, 1966; Webster *et al.*, 1966) would lead one to predict that they should differ from the corresponding carrier proteins lacking this group, for instance by showing a slightly lower anionic mobility upon electrophoresis. Actually the present study has shown that most of the synthesized ^{14}C protein, when subjected to polyacrylamide gel electrophoresis in 8 M urea at pH 3.8, moves further toward the anode than the carrier protein.

An alternate method of identifying the products of cell-free protein synthesis resides in comparing the peptides resulting from their trypsin degradation with digests of the viral coat protein (Nathans *et al.*, 1962; Nathans, 1965). The present study has employed column chromatography rather than two-dimensional paper mapping for this purpose. Clear evidence was obtained that most of the incorporated label which remained associated with coat protein upon DEAE Sephadex chromatography, *i.e.*, 20% of the total, remained associated with those characteristic peptides of the MS2 digests which can readily be isolated and further purified. However, the bigger peptides do not lend themselves to easy chromatographic purification. Thus neither the N-terminal peptide, containing 38 residues, nor its formylmethionyl derivative have, as yet, been identified among the products. The C-terminal hexadecapeptide has been detected, but only about 40% as much was found to be synthesized of this peptide than of the small peptides.

On the basis of these two sets of data it is suggested that much or most of the coat protein formed under

* From the Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720. *Received June 12, 1967.* This investigation was supported by Research Grant GB 3017 from the National Science Foundation.

† Taken in part from a dissertation submitted by J.-Y. L. in partial satisfaction of the requirements for the degree of Doctor of Philosophy, Department of Molecular Biology, University of California, 1967.

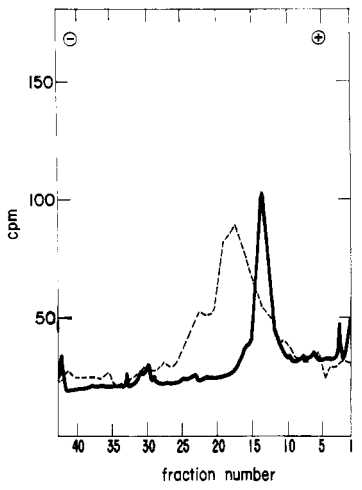

FIGURE 1: Polyacrylamide gel electrophoresis of reaction products and carrier protein (Duesberg and Rueckert, 1965). The monomer solution contained 4 g of ethylene diacrylate as cross-linker (Choules and Zimm, 1965) and 15 g of acrylamide in 50 ml of 8 M urea. The pH 3.8 buffer concentrate consisted of 100 ml of 8 M urea, 3 g of KAc, 12 ml of HAc, and 2.5 ml of tetramethylethylenediamine. The monomer (1 ml), buffer (0.4 ml), and 10 M urea (4.3 ml) were deaerated mixed with 1.8 ml of 0.3% ammonium persulfate, and 1 ml of this was polymerized/tube at 45° (30 min) (5 × 0.5 cm). Electrophoresis at 120 v, or 5 ma/tube, at room temperature for 30–60 min. The extruded gels were stained with Amido Black (1% solution 7% HAc), and the excess dye was removed by diffusion in 7% HAc. The stained bands were recorded by means of a Joyce-Loebl microdensitomer. The gels were then sliced into $^1/_{16}$-in. slices, which were placed in scintillation vials with 1 ml of a solution consisting of one volume of 10% hydroxide of Hyamine (10 − x) and 9 ml of 1 M piperidine. The vials were closed and shaken for 1 hr at 37° prior to addition of 10 ml of Bray's solution and counting. Slice 1 includes the origin. Dashed line: radioactivity; solid line: Amido Black tracing, the location of the peak being confirmed visually.

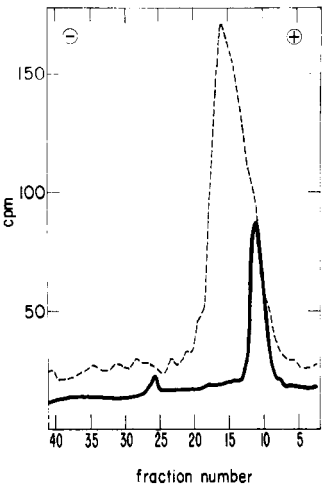

FIGURE 2: Same as Figure 1, with carboxymethylated products and protein.

our experimental conditions in cell-free extracts differs not only in regard to its N-terminus, but also in regard to its C-terminal sequence, the latter alteration causing it to be less acidic and probably either shorter or longer than the functional MS2 coat protein.

Experimental Section

S-30 extracts of *E. coli* A-19 were prepared by standard procedures (Nirenberg and Matthaei, 1961). The extract was preincubated (20 min at 37°) in 0.01 M pH 7.8 Tris, 0.01 M $MgAc_2$, 0.03 M NH_4Cl, and 0.006 M mercaptoethanol. The final reaction mixture contained per milliliter: 0.25 ml of S-30, 10 μmoles of $Mg(AC)_2$ 3 μmoles of ATP,[1] 0.2 μmole of GTP, 5 μmoles of phosphoenolpyruvate, 20 μg of pyruvate kinase, 75 μmoles of NH_4Cl, 50 μmoles of pH 7.8 Tris, 10 μmoles of glutathione, and 0.04 μmole of each except the labeled amino acid (2 μc, 0.01 μmole). The incorporation mixtures were shaken at 37° for 40 min unless otherwise stated. For determination of incorporated radioactivity, the proteins were precipitated with 5% TCA at 90° for 15 min, filtered on Millipore filters (type AA), washed with 5% TCA, and counted on planchets in the gas-flow counter or in the scintillation spectrophotometer. If little protein were present 0.1 mg of BSA was added prior to TCA precipitation. Polyacrylamide gel electrophoresis was performed by the method of Duesberg and Rueckert (1965). For details, see legend to Figure 1.

The isolation of coat protein like material from [^{14}C]alanine containing incorporation mixtures was performed as follows. To the mixture (1 ml) was added 3 ml of 10 M urea, 1 ml of 1 M pH 8.6 Tris, 0.1 ml of mercaptoethanol, and 1.5–15 mg of coat protein. Frequently the SH groups of the proteins were aminoethylated or carboxymethylated at this stage (Lin *et al.*, 1967). Macromolecules were twice precipitated with two volumes of ethanol, treated with RNase (50 μg) for 1 hr at 37°, and dialyzed against 0.002 N KOH at 4° overnight. The lyophilized material was then passed through a column (2.5 × 60 cm) of DEAE Sephadex 50A, bead form, with 0.004 M pH 7.8 Tris in 8 M urea, developing it with an exponential gradient of 0–0.25 M NaCl in the same solvent (20 ml/hr, 5-ml fractions). The tubes containing the coat protein and most of the radioactivity in a single peak (tubes 21–27) were pooled, dialyzed, lyophilized, and the protein

[1] Abbreviations used: ATP, adenosine triphosphate; GTP, guanosine triphosphate; TCA, trichloroacetic acid; BSA, bovine serum albumin; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

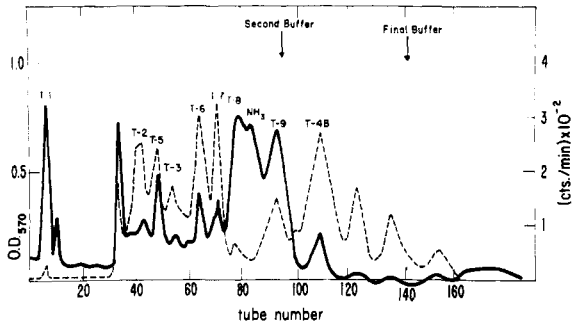

FIGURE 3: Pattern of soluble tryptic peptides of carrier and purified incorporation products with [^{14}C]lysine and [^{14}C]arginine. Solid line: ninhydrin color; dashed line: radioactivity on 0.2 ml/2-ml fraction.

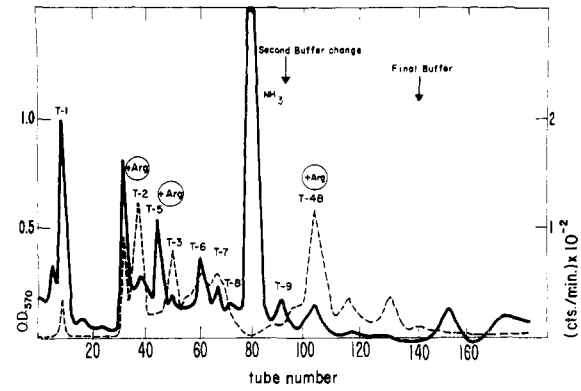

FIGURE 4: Same as Figure 3 with only [^{14}C]arginine; 0.3 ml/2 ml counted; arginine-containing peptides indicated.

was twice dissolved in dilute alkali and precipitated at pH 8 at 0°. It then showed an A_{max} at 278 mμ and a minimum of 243 mμ (max/min = 1.70). The [^{14}C]-lysine, -arginine, and -tyrosine reaction products were not fractionated on Sephadex columns.

The protein was digested at 37° with TPCK–trypsin (1:50) in a pH-Stat. The digest was brought to pH 4.0 with 1 N HAc and centrifuged. The soluble fraction was lyophilized, redissolved at pH 2.0, and subjected to peptide fractionation on an Amberlite IR-120 column (0.9 × 15 cm) utilizing the automatic amino acid analyzer and an autograd (Tsung and Fraenkel-Conrat, 1965b) (temperature 52°, flow rate 60 ml/hr). One-twentieth was analyzed by ninhydrin and the rest was passed through a fraction collector, and the radioactivity was determined in each fraction.

Results

The addition of 0.02–0.2 mg of MS2 RNA to 1 ml of incorporation mixture caused an approximately linear increase in incorporation of [^{14}C]lysine and [^{14}C]arginine, which in a typical experiment reached 37-fold that of the control sample lacking RNA. The incorporation of [^{14}C]alanine was similarly increased, but that of [^{14}C]histidine only a tenfold.

The gel electrophoretic pattern of the mixture of products (obtained with 0.125 mg/ml of MS2 RNA) consisting to about 75% of the intact 28S molecule and unlabeled coat protein, with or without prior carboxymethylation (Lin *et al.*, 1967) in 8 M urea, are shown on Figures 1 and 2. It is evident that in both cases the main radioactive peak had progressed appreciably further toward the cathode than the carrier protein peak. This was in contrast to the behavior of the aminoethylated material (Lin *et al.*, 1967) which moved very rapidly through the gel at pH 3.8, and under such conditions showed a near coincidence of carrier and ^{14}C products, the sharp maxima occurring in adjacent fractions. The facility with which misleading results can be obtained by this technique is thereby illustrated.

The broad peak of biosynthesized protein differs from the patterns obtained by Nathans *et al.* (1966) and others. The possibility must be considered that the use of buffers containing anionic detergents makes polyacrylamide gel act mainly as a molecular sieve, and that only in neutral denaturants, such as urea, does it show electrophoretic discrimination, and thus reflects the charge heterogeneity of the biosynthesized proteins which are minimized by the anionic detergents.

The radioactive peptide patterns given by tryptic digests of purified coat protein like material are shown in Figures 3–6. It is evident that with [^{14}C]lysine and arginine as labels all peptides except T1, which lacks these amino acids, appear to be labeled, whereas, the use of only one [^{14}C]amino acid, be it arginine, alanine, or tyrosine, leads to label appearing predominantly in the peptides containing the respective amino acid. However, the presence of other proteins in this coat protein like material is indicated by the appearance of a few nonidentifiable labeled peptide peaks. The correspondence is better with the more carefully purified [^{14}C]alanine-containing products (Figure 5), than with the others.

Since previous attempts to determine the sequential order of labeling from the N terminus to the C terminus (Nathans, 1965) were handicapped by incomplete knowledge of the sequence of peptides in the MS coat protein it was attempted to obtain definitive data on this question, now that the complete amino acid sequence of this protein is known (Weber *et al.*, 1966). Two incorporation mixtures containing MS2 RNA (0.125 mg/ml) were prepared, differing only in that in one [^{3}H]alanine was diluted after 5 min by a 100-fold of unlabeled alanine, while in the other only [^{14}C]alanine was present throughout. After 40 min at 37° the two samples were mixed, and subjected to the usual aminoethylation, ribonuclease, and alkali treatments, followed by DEAE Sephadex fractionation and isoelectric precipitation as described above. Table I gives the recovery of ^{3}H and ^{14}C throughout this purification procedure. The fact that of the [^{3}H]-

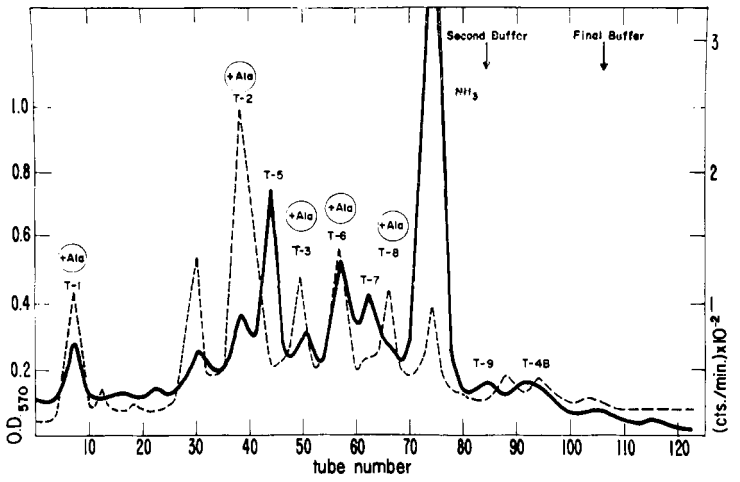

FIGURE 5: Same as Figure 3 with [^{14}C]alanine; 0.2 of 2.5 ml counted; alanine-containing peptides indicated.

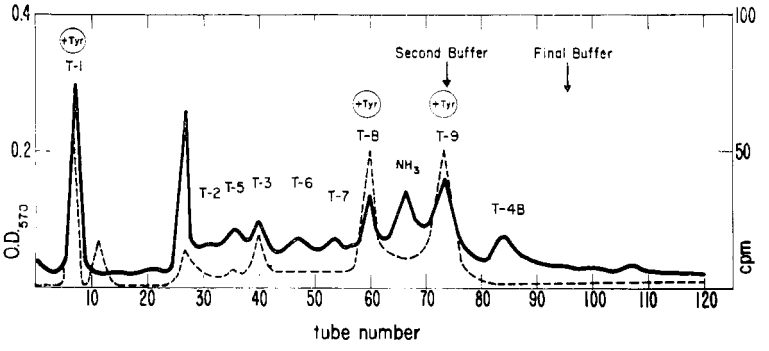

FIGURE 6: Same as Figure 3 with [^{14}C]tyrosine; 0.35 of 2.5 ml counted; tyrosine-containing peptides indicated.

alanine incorporated in 5 min about 40% was found in the purified coat protein fraction, slightly more than after the 10-min pulse or the 40-min incorporation with [^{14}C]alanine, suggests that most of the coat protein is finished in the first 5 min.

The doubly labeled protein was trypsin digested. The soluble fraction of the peptides contained about 80% of the total counts, although only 64% of the alanine is located in the soluble peptides of the protein. Fractionation of these peptides yielded the typical pattern (Figure 5) with most of the counts associated with peptides T1, T2, T3, T6, and T8. In one experiment each of these peptides was further purified by paper chromatography (butanol–acetic acid–water–pyridine, 30:6:24:20). In each instance the main or one of the main radioactive peaks coincided with the carrier peptide, detected by ninhydrin or, for T3, by the starch iodine test. The purity and recovery of each of these peptides are indicated by their amino acid compositions, which together with their ^{3}H and ^{14}C contents are listed on Table II. For obvious reasons the additional purification step causes losses. When the ratio of ^{3}H:^{14}C counts is plotted against the location of each peptide along the chain (Figure 7), a slope is noted which conforms to expectation in that the relative ^{3}H content decreases from the N terminus toward the C terminus. These differences, however, are not great—presumably because 5 min is too long a pulse, a high proportion of the coat protein being finished at that time. However, a new fact seems to emerge from these

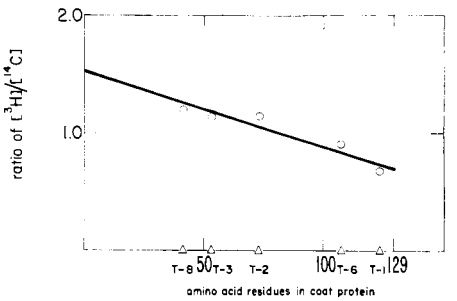

FIGURE 7: Sequential synthesis of MS2 coat protein. The ^{3}H pulse:^{14}C 40-min alanine incorporation data from Table II plotted against the approximate location of the alanine residues on the peptide map (Lin *et al.*, 1967).

TABLE I: Purification of *in Vitro* Proteins with Carrier Coat Protein.

Stage of Purification	Total Incorporated Radioactivity (cpm × 10^{-4})					
	5-min [^{3}H]Alanine Pulse				10-min [^{3}H]Alanine	
	Preparation A[a]		Preparation B[a]		Preparation C[a]	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
1 Starting material	280	174	922	68	820	230
2 Aminoethylation, alcohol precipitation	194	170				
3 Ribonuclease and alkali treatment	190	170			450	120
4 DEAE Sephadex fractionation	150	160	600	65	330	66
5 Isoelectric precipitation						
First	80	81	340	46	240	84
Second	60	66	230	28	160	34
6 Soluble fraction in tryptic digest	52	58	180	22	130	25

[a] Preparation A contained 0.125 and 1.5 ml of the [^{14}C]alanine- (40-min incorporation) and [^{3}H]alanine-pulsed reaction mixtures, respectively; B and C contained 0.5 ml of each component.

TABLE II: Characterization of Purified Alanine Containing MS2-Coat Protein Peptides Isolated with Incorporation Mixture (5-min [^{3}H]Ala pulse, 40-min [^{14}C]Ala).[a]

Peptide	Composition[a]	cpm/μmole of Peptide	^{3}H:^{14}C	cpm/μmole of Ala
T8	$Ser_{0.8}Glu_{1.1}\mathit{Ala}_{1.0}Tyr_{0.6}Lys_{0.9}(Gly_{0.1})$	^{3}H: 12,500		12,500
		^{14}C: 10,400	1.20	10,400
T3	$Asp_{1.1}Ser_{1.3}Glu_{2.1}\mathit{Ala}_{1.0}Arg_{1.2}(Gly_{0.1})$	^{3}H: 12,100		12,100
		^{14}C: 10,700	1.13	10,700
T2	$Thr_{1.9}Glu_{2.1}Pro_{1.0}Gly_{2.0}\mathit{Ala}_{3.0}Val_{3.9}Leu_{1.1}Arg_{0.9}(Ser_{0.2}$-$Ile_{0.1})$	^{3}H: 37,200		12,400
		^{14}C: 32,400	1.15	10,800
T6	$Glu_{1.0}Gly_{1.0}\mathit{Ala}_{1.0}Met(O)_{0.8}Leu_{1.9}Lys_{1.0}(Ser_{0.1}Val_{0.1})$	^{3}H: 9,400		9,400
		^{14}C: 10,300	0.91	10,300
T1	$Asp_{2.9}Ser_{1.8}Pro_{1.9}Gly_{2.1}\mathit{Ala}_{3.0}Ile_{2.9}Tyr_{0.9}(Glu_{0.1})$	^{3}H: 9,600		3,200
		^{14}C: 13,900	0.68	4,600

[a] Not corrected for destruction of serine, etc.: Met (O) represents the sum of methionine and its sulfoxide; traces below 0.1 mole are not listed; Trp (occurring in T2) was not analyzed for. The recovery of peptides, derived from the composition ranged from 12 to 14%, except for T6 (8%).

data if the absolute amount of radioactivity, both as ^{14}C or ^{3}H, per micromole of recovered peptide-bound alanine is considered. For it then appears that peptides T8, T3, T2, and T6 are labeled at a similar level, but that the alanines in T1, the C-terminal peptide, carry in average only about one-third as much label (Table II, last column). A similar though quantitatively lesser depression of label in the C-terminal peptide was observed by Nathans (1965).

The finding that less label is associated with the C-terminal carrier peptide than with the others (the N-terminal T11 not having been studied in these experiments) can be interpreted in various ways. Some of these hypotheses would also supply explanations for the different gel electrophoretic behavior of the labeled as compared to *bona fide* MS2 coat protein. Thus, it is possible that the mechanism of chain termination is malfunctioning, resulting in shorter or longer chains which might well move faster upon gel electrophoresis, and yield less T1 after tryptic digestion. Alternatively, the C-terminal peptide might be correctly produced, but might be particularly susceptible to the proteases or peptidases which were definitely shown to be operative in the *E. coli* extracts. Thus unpublished data by C.-M. Tsung have shown the appearance of degradation products, noticeable in a sucrose gradient, upon incubation of MS2 coat protein with the typical incorporation mixture for 30 min, as well as the complete degradation of an added tripeptide into its component amino acids.

Of the radioactivity added to the gels in the experi-

ment illustrated on Figures 1 and 2, about 6% was found associated with the carrier protein which corresponded to about 20% of the total radioactivity recovered from the gels. From the recovery of the T1 peptide (Tables I and II), as related to the total incorporated radioactivity, it can be calculated that about 7% of that amount (40% of 18%) is present in complete MS2 coat protein, if one disregards the N-terminal situation.

It is evident that the *in vitro* synthesis of complete proteins remains problemetical and, at best, inefficient, although it must be noted that the experimental conditions, with particular reference to the magnesium concentration have been arbitrarily adopted from previous related studies and were not optimized in regard to product fidelity in the present experiments.

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