line, phenylalanine → serine), as would be expected for those exchanges caused by definite chemical reactions (e.g., deamination), but not for spontaneous and/or random changes in the coding behavior of bases, which are held to be responsible for some of our and Wittmann's data.

However, as stated, the testing of our virus preparations has been plagued by a considerably higher level of spontaneous mutation or contamination in the past 2 years than at earlier stages of this study. Thus we cannot safely assume that all mutants isolated are actually the consequence of the chemical agent to which the RNA (or the virus) was exposed. It is for this reason that we have not isolated new mutants produced by the low-efficiency mutagenic reactions (bromination or methylation) but have concentrated our efforts on nitrous acid mutants which with high probability are the actual result of deamination. The localization of exchanges was in good part performed on mutants isolated several years ago. The preparation of progeny of such mutants showing the original amino acid exchanges has not been affected by the problems encountered with the isolation of new mutants.

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Further Attempts to Characterize Products of TMV-RNA-directed Protein Synthesis*

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Radioactive peptides or proteins synthesized in a cell-free *E. coli* amino acid–incorporating system under the direction of TMV-RNA were characterized in order to assess possible similarities to TMV-specific products. Chromatographic separations of proteins and tryptic peptides as well as serological methods were used to determine whether TMV-coat protein or other unknown TMV-specific protein products were formed. No clear evidence was obtained that a significant fraction of the amino acids incorporated in soluble proteins or peptides could be identified with such products.

In some preceding experiments the thesis was tested whether purified TMV-RNA could direct the cell-free synthesis of TMV protein in *Escherichia coli* extracts under conditions optimal for amino acids incorporation (Tsugita *et al.*, 1962). Different methods used in those experiments for the identification of the products which were synthesized by the *E. coli* system gave somewhat conflicting results. While one serological experiment as well as certain chemical tests seemed to support that thesis, reconstitution experiments with the *in vitro*–synthesized proteins failed to do so. Therefore the possibility was considered that under our experimental conditions protein might be synthesized *in vitro* in such a manner that it was unable to form its proper 3-dimensional structure. This would be expected if the product of cell-free protein synthesis represented either denatured or incomplete TMV protein.

Additional experiments employing both chemical and serological methods have now been performed to attempt substantiation of the conclusion that soluble products synthesized by the *E. coli* system were related to TMV-coat protein, or to other proteins synthesized in TMV-infected tobacco leaf cells. The results of these experiments were essentially negative, so that the nature of the proteins synthesized in the cell-free *E. coli* system under the influence of TMV-RNA must at present be regarded as uncertain.

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Results

Chromatographic Separation of ^{14}C-Labeled Protein Products from Native TMV Proteins.—The experimental conditions used for incorporation of labeled amino acids were in principle the same as used in previous studies (Nirenberg and Matthaei, 1961). However, both the preparations of DNAase-treated "preincubated S-30" *E. coli* extracts and the components of reaction mixtures were modified as described recently (Nirenberg, 1963). [^{14}C]Amino acid–incorporation reaction mixtures were incubated at 37° for 90 minutes, and then were chilled and diluted with equal volumes of cold buffer containing 0.01 M Tris, *p*H 7.8, 0.014 M magnesium acetate, 0.05 M KCl, and 0.006 M mercaptoethanol. The diluted reaction mixtures then were centrifuged at 105,000 *g* for 2 hours at 0°. The ribosomal pellets were discarded since only the [^{14}C] protein products in supernatant solutions were used in this study. These solutions were stored and shipped at −60 to −80°. Insoluble material appearing upon thawing was centrifuged off. Native TMV protein was then added to the solution (about 3–5 mg/ml) and reisolated by two cycles of isoelectric precipitation (*p*H 4.6). The material sometimes contained considerable amounts of RNA at this stage. It was therefore treated with pancreatic RNAase (about 4 μg/ml original reaction mixture, 1–4 hours, 25°) and passed through a Sephadex G25 or G50 column with water as eluent. The first ultraviolet-absorbing material to emerge usually showed a typical protein spectrum corresponding in amount to 50–80% of the added virus protein, and contained in different experiments from 400 to 6000 cpm/mg, based on the absorbance of 1.3 (at 280 mμ) for an 0.1% solution of TMV protein. The TMV protein reisolated from control reaction mixtures (incubated either without added RNA, or with yeast RNA) contained from 30 to 70% of the counts found in corresponding reaction mixtures incubated with TMV-RNA.

Some of the protein so isolated was treated with bentonite to free it from RNAase (Singer and Fraenkel-Conrat, 1961), and then used in reconstitution experiments with TMV-RNA. As previously noted (Tsugita *et al.*, 1962), poor yields in infective and sedimentable virus were obtained. Upon repeated ultracentrifugation of this reconstituted virus the radioactivity disappeared from the pelletable material.

The bulk of the protein was subjected to purification. Upon DEAE-cellulose chromatography at *p*H 6.8 native TMV protein appears soon after the holdup volume. Elution with *p*H 9.5 carbonate yields a small peak, and upon addition of 0.1 N NaOH a final peak of varying height appears which is believed to be due to denatured material because its amount seems to be a function of the age and storage conditions of a given preparation of the protein (Fig. 1, top pattern). It is evident from the other elution patterns shown on Figure 1 that most of the [^{14}C]serine-containing products of TMV-RNA and yeast RNA–induced protein synthesis are eluted only by alkali. The native protein is almost free of counts and the small peak that is eluted at *p*H 9.5 (probably representing partly denatured carrier protein) contains only a small fraction of the counts. This was found the case also for incorporation mixtures containing ^{14}C-labeled phenylalanine or tyrosine. The radioactivity remaining associated with the native protein was maximally 0.2% of that subjected to fractionation. We are thus forced to conclude from these experiments that the product of cell-free protein synthesis which follows

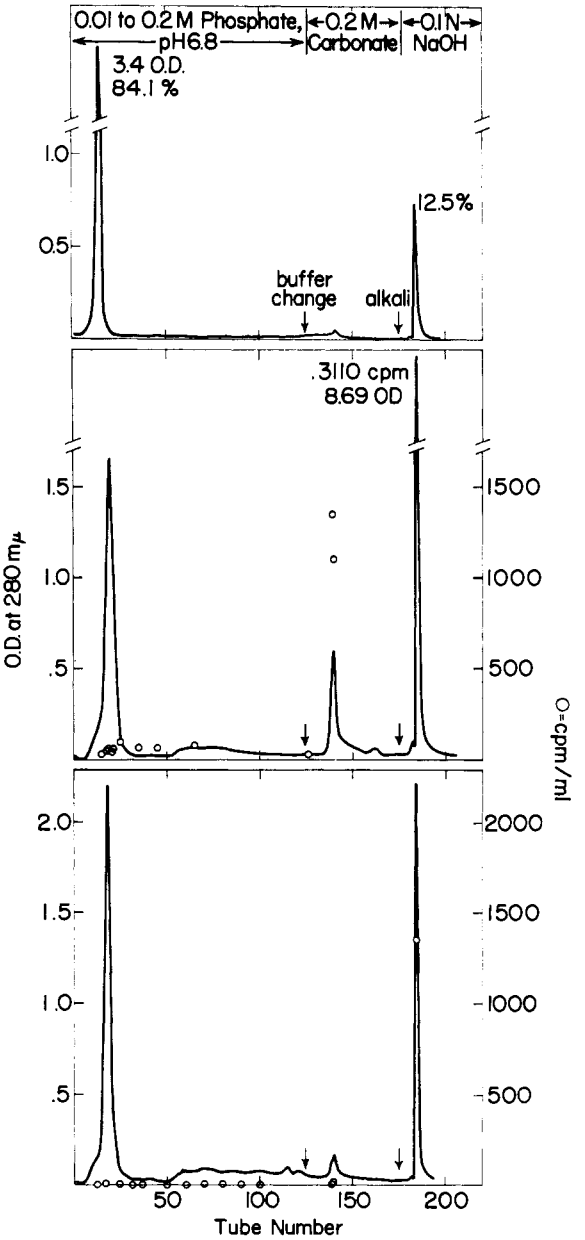

FIG. 1.—Chromatographic separation of native TMV protein from *p*H 4.5-insoluble biosynthetic macromolecular material. To a DEAE-cellulose column (1.1 × 13 cm), equilibrated with 0.01 M, *p*H 6.8 sodium phosphate, was added the protein sample (about 30 mg) which had been equilibrated by dialysis against the same buffer. The column was developed at 4° with a linear gradient of 0.01–0.2 M, *p*H 6.8 phosphate (200 ml each), followed by 0.2 M, *p*H 9.5 carbonate-bicarbonate and finally 0.1 N sodium hydroxide. The absorbance at 280 mμ was determined for all fractions (3 ml). The top graph represents the elution pattern for a typical preparation of TMV protein showing the main peak of native protein and a small amount of denatured material eluted only by alkali. The two graphs below are for carrier-TMV protein, reisolated from a TMV-RNA and a yeast RNA containing amino acid–incorporation experiments [^{14}C] serine, respectively. These show greater but varying amounts of ultraviolet-absorbing material eluted only with alkali. The radioactivity (cpm/ml, indicated by open circles) was associated almost exclusively with these latter peaks.

TMV protein through 2 isoelectric precipitations as well as Sephadex chromatography can be separated from it by DEAE-cellulose chromatography.

Characterization of Tryptic Peptides of TMV Protein and ^{14}C-Labeled Product.—In view of the separa-

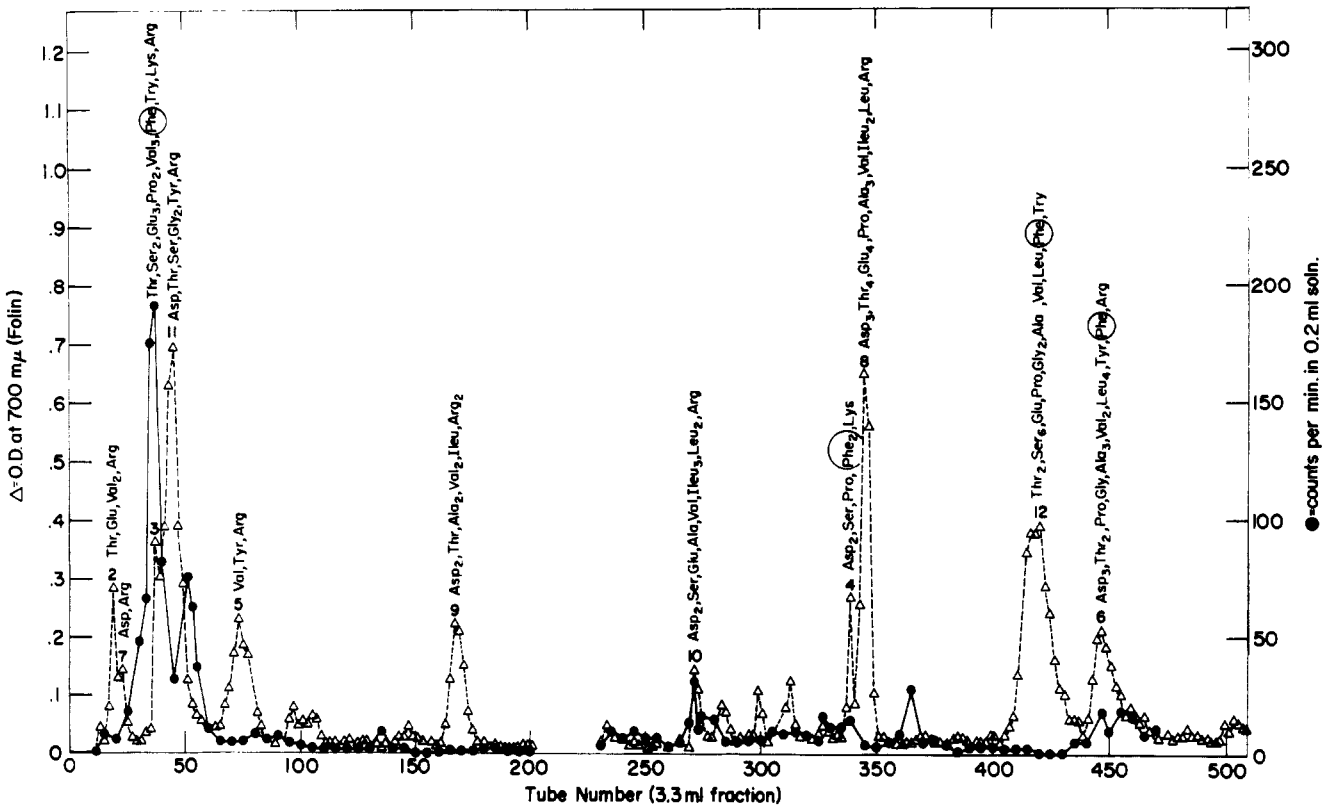

FIG. 2—Chromatographic separation of tryptic peptides of a mixture of TMV protein with the *p*H 4.5-insoluble biosynthetic macromolecular material. The Dowex 1 × 2 separation is done according to Funatsu (1964). The typical TMV-peptide peaks detected by Folin color (Δ) are identified by their position. Tubes 200–250 were not collected due to mechanical difficulties. No TMV tryptic peptides are eluted in this region. The numbers above the peaks indicate the sequential position of each peptide on the amino acid–sequence map. (Peptide 1 is removed from the digest by precipitation at *p*H 4.6 prior to chromatography.) The amino acid composition of each typical peptide is given, circling the content of phenylalanine, since [^{14}C] phenylalanine was used as label for the amino acid incorporation in this experiment. The location of the only marked radioactivity peaks located in the vicinity of peptide 3 (as shown by solid circles, ●) was also noted with [^{14}C] serine as label, and that radioactivity was separated from peptide 3 by the 2-dimensional mapping.

bility of native TMV protein from the radioactive material the working hypothesis was entertained that the biosynthesized material represented largely denatured TMV protein. This seemed possible since amino acid analysis of the small amount of material in the ^{14}C-labeled peak eluted at *p*H 9.5 (Fig. 1, middle pattern) indicated a composition similar to that of TMV protein, even though it was eluted from the column much later than that carrier protein. To test this possibility, the radioactivity peaks of [^{14}C]phenylalanine, tyrosine, or serine-labeled reaction mixtures were again mixed with carrier TMV protein, and the mixtures were digested with trypsin in the usual manner (Tsugita *et al.*, 1960). The *p*H 4.5 insoluble peptide 1 (the peptides are numbered sequentially starting with the acetyl-N-terminal peptide) was separated and was found to contain, after repeated isoelectric precipitations, from 30 to 50% of the total radioactivity. The soluble peptides of the digest were separated by an improved technique of Dowex 1 chromatography (Funatsu, 1964). If part or all of the ^{14}C-labeled material were identical with TMV protein then some or all of the peptides containing the labeled amino acid under investigation should be labeled in proportion to their content of that amino acid. As illustrated by one experiment with [^{14}C]phenylalanine (Fig. 2), most of the counts occur in the approximate position of peptide 3. This was the case also with serine as the labeled amino acid. The absence of appreciable counts from other typical phenylalanine- or serine-containing peptide peaks differs from the results of earlier experiments based upon an elution schedule which did not separate the peptides as well as the system employed in this study. The present data suggest that most of the product is not structurally related to TMV-coat protein.

The question remained whether the radioactive peak coinciding with peptide 3 actually represented peptide 3. Rechromatography in butanol–acetic acid–water–pyridine (40:6:24:20) gave partial separation of the ^{14}C material, as detected by radioautography, from peptide 3 and the conclusion that these were not identical was clearly substantiated by subsequent electrophoresis.

It appears surprising that the [^{14}C]phenylalanine- as well as the [^{14}C]serine-containing peptides present in the *p*H 4.6–soluble fraction of these tryptic digests chromatograph on Dowex predominantly as single peaks at least one of which, carrying the phenylalanine label, appeared to consist of one main component, also upon paper chromatography and electrophoresis, and which was not identifiable with any tryptic fragment of TMV protein. The position of this material in the elution pattern suggests that it represents basic peptides. Further studies of the nature of this and other labeled peptides are in progress.

Most of the radioactive material which is insoluble at *p*H 4.6 remained associated with peptide 1 during the reduction and aminoethylation of its cysteine residue (Tsung and Fraenkel-Conrat, 1963). Upon subsequent tryptic digestion and Dowex 1 × 2–column separation of the products, the label was dispersed almost equally over all tubes.

Serological Tests for Denatured TMV Protein.—

TABLE I
PRECIPITIN REACTION OF ^{14}C-LABELED PROTEIN PRODUCT WITH ANTISERUM AGAINST DENATURED TMV PROTEIN

RNA Added to Reaction Mixture:	[^{14}C]Phenylalanine TMV-RNA	[^{14}C]Phenylalanine Yeast-RNA	[^{14}C]Arginine TMV-RNA	[^{14}C]Arginine Yeast-RNA
Protein-bound [^{14}C]amino acids (cpm/ml)[a]	28,800 (100%)	13,700 (100%)	11,600 (100%)	5,200 (100%)
Washed immune precipitate				
(a) Without preceding Sephadex treatment of the reaction mixture (cpm/ml)[a]	10,900 (39.3%)	10,500 (77.0%)		
(b) After purification of the reaction mixture on a Sephadex column (cpm/ml)[a]	2,900 (10.6%)	2,080 (15.2%)	850 (6.7%)	670 (12.9%)

[a] Based on volume of original incorporation mixture, diluted 1:1, and centrifuged to remove ribosomes.

Serological precipitation tests, similar to those reported in an earlier paper (Tsugita *et al.*, 1962), were performed with the exception that a strong antiserum against denatured TMV protein was used rather than an antiserum against the virus. This should be advantageous for the purpose of detecting small amounts of biosynthesized TMV protein or denatured TMV protein.

The protein used as antigen was a preparation which had partly precipitated during a long period of cold storage. It was treated with 0.1% mercaptoethanol in 6 M urea and renatured by the treatment indicated by Anderer (1959), but it differed from native TMV protein in being partly insoluble in 0.45% NaCl solution. In line with older observations (Aach, 1959; Takahashi and Gold, 1960), the antisera gave stronger precipitates with the homologous protein and with native TMV protein than with the same amounts of TMV.

Proteins were isolated from amino acid incorporation mixtures after ribosomes had been removed by centrifugation as described earlier. Frozen solutions were thawed and small sediments were separated by centrifugation. Each supernatant was treated with 10 μg pancreatic RNAase per ml solution to destroy RNA. The sediment, which might contain denatured proteins, was solubilized by dialysis from 8 M urea solution, digested by RNAase as given, and added to the supernatant after addition of 0.3 mg of unlabeled TMV protein. Some of the samples were fractionated by means of a Sephadex G-50 column which was equilibrated with 0.2% NaCl buffered with 0.002 M sodium phosphate at *p*H 7.6. The early effluent fractions containing predominantly the proteins (as indicated by their ultraviolet-absorption maxima) were combined and reduced to the starting volume in a desiccator.

Antiserum (0.5 ml) against denatured TMV protein was added in an ice bath. After thorough agitation the mixture was kept in a refrigerator and occasionally shaken. After 48 hours the precipitate was centrifuged and washed five times by centrifugation using buffered saline. The washings were continued three times on a Millipore filter. The remaining protein was redissolved in 0.1 M NaOH, reprecipitated with trichloroacetic acid, washed, and plated as indicated by Siekevitz (1952). The supernatant of the immune precipitation was treated a second and a third time with 0.3 ml of the same antiserum until no precipitation occurred. The combined precipitates were washed, dissolved, reprecipitated by trichloroacetic acid, washed, and plated as before. To avoid errors by self-adsorption of the protein precipitates, they were redissolved and checked by counting in a liquid scintillation spectrometer. These counts were consistently 210–220% of the plated counts.

Table I summarizes the results of two of our experiments in which [^{14}C]amino acids were used as indicators. The data show that TMV-RNA stimulated more

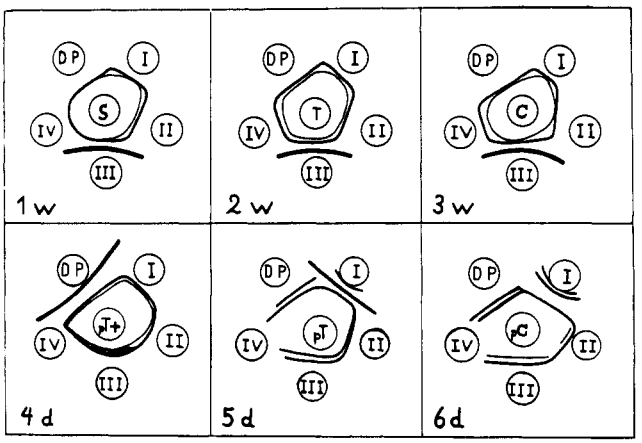

FIG. 3—Typical immune-precipitation patterns obtained on Ouchterlony plates. For patterns 1–3, denoted by w, antisera against plant-tissue fractions obtained 3 weeks after inoculation were used; for patterns 4–6, denoted by d, that time was 3 days. Numbers I–IV denote the wells containing antisera against the various types of tissue fractions (see text), DP denotes the antiserum against denatured TMV protein. The *E. coli*—incorporation mixtures containing TMV-RNA and [^{14}C] serine (S), or tyrosine (T), or the macromolecular components of the latter (pT), or the same plus added TMV protein (pT+), or the corresponding [^{14}C] tyrosine control incorporation mixtures lacking TMV-RNA (C, pC) are placed in the center wells. A total of 0.25 ml of the undiluted antigens and antisera were pipetted in five 0.05-ml aliquots into each well. Of the antisera, no. III, obtained from 3-week-infected plants and no. I from 3-day-infected plants gave precipitates with all antigens which were absent from the other antisera.

[^{14}C]amino acid incorporation into protein than did yeast RNA. However, TMV antiserum precipitated proportionally fewer [^{14}C]protein counts, compared to total counts incorporated, when added to the reaction mixtures containing proteins synthesized in the presence of TMV–RNA than when added to the controls containing yeast RNA. The present results therefore, gave no indication for the *in vitro* synthesis of TMV protein, native or denatured, by the *E. coli* system subjected to the influence of purified TMV-RNA.

Serological Tests for TMV-specific Proteins Other than the Coat Protein of the Particle.—It appears probable that TMV infection induces within the plant cell the synthesis of other proteins simultaneously or prior to the synthesis of the TMV-coat protein. To test for such products in the *E. coli in vitro* system directed by TMV-RNA, the Ouchterlony gel-diffusion technique was employed. A batch of Connecticut Broadleaf tobacco plants was harvested 3 days after thorough inoculation with common TMV; another batch of the same kind of plants was harvested 3 weeks after the inoculation. The leaves of the two plant groups were

homogenized in a cold room using a Waring Blendor, and fractionated by differential centrifugation (Aach and Melchers, 1957), without discarding any part of the leaf brei. Fraction I, sedimenting by centrifugation at 1500 × *g*, was rich in chloroplasts. Fraction II (13,000 × *g*) was rich in mitochondria, fraction III (45,000 × *g*) contained most of the microsomes, and fraction IV contained the unsedimented proteins. Antisera to these four fractions of the two batches of infected plants were obtained by injecting rabbits intravenously eight times on alternating days, followed by two subcutaneous injections with Freund's adjuvants. The rabbits were bled 4 weeks after the last injection. The antisera to tobacco fractions I–IV were also termed I–IV, respectively.

Each of the eight antisera against fractions of infected plants and one antiserum against denatured TMV protein was tested in agar plates of 5 cm diameter against the following five antigens: (1) soluble protein labeled with [^{14}C]tyrosine in the presence of TMV-RNA (5 ml of the diluted 100,000 × *g* supernatant solution was treated with pancreatic RNAase, and incubation was followed by two consecutive separations on Sephadex G-50 and by reduction of the final volume to 5 ml [pT]); (2) soluble protein labeled with [^{14}C]tyrosine in the absence of TMV-RNA and isolated as described under (1) (pC); (3) centrifuged and diluted incorporation mixture containing soluble protein, labeled with [^{14}C]tyrosine in the presence of TMV-RNA (T) (the protein was not purified as described under [1]); (4) centrifuged and diluted incorporation mixture, containing soluble protein, labeled with [^{14}C]serine in presence of TMV-RNA (S) (the protein was not purified); (5) centrifuged and diluted incorporation mixture, containing soluble protein control, labeled with [^{14}C]tyrosine in absence of TMV-RNA (C) (the protein was not purified).

A few typical patterns of the precipitin bands are shown in Figure 3. All antisera showed some precipitation bands with all the incorporation mixtures (possibly attributable to antibodies against bacterial proteins) with the exception of antiserum IV, which did not react with the Sephadex-separated proteins. The number and the characteristics of the bands, however, were the same in samples and controls (1, 3, 4 above versus 2 and 5). There were also no significant differences between the band patterns given by the separated proteins and the untreated reaction mixture.

While all the antisera, which were prepared against fractions I–IV from 3-week-infected plants reacted with TMV protein (not shown), this was not the case with the antisera, which were prepared with tissue fractions isolated, 3 days after infection. Thus when TMV protein was added to the protein of the reaction mixture in the center well, this resulted in a distinct band near the "DP" hole, but not in corresponding position near the antisera I–IV. The location of this band indicates where a precipitation band of TMV protein should be expected if TMV protein were present in the reaction mixture. A test for the sensitivity of the technique using the reaction of the antiserum against denatured TMV protein with native TMV protein showed the limit of estimation of this protein to lie between 0.1–0.3 μg. These values compared with calculations based on amino acid incorporation in *E. coli* extracts suggest that it should be possible to detect by serological techniques a specific protein synthesized by an *in vitro* system, unless it were a very minor component, or particularly sensitive and thus destroyed by procedures necessary for its demonstration.

As reported previously (Tsugita *et al.*, 1962), TMV-RNA markedly stimulates amino acid incorporation into protein in *E. coli* extracts. However, further characterization of the [^{14}C]protein product formed in reaction mixtures suggests that little if any soluble TMV-coat protein, nor major peptide fragments thereof, nor other TMV-specific proteins serologically detectable by our methodology are synthesized under the conditions described. Further studies are required to determine the nature of the protein products formed.

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