*STUDIES ON THE FORMATION OF TOBACCO MOSAIC VIRUS RIBONUCLEIC ACID, IV. RATE OF SYNTHESIS OF VIRUS-INDUCED PROTEINS AND RIBONUCLEIC ACID FOLLOWING INFECTION**

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TMV,[1] consisting of protein subunits of known composition and sequence of amino acids[2, 3] and an RNA possessing a characteristic nucleotide composition significantly different from that of the RNA's of the host cell,[4] is ideally suited to study the synthesis of protein and RNA. According to the present concept of protein synthesis, a replica of DNA is made in the form of RNA with uracil replacing thymine. This replica called "messenger RNA," which is short-lived in bacteria, attaches itself to ribosomes existing in the cytoplasm and acts as a template for the synthesis of cellular proteins.[5–7] Thus, the site of synthesis of most of the cell proteins is recognized as ribosomes present in the cytoplasm.

The replication of TMV-RNA is not directed by host cell DNA.[8] Since TMV-RNA represents the genetic material, all of the information needed for making virus-specific proteins must be present in its nucleotide sequence. Hence, it would seem that *in vivo* viral nucleic acid acts as a template for the synthesis of proteins necessary for the formation of TMV. A direct demonstration of this function was attempted by using the cell-free protein-synthesizing system of *Escherichia coli*.[9] Even though claims[10] relating to the synthesis of TMV protein have been put forward, these are currently withdrawn by one of the authors.[11]

The present paper is concerned with the rate of synthesis of virus-induced proteins and RNA *in vivo* following infection with TMV and also with the examination of the possible role of microsomes of the cytoplasmic fraction of the infected leaf cell in the synthesis of virus-specific proteins. The results presented in this paper show a continuous synthesis of protein and RNA following infection with TMV; the amount of protein synthesized is in excess of the amount needed to form the virus rod. Furthermore, the microsomes of the cytoplasmic fraction appear to be free from the RNA resembling the composition of TMV-RNA. It is concluded from these results that *in vivo* the microsomes of the cytoplasmic fraction are not the site for the synthesis of virus-induced proteins.

Experimental.—TMV inoculum for infecting tobacco plants: The common strain of TMV used in this investigation was kindly supplied by Dr. F. O. Holmes of The Rockefeller Institute. TMV inoculum used for infecting the tobacco plants was prepared as previously described.[4]

Plants: Tobacco plants (*Nicotiana tabacum* var. Turkish) used in these studies were grown in a greenhouse. These were kindly provided by Dr. Armin C. Braun of The Rockefeller Institute.

The plants, approximately 12 weeks old, were transferred from the greenhouse to a room in which the temperature was maintained at 26°. They were illuminated for 14 hr a day. Plants of the same size and general appearance were selected, and bottom leaves were removed and discarded. The remaining leaves were dusted with carborundum and were rubbed with TMV inoculum prepared as described previously.[4] Control plants were rubbed with carborundum and 0.1 *M* phosphate buffer at pH 7.0. The leaves were afterward rinsed with water. Leaves of the same size and general appearance were harvested at intervals of time up to 20 days, and protein and TMV-RNA (TMV) present in the cytoplasmic fraction of the leaf were estimated as described below.

Determination of protein and TMV-RNA in 105,000 g pellet: Unless otherwise stated, all operations were conducted at 0–4°. Immediately after harvesting the leaves, their midribs were removed, and the leaf blades were quickly cut into small pieces. Two-gram portions were placed in a homogenizer tube of 15-ml capacity, fitted with a Teflon pestle. The leaves were ground for 5 min with 5 ml of 0.5 *M* sucrose containing 0.01 *M* Tris-HCl buffer at pH 8.0 and 0.002 *M* $MgCl_2$. The ground material was centrifuged for 20 min at 20,200 *g* in the refrigerated Servall centrifuge. The residue was washed twice with 3 ml of above grinding medium. The combined supernatants were centrifuged for 2 hr at 105,000 *g* in the no. 40 rotor of the Spinco Model L ultracentrifuge. After draining the supernatant thoroughly, the pellet was suspended in 1.5 ml of 0.1 *M* sodium phosphate buffer at pH 7.4 containing $4 \times 10^{-3} M$ EDTA and centrifuged for 20 min at 12,100 *g* in the refrigerated Servall centrifuge. The residue was extracted twice with 1.5-ml portions of the above phosphate buffer. In an aliquot of the combined extract, TMV-RNA was determined as previously described,[4] and in another aliquot protein was determined according to the biuret method[12] with some modification.

To 2 ml of the above extract at 0° was added 0.66 ml of 40% TCA with mixing. After 15 min, the mixture was centrifuged for 10 min at 7,900 *g* in the refrigerated Servall centrifuge. The precipitate was washed two times with 2 ml of cold 10% TCA and twice with absolute alcohol. It was finally washed once with absolute alcohol and once with ether at room temperature. The precipitate was air-dried and dissolved in 1 ml of 3% NaOH by incubating at 30° for 4 hr. The volume was made up to 9 ml with 3% NaOH, and to this was added 0.25 ml of 20% $CuSO_4$ solution. The mixture was shaken vigorously at room temperature for 1 min. After 10 min at room temperature, it was centrifuged in the Clinical centrifuge at top speed for 10 min. The supernatant was drawn out with a capillary pipette, and the color intensity was measured at 550 mμ in the Beckman spectrophotometer against a blank prepared with reagents only. The amount of the protein was calculated from a standard curve obtained with TMV protein.

Determination of radioactivities of microsomal RNA, soluble RNA, and TMV-RNA isolated from infected tobacco leaves exposed to Pi^{32} for short period: Plants, 12 weeks old, were infected with TMV as described previously.[4] At intervals of time, 0, 12, and 22 hr after infection, the leaves were detached from the plants, and their petioles were dipped in distilled water containing 10 μc Pi^{32} per ml for 2 hr. The leaves were illuminated during the period they were in the Pi^{32} medium. Immediately after removal from the medium the midribs were removed, and their leaf blades were cut into small pieces. Microsomal RNA, soluble RNA, and TMV-RNA were isolated, and their radioactivities were determined according to the procedure described previously.[13]

Determination of radioactivities of microsomal RNA, soluble RNA, and TMV-RNA isolated from tobacco leaves grown for 5 days in Hoagland's medium containing Pi^{32}: Tobacco plants, 4–5 weeks old, were transferred from the greenhouse to a room in which the temperature was maintained at 26°. Plants of the same size and general appearance were selected, and bottom leaves were removed and discarded. The remaining leaves were dusted with carborundum. One group (control) was rubbed with 0.1 *M* sodium phosphate buffer at pH 7.0, and the other group was rubbed with TMV inoculum. The leaves were afterward rinsed with water. The plants were immediately removed from the pots, and their roots were washed with tap water. One plant was placed in each bottle containing 80 ml of Hoagland's nutrient solution to which was added 0.6 μc Pi^{32} per ml. The phosphate content of Hoagland's nutrient solution was reduced to one tenth of that prescribed to ensure maximum uptake of Pi^{32} by the plants. The rest of the culture conditions were exactly the same as previously described.[13] The leaves, were harvested after 5 days. The microsomal RNA and soluble RNA from the healthy leaves, and microsomal RNA, soluble RNA, and TMV-RNA from the infected leaves were isolated, and their radioactivities were determined according to the procedures previously described.[13]

Determination of total amounts of radioactivities in the nucleotides of RNA's associated with microsomal and soluble cytoplasmic fractions and TMV isolated from infected tobacco leaves grown for 5 days in Pi^{32} medium: Tobacco plants, 4–5 weeks old, were infected with TMV inoculum. Immediately after inoculation, they were removed from pots, their roots were washed with tap water, and transferred to Hoagland's nutrient solution containing 0.3 μc Pi^{32} per ml. The rest of the culture conditions were exactly the same as described previously.[13] The leaves were harvested after 5 days. Microsomal RNA, soluble RNA, and TMV-RNA were isolated, and the

radioactivities of their nucleotides obtained by alkali hydrolysis were determined as previously described.[13]

Determination of extent of retention of P^{32} TMV-RNA added to cycloplasmic fraction of healthy tobacco leaves and the radioactivities of mononucleotides obtained by alkali hydrolysis of RNA associated with microsomal and soluble cytoplasmic fractions: Unless otherwise stated, all operations were conducted at 0–2°. Immediately after harvesting the leaves from healthy tobacco plants, their midribs were removed, and their leaf blades were cut into small pieces. Two-gram portions were placed in a homogenizer tube of 15-ml capacity, fitted with a Teflon pestle. The leaves were ground for 5 min with 5 ml of 0.5 *M* sucrose containing 0.01 *M* Tris-HCl buffer at pH 8.0 and 0.002 *M* $MgCl_2$. The ground material was centrifuged for 20 min at 20,200 *g* in the refrigerated Servall centrifuge. The residue was washed twice with 3-ml portions of above grinding medium. To the combined supernatants were added 1.25 mg P^{32} TMV-RNA having 359,285 cpm. P^{32} TMV-RNA was prepared from radioactive TMV using phenol procedure.[14] The combined supernatants were centrifuged for 2 hr at 105,000 *g* in the no. 40 rotor of the Spinco Model L ultracentrifuge.

The supernatant was poured into a beaker at 0°. To this was added cold 40% TCA with mixing (final concentration of TCA was 6%). After 15 min, precipitate was collected by centrifugation in the refrigerated Servall centrifuge at 7,900 *g* for 10 min. The precipitate was washed, dried, and hydrolyzed with alkali. The radioactivities of RNA and mononucleotides were determined as previously described.[13]

After draining the supernatant thoroughly, the microsomal pellet was suspended in 1 ml of 0.1 *M* sodium phosphate buffer at pH 7.4 containing 4×10^{-3} *M* EDTA and centrifuged for 20 min at 12,100 *g* in the refrigerated Servall centrifuge. The residue was extracted twice with 1-ml portions of the above phosphate buffer. From the combined extracts TCA precipitate was obtained, washed, air-dried, and hydrolyzed with alkali. The radioactivities of RNA and mononucleotides were determined as previously described.[13]

Reagents: Pancreatic RNAase was purchased from the Worthington Biochemical Corp., Freehold, New Jersey, and sea sand from Merck and Co., Inc., Rahway, New Jersey. Pi^{32} was purchased from the Oak Ridge National Laboratory. TMV antiserum was kindly supplied by Dr. A. Siegel of the University of Arizona.

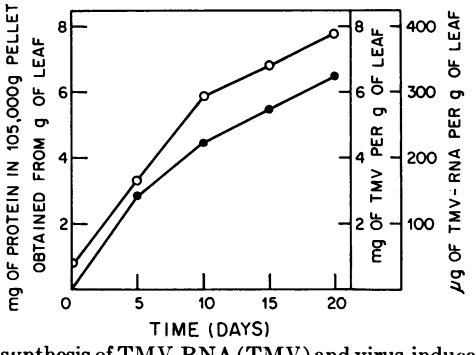

FIG. 1.—Rate of synthesis of TMV-RNA (TMV) and virus-induced proteins. O–O, total protein in the pellet obtained by centrifugation of cytoplasmic fraction of infected tobacco leaf at 105,000 *g*; ●—●, TMV-RNA (TMV). For details see text. The values are the averages of two determinations performed on two groups of tobacco plants grown under identical conditions. Protein content of the pellet obtained from the cytoplasmic fraction of the healthy tobacco leaves remained the same throughout the experimental period.

Results.—Rate of synthesis of TMV-RNA (TMV) and virus-induced proteins: The results presented in Figure 1 show a continuous increase in the synthesis of TMV-RNA (TMV) and virus-induced proteins throughout the experimental period. The total protein synthesized, following infection with TMV, was in excess of the amount needed to form TMV, which is made up of 95 per cent protein and 5 per

cent RNA. The synthesis of excess protein is discernible throughout the experimental period. The excess protein might represent so-called X-protein[15-18] in a highly aggregated state or a mixture of several proteins neeeded in the biosynthesis of TMV. TMV-RNA containing approximately 6,400 nucleotides is enough for coding at least 12 proteins of the size of TMV protein subunit, assuming that 3 nucleotides code for one amino acid.[19] Even though all the protein extracted from the microsomal pellet was precipitated with TMV antiserum, this is, however, not an infallible test to establish the identity or the chemical nature. Experiments to establish their chemical nature are in progress.

Since there is a continuous synthesis of proteins, the template RNA on which these are synthesized might not be of a transient nature, as was observed in bacteria.[6, 7] The template RNA (TMV-RNA) might be found attached to the microsomes involved in its synthesis. To examine this possibility and to identify the template RNA, if present, attached to the microsomes of the cytoplasmic fraction, the following experiments were carried out.

Specific radioactivities and the nucleotide composition of RNA's associated with microsomal and soluble cytoplasmic fractions and TMV: The results presented in Table 1 show no differences in the radioactivities associated with the microsomal

TABLE 1

SPECIFIC RADIOACTIVITIES OF MICROSOMAL RNA, SOLUBLE RNA, AND TMV-RNA ISOLATED FROM TOBACCO LEAVES EXPOSED TO Pi ^{32} FOR 2 HR

Time (hr) after infection	Counts/Min/10 μg RNA		
	Microsomal	Soluble	TMV
0	54	358	
12	53	319	
24	56	252	1,310

After infection with TMV the leaves of same size were detached from the plants at intervals of time given in the table, and their petioles were dipped in H_2O containing Pi^{32} (10 μc/ml) for 2 hr. The rest of the conditions were given in the text. For the isolation of TMV, 10 gm of leaves were homogenized instead of 2 gm used in other cases.

RNA of the leaves exposed to Pi^{32} at 0, 12, and 22 hr after infection. In the case of soluble RNA there was indeed a decrease in the uptake of P^{32}. The radioactivity of soluble RNA obtained from leaves harvested 24 hr after infection was about 30 per cent less than that of the soluble RNA of the leaves harvested 0 time after infection. On the other hand, the radioactivity of TMV-RNA (TMV) isolated from leaves harvested 24 hr after infection was about 25 times more than that of the microsomal RNA. If the TMV protein is synthesized on a template (TMV-RNA) bound to the microsomes of the cytoplasmic fraction, there should have been an increase at least in the radioactivity of the microsomal fraction obtained from 24-hr sample. The amount of TMV synthesized in 24 hr was about 200 μg/gm of leaf. It is likely that the number of microsomes taking part in the synthesis of protein during this period might be small, since it was shown under a variety of conditions that the rate of protein synthesis is proportional to the microsome concentration.[20-22] For this reason the infected tobacco plants were grown in Pi^{32} medium for 5 days, during which time there was a considerable amount of protein synthesis (see Fig. 1). The radioactivities of microsomal RNA, soluble RNA, and TMV-RNA isolated from infected tobacco leaves grown in Pi^{32} medium for 5 days, were compared with those of microsomal RNA and soluble RNA obtained from healthy leaves grown

under identical conditions. In addition, their nucleotide composition was obtained by analyzing the distribution of the radioactivity in their nucleotides. The results are presented in Tables 2 and 3.

TABLE 2

SPECIFIC RADIOACTIVITIES OF MICROSOMAL RNA, SOLUBLE RNA, AND TMV-RNA ISOLATED FROM TOBACCO LEAVES GROWN IN Pi^{32} MEDIUM FOR 5 DAYS

RNA's	Counts/Min/μg RNA	
	Healthy leaves	Infected leaves
Microsomal	128	103
Soluble	145	141
TMV		729

Plants were grown in Hoagland's medium containing 0.6 μc Pi^{32} per ml for 5 days. The rest of the procedures were described in the text.

TABLE 3

TOTAL AMOUNTS OF RADIOACTIVITIES IN THE NUCLEOTIDES OF RNA'S ASSOCIATED WITH MICROSOMAL AND SOLUBLE CYTOPLASMIC FRACTIONS AND TMV ISOLATED FROM INFECTED TOBACCO LEAVES GROWN IN Pi^{32} MEDIUM FOR 5 DAYS

RNA's	Counts/Min			
	Cytidylic acid	Adenylic acid	Guanylic acid	Uridylic acid
Microsomal	905 (0.75)	942 (0.78)	1,207 (1.00)	862 (0.71)
Soluble	1,525 (0.90)	1,180 (0.70)	1,690 (1.00)	982 (0.58)
TMV	2,842 (0.72)	4,524 (1.15)	3,928 (1.00)	4,229 (1.08)

Figures in parentheses are the ratios of total amounts of radioactivity in the nucleotides. Soon after infection, plants were removed from pots and transferred to Hoagland's medium containing 0.3 μc Pi^{32} per ml. They were in this medium for 5 days. The rest of the procedures were described in the text.

The results presented in Table 2 show no differences in the radioactivities associated with the soluble RNA obtained from healthy and infected leaves. On the other hand, the radioactivity of microsomal RNA obtained from the infected leaves was about 23 per cent less than that of the microsomal RNA obtained from the healthy leaves. The radioactivity of TMV-RNA was about 7 times more than that associated with the microsomal RNA and about 5 times more than that associated with the soluble RNA isolated from infected leaves. The distribution of radioactivity in the nucleotides of RNA associated with the microsomal fraction is similar to the nucleotide composition of microsomal RNA.[4] The distribution of radioactivity associated with the soluble RNA fraction is similar to the nucleotide composition obtained by alkali hydrolysis of soluble RNA (composition of soluble RNA, isolated from infected tobacco leaf—unpublished results). Lastly, the radioactivity associated with TMV-RNA is typical of TMV-RNA composition[4] (Table 3). As was reported in the case of hemoglobin synthesizing system of reticulocytes,[23] if the microsomal fraction in the present case consists of clusters of microsomes (pentamers) held together by a template RNA (TMV-RNA), the average nucleotide composition of such an aggregate would be guanylic acid, 1.10; adenylic acid, 0.93; cytidylic acid, 0.71; and uridylic acid, 0.90. Thus, the microsomal fraction and the soluble cytoplasmic fraction did not reveal the presence of a template RNA resembling the composition of TMV-RNA.

It might still be possible that TMV-RNA was indeed present, attached to the microsomes, and was destroyed during the isolation by the action of nucleases[24] present in the leaf cell. To test this possibility, P^{32} TMV-RNA was added to the cytoplasmic fraction of healthy tobacco leaves, and the amounts of labeled RNA

associated with the microsomal and soluble cytoplasmic fractions were determined. The results presented in Table 4 show that about 35 per cent of the added P^{32} TMV-RNA was associated with the microsomal fraction, and about 29 per cent was associated with the soluble RNA fraction. In addition, the distribution of radioactivity in the nucleotides of RNA associated with the microsomal and soluble RNA

TABLE 4

RECOVERY OF TMV-RNA ADDED TO CYTOPLASMIC FRACTION OF HEALTHY TOBACCO LEAF AND THE NUCLEOTIDE COMPOSITION OF RNA RETAINED BY MICROSOMAL AND SOLUBLE CYTOPLASMIC FRACTIONS

Fractions	Radioactivity recovered (cpm)	Per cent recovered	Counts/Min			
			Cytidylic acid	Adenylic acid	Guanylic acid	Uridylic acid
Microsomal	126,720	35.3	4,117 (0.74)	6,149 (1.10)	5,575 (1.00)	5,844 (1.05)
Soluble cytoplasmic	105,360	29.3	4,117 (0.73)	6,395 (1.14)	5,604 (1.00)	5,998 (1.07)
Total recovery	232,080	64.6				

1.25 mg TMV-RNA having 359,285 cpm were added to the cytoplasmic fraction obtained from healthy tobacco leaves. The rest of the procedures were described in the text. Figures in parentheses are the ratios of total amounts of radioactivity in the nucleotides.

fractions is similar to the nucleotide composition of TMV-RNA.[4] Thus, it should be possible, using the procedure described in this paper, to reveal the presence of TMV-RNA if it is present, attached to the microsomes.

Discussion.—As shown in this paper, the synthesis of TMV-RNA (TMV) and virus-induced proteins continued throughout the experimental period. The total protein synthesized was in excess of the amount needed in the formation of TMV. The excess protein might be the so-called X-protein[15–18] or a mixture of several proteins, since TMV has enough RNA to code for at least 12 proteins of the size of TMV protein subunit, assuming that one amino acid is coded by three nucleotides.[19] All of the genetic functions of TMV-RNA are not yet known. However, it is possible that TMV-RNA, in addition to directing the synthesis of coat protein, might also direct the formation of several proteins, some of which have enzymatic functions essential in the infection and replication processes.

The microsomes of the cytoplasmic fraction have been suggested as the site of the formation of TMV protein.[25] In the case of fowl plague virus whose genetic material is RNA, the g-antigen is first formed in the nucleus and this diffuses later into the cytoplasm.[26] Even though the TMV-induced proteins are present in the cytoplasmic fraction of the infected leaf cell, the microsomes present in this subcellular fraction might not be involved in their formation. This conclusion is based on the following experimental evidence. The microsomes of the cytoplasmic fraction are rapidly degraded following infection with TMV.[4] If these are involved in the synthesis of viral proteins, they should be conserved rather than degraded during the rapid synthesis of proteins, for it was shown under a variety of conditions that the rate of protein synthesis is proportional to the microsome concentration.[20–22] The results presented in this paper further show that the microsomes of the cytoplasmic fraction obtained from infected tobacco leaf contained no detectable template RNA resembling the composition of TMV-RNA. It is likely, therefore, that the virus-induced proteins are synthesized at the same site as the viral RNA is formed, and these later diffuse into the cytoplasm.

In the case of fowl plague virus, the RNA containing g-antigen appears first in

nucleus,[26] and it was further shown that the RNA itself is synthesized in the nucleus;[27] infective RNA extractable from Krebs ascites tumor cells by phenol was shown to be at first mostly located in the nuclear fraction.[28] The presence of a high proportion of the double-stranded RNA (replicative form) in the nucleus of Kreb-II ascites tumor cells infected with encephalomyocarditis virus, suggests that the replication of viral RNA occurs in the cell nucleus.[29] Thus, in the case of several RNA-containing viruses, the nucleus appears to be the site of formation of their genetic material. The findings of Zech and Vogt-Kohne[30] with the help of ultraviolet microscopy indicated the nucleus as the site of formation of TMV-RNA. We have preliminary evidence indicating that TMV is synthesized in the nucleus.[31]

Summary.—The rate of synthesis of TMV-RNA (TMV) and virus-induced proteins in tobacco leaves infected with TMV was studied. Following infection with TMV, there was a continuous synthesis of virus-induced proteins and RNA. The total protein synthesized was in excess of the amount needed for the formation of virus rods. This excess protein might be the so-called X-protein or a mixture of several proteins, for TMV has enough RNA to code for at least 12 proteins of the size of a TMV protein subunit, assuming that one amino acid is coded by 3 nucleotides.

The microsomes of the cytoplasmic fraction obtained from infected tobacco leaf were examined for the presence of TMV-RNA template, needed for viral protein synthesis. These particles contained no detectable RNA resembling the composition of TMV-RNA. On the basis of the evidence presented in this paper and in the previous publications of this series, it is concluded that *in vivo* the microsomes of the cytoplasmic fraction are not involved in the synthesis of virus-induced proteins.

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[1] Abbreviations: TMV, tobacco mosaic virus; TMV-RNA, tobacco mosaic virus ribonucleic acid; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetate; TCA, trichloroacetic acid; RNAase, ribonuclease.

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*A UNIFORM COHOMOLOGY THEORY FOR ALGEBRAS**

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The deformation theory for algebras[1] suggests that given any category $\mathfrak{C}$ of equationally defined algebras and homomorphisms (e.g., associative algebras, Lie algebras, Jordan algebras, commutative associative algebras, nilpotent algebras with fixed finite index), there exists a natural cohomology theory appropriate to $\mathfrak{C}$. This paper gives a definition of a cohomology theory for all such $\mathfrak{C}$ in a uniform way. However, only the groups H^n for $n \geq 2$ are defined, H^2 being introduced as a group of "singular" extensions. Fixing a suitable concept of extension normalizes H^2 and therewith the theory. (The coefficient ring need not be a field, nor extensions additively split.) The axioms[2] for a cohomology theory are satisfied, beginning with H^2, including the vanishing of H^n for $n > 2$ when the coefficient module is injective. It follows that in the presence of sufficiently many injectives, the theory is unique, i.e., any other satisfying the axioms and with the same H^2 must coincide with the present one.

1. *Definition of the Cohomology Groups.*—Given an algebra A in the category $\mathfrak{C}$, by a two-sided module P over A is meant a module over the coefficient ring together with linear maps $A \otimes P \to P$ and $P \otimes A \to P$ (the images under which of $a \otimes p$ and $p \otimes a$ will be denoted simply by ap and pa, respectively) such that the module direct sum $A + P$ with multiplication given by $(a_1, p_1)\ (a_2, p_2) = (a_1a_2, a_1p_2 + a_2p_1)$ is again an algebra of the category $\mathfrak{C}$. A *singular extension* $\mathbf{E}$ over A with kernel C is a short exact sequence $0 \to C \to B \to A \to 0$ with B in $\mathfrak{C}$ and $C^2 = 0$. It follows that C is in a natural way a two-sided module over A. An *n-fold extension* over A with initial term a two-sided A module P is, for $n = 2$, just a singular ex-