# THE KINETICS OF TMV-RNA SYNTHESIS AND ITS CORRELATION WITH ACCUMULATION OF COMPLETE VIRUS IN INOCULATED TISSUE CULTURE AND THE EFFECT OF KINETIN ON VIRUS SYNTHESIS

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ABSTRACT

THE KINETICS OF TMV-RNA SYNTHESIS AND ITS CORRELATION WITH ACCUMULATION OF COMPLETE VIRUS IN INOCULATED TISSUE CULTURE AND THE EFFECT OF KINETIN ON VIRUS SYNTHESIS

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Lawrence E. Pelcher

Tobacco mosaic virus ribonucleic acid (TMV-RNA) synthesis and the formation of complete virus were studied at 12-hr intervals after inoculation of tobacco (Nicotiana tabacum L. var. Havana 38) tissue culture. Polyacrylamide gel electrophoresis was used to follow the kinetics of viral RNA synthesis and to determine its relation to cellular ribosomal RNA synthesis. The rate of TMV-RNA synthesis, as measured by the incorporation of uridine-$^{3}H$ into viral nucleic acid, increased in a near linear manner during the first 60 hr after inoculation. During the 48-60-hr period, the rate of viral RNA synthesis was approximately six times greater than during the 12-24-hr period. The rate of viral RNA synthesis then declined, except for a period 72-84 hr after inoculation when the rate increased slightly. Cellular ribosomal RNA synthesis, as measured by its specific activity

(cpm/μg), remained relatively constant through the entire incubation period; however, the specific activity of the viral RNA decreased. The peak rate of accumulation of complete virus, as measured by incorporation of uridine-$^{3}H$ and leucine-$^{14}C$ into complete virus, occurred during the 48-60-hr period, the period of most rapid viral RNA synthesis. After this period the rate of accumulation of complete virus declined, except during the 84-120-hr period when a slight increase was observed. Up to 48 hr after inoculation, crystalline inclusions were rarely observed, but at 72 hr large aggregates of crystal-bearing cells were present. Crystal-bearing cells were observed to undergo division with the crystals being distributed between daughter cells.

When virus-infected cells were exposed to 2.5 x $10^{-4}$ M kinetin during the 48-60-hr period, the rate of incorporation of uridine-$^{3}H$ into cellular ribosomal RNA was inhibited by approximately 70%; however, uridine-$^{3}H$ incorporation into viral RNA was virtually unaffected. Studies employing density gradient analysis of virus synthesized during the kinetin treatment indicated that accumulation of complete virus and incorporation of uridine-$^{3}H$-TMV-RNA into complete virus was inhibited by approximately 30-50%. However, the specific activity (cpm/μg complete virus) was approximately the same in kinetin-treated and non-treated infected cells. Incorporation of leucine-$^{14}C$ into complete virus appeared to be stimulated (30-50%) in kinetin-treated infected cells.

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This dissertation is dedicated

to my wife, Marilyn

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PART ONE

# THE KINETICS OF TMV-RNA SYNTHESIS AND ITS CORRELATION WITH ACCUMULATION OF COMPLETE VIRUS IN INOCULATED TISSUE CULTURE

## Introduction

The development of a plant virus-plant tissue culture system suitable for the study of virus replication and host cell-virus interaction has been the goal of many virologists. Although the successful infection of plant tissue culture has been reported (Kassanis *et al.*, 1958; Wu *et al.*, 1960; Hirth and Lebeurier, 1965; Murakishi, 1968; Motoyoshi and Oshima, 1968), only a low percentage of the cells were infected and the final virus titer attained was generally lower than in the whole leaf (Kassanis, 1957). The low efficiency of infection precluded biochemical studies of virus replication or host cell-virus interaction in *in vitro* inoculated plant tissue culture (Quak, 1965).

Recently a plant virus-plant tissue culture system with an improved efficiency of inoculation resulting in a high virus titer has been developed (Murakishi *et al.*, 1970). In this system the growth curve and yield of tobacco mosaic virus was comparable to that attained in the tobacco

leaf (Murakishi et al., 1971). In inoculated tissue cultures derived from hypersensitive tobacco, a necrotic reaction occurs which is similar in appearance to that observed following inoculation of a hypersensitive tobacco leaf (Beachy and Murakishi, 1971a,b).

The development of a plant virus-plant tissue culture system with a virus titer and response comparable to that obtained in the tobacco leaf system would enable virologists to study virus replication and host cell-virus interaction in plant tissue culture.

This thesis reports the first detailed study of viral RNA synthesis in inoculated plant tissue culture. The kinetics of viral RNA synthesis was correlated with the incorporation of viral RNA into complete virus and with the accumulation of complete virus.

A correlation between viral RNA and viral coat protein synthesis and the incorporation of these components into complete virus was also made. A study was made of the temporal relation of viral crystalline inclusion formation and the synthesis of viral RNA and complete virus. Cellular RNA and protein synthesis in inoculated plant tissue culture were studied to determine if there was a relationship between the rate of synthesis of these cellular components and the rate of virus synthesis.

# Literature Review

## Introduction

The intent of this review is to give future researchers, who may use plant tissue culture to study aspects of virus replication and host cell-virus interaction, a background in the work that has been performed in the plant system. It is hoped that such knowledge will be an aid in selection and design of experiments which will advance our knowledge of the many aspects of host cell-virus interaction.

Although there are many excellent texts which deal with various aspects of host cell-virus interaction (Mundry, 1963; Schlegel et al., 1967; Esau, 1968; Matthews, 1970), the material dealt with in this review is mainly concerned with the possible sites and modes of virus synthesis and the effects of virus infection on nucleic acid metabolism of plant cells.

## Site of Viral RNA Synthesis and the Effect of TMV Infection on Nucleic Acid Metabolism

### I. The Nucleus

The nucleic acid content of the nuclear-chloroplast fraction from inoculated tobacco leaves was reported to increase 96-100 hr after infection. (Basler and Commoner, 1956). The nucleic acid content of this fraction declined

during the period of rapid accumulation of complete virus, 120-138 hr post inoculation (p.i.).

Using microspectrophotometric techniques, Zech (1963) studied nucleic acid changes in TMV-infected leaf hair cells. In the initially inoculated hair cell, the nucleic acid content of the nucleus and surrounding cytoplasm increased rapidly 2-8 hr p.i. After this time, the nucleic acid content of the nucleus decreased, returning to control levels 24 hr p.i. The nucleic acid content of the cytoplasm surrounding the nucleus increased rapidly 2 hr after inoculation reaching 25 times the control level by 8 hr p.i. and then decreased slowly during the next 22 hr. By the 7th hr post inoculation, a change in the extinction ratio at 265 nm and 280 nm occurred. This change was interpreted to indicate the onset of large amounts of protein synthesis. Mundry (1963), reviewing Zech's work, attributed the change in extinction ratios to incorporation of the excess nucleic acid into nucleoprotein particles--presumably complete virus.

Bald (1964), using specific staining techniques, characterized the increased nuclear nucleic acid as ribonucleic acid (RNA). At short times after inoculation there appeared to be an increase in the nucleolar mass of RNA. Much of the RNA originating in the nucleolus was observed migrating out of the nucleus "en mass" into the cytoplasm, where it was observed to mix with the "virus protein gel."

The incorporation of tritiated uracil into epidermal cells was studied by Yasuda and Hirai (1964). Using radioautographic techniques, an increased accumulation of uracil-$^{3}H$ in the nucleus during the first 24 hr p.i. was observed. The amount of uracil-$^{3}H$ in the nucleus remained constant from 24-48 hr after inoculation and then began to decline. Beginning approximately 30 hr after inoculation, accumulation of uracil-$^{3}H$ in the cytoplasm of infected cells exceeded that of control cells.

Hirai and Nakagaki (1966), using differential staining and microspectrophotometric techniques, measured DNA, RNA, histone and protein contents in nuclei of epidermal cells after inoculation with TMV. Six to eight hours after inoculation, a 20% increase in nuclear RNA was observed, after which time the RNA content returned to near control levels.

As pointed out by Bald (1964), there appears to be a clear effect on nuclear RNA metabolism following infection with TMV. However, these techniques were not capable of distinguishing between cellular and viral RNA synthesis. Hurwitz et al. (1962) demonstrated that actinomycin-D prevented the synthesis of RNA in treated cells, presumably by the binding of the drug to the DNA template, thus preventing transcription. Franklin and Baltimore (1962) reported that while actinomycin-D severely inhibited cellular RNA synthesis, it had little effect on RNA synthesis of

RNA-containing animal viruses. Using this information, Sänger and Knight (1963) studied the effect of this drug on cellular and viral RNA synthesis in tobacco leaves. Actinomycin-D inhibited cellular ribosomal RNA synthesis but appeared to have no direct effect on viral RNA synthesis. These results indicated that a DNA template is not directly involved with the synthesis of TMV-RNA and that actinomycin-D offered a means of distinguishing between cellular RNA synthesis and viral RNA synthesis in infected cells.

Smith and Schlegel (1965) used actinomycin-D and radioautographic techniques to follow tritiated uridine incorporation into healthy and virus-infected cells. Actinomycin-D treatment greatly reduced uridine incorporation into the nucleolus and cytoplasm of healthy cells; however, virus-infected cells incorporated uridine into both the nucleus and cytoplasm. During short labeling periods, there was a large amount of nucleolar incorporation. With longer periods of labeling, both the nucleus and the cytoplasm exhibited label incorporation. With very long labeling periods, incorporation into virus inclusion bodies occurred. Similar patterns of labeling were also observed in Vicia faba root cells infected with clover yellow mosaic virus (CYMV) after actinomycin-D treatment. These observations were interpreted to mean that the nucleolus was the site of synthesis of both TMV and CYMV-RNA.

De Zoeten and Schlegel (1967), using light microscope and electronmicroscope autoradiography, followed uridine incorporation into actinomycin-D treated CYMV-infected cells and broad bean mottle virus-infected cells. The nuclear and cytoplasmic labeling patterns observed earlier by Smith and Schlegel (1965) with TMV-infected cells also occurred with cells infected with CYMV. Broad bean mottle virus-infected cells did not exhibit the nuclear uridine incorporation in the presence of actinomycin-D but did exhibit cytoplasmic uridine incorporation, suggesting that the cytoplasm was the site of synthesis of this virus.

## II. The Chloroplast

Holmes (1928) and Fulton (1950) have demonstrated that yellow areas of mosaic leaves contain more TMV than green areas of the same leaves. Numerous electron microscopic studies have indicated the presence of TMV in chloroplasts (Shalla, 1964; Milne, 1966a,b). Although these authors did not imply that the chloroplast was the site of TMV synthesis, the association of TMV with the mosaic areas of the leaves and chloroplasts suggests that virus infection may in some way alter their metabolism.

Basler and Commoner (1956) reported that the nucleic acid content of a buffer-insoluble fraction from leaf homogenates increased 96-100 hr after inoculation before complete virus could be detected. The buffer-insoluble material was

collected by centrifugation which would have sedimented nucleii and chloroplasts. The excess nuclei acid in this fraction decreased 120-200 hr p.i. at a time when the amount of extractable virus was increasing. The nature of the increased nucleic acid was not determined but was considered to represent the de novo synthesized precursor of tobacco mosaic virus-ribonucleic acid (TMV-RNA).

Babos (1966b) studied ribonucleic acid turnover in tobacco leaves infected with TMV. The nuclear-chloroplast fraction from infected leaves 48-72 hr after inoculation exhibited a 15% increase in RNA content relative to control leaves. The rate of phosphorus$^{32}$ ($^{32}$P) incorporation by the nuclear-chloroplast fraction from infected leaves increased with time of incubation and was about 40% above the same fraction from uninoculated leaves after 120 min of incubation. However, the nucleotide composition of the RNA isolated from this fraction was not that expected of TMV-RNA.

The findings of Hirai and Wildman (1969) and Fraser (1969, 1972) are contrary to the reports of a stimulated RNA synthesis associated with the chloroplast fraction from infected leaves. When $^{32}$P was supplied to young tobacco leaves during the period of rapid TMV synthesis and then the total leaf ribonucleic acid was analyzed by polyacrylamide gel electrophoresis, $^{32}$P incorporation into chloroplast RNA was severely inhibited. Isolated chloroplasts exhibited an incorporation of radioactive RNA precursors into RNA as well

as reduced capacity to incorporate labeled amino acids into protein (Hirai and Wildman, 1969). Hirai and Wildman suggested that, due to the inability of the chloroplasts to exhibit normal metabolic functions, the chloroplast could not be the initial site of TMV multiplication. Hirai and Wildman (1967b) reported that actinomycin-D, when applied to healthy leaves, produced symptoms similar to those produced by TMV infection. Based on these similarities, they suggested that TMV infection in some way represses transcription of chloroplast DNA and that this repression accounts for the decreased metabolic activity of chloroplasts in TMV-infected leaves.

Fraser (1969) confirmed the results of Hirai and Wildman (1969) but found that the particular strain of TMV used in a large degree determined the effect of virus infection on synthesis and stability of chloroplast ribosomal RNA in tobacco leaves. The flavum strain of the virus, which causes severe yellowing of the leaf, also caused severe inhibition of chloroplast RNA synthesis. The vulgare strain, which only causes light green symptoms, produced a less severe inhibition of chloroplast RNA synthesis. Fraser reported that Peterson and McKinney (1939) demonstrated that leaves infected with the flavum strain of the virus attained a much higher chlorophyllase activity than vulgare-infected leaves. Fraser also studied chlorophyll content in relation to the symptoms produced by the two strains of the virus.

He found that chloroplast ribosomal RNA breakdown occurred much earlier than that of chlorophyll in flavum-infected leaves. This observation tended to argue against breakdown in the chloroplast structure leading to release of ribonuclease which would result in chloroplast ribosomal RNA degradation, but rather indicated that TMV infection has a specific and direct effect on chloroplast ribosomal RNA synthesis.

Fraser (1972) further studied the effects of TMV replication on chloroplast ribosomal RNA synthesis in relation to the age of the leaf at the time of inoculation. Younger leaves (1.5 cm) exhibited a reduction in chloroplast ribosomal RNA content until approximately 20 days after inoculation. After this time the chloroplast ribosomal content was slightly greater than that in control leaves of the same age. Leaves which were older (5 cm) at the time of inoculation reacted much less severely to TMV infection. The chloroplast ribosomal RNA content increased after inoculation at a time when the chloroplast ribosomal RNA content of control leaves was also increasing. The chloroplast ribosomal RNA content of infected leaves reached approximately 75% that of control leaves. After reaching maturity, the chloroplast ribosomal RNA content of both infected and control leaves declined at about the same rate.

Randles and Coleman (1970) studied the effect of lettuce necrotic yellows virus on chloroplast ribosomal RNA synthesis and stability of chloroplasts in tobacco leaves. The concentration of 70 S chloroplast ribosomes declined within one day of appearance of symptoms in systemically infected leaves and could not be detected 1-3 days later. Chloroplast ribosomal RNA could only be detected in trace amounts and $^{32}P$ incorporation into chloroplast ribosomal RNA was extremely low as compared to healthy leaves. These observations suggest not only a rapid decline in chloroplast RNA synthesis but also a rapid degradation of pre-existing chloroplast ribosomal RNA. However, observations on chloroplast structure indicate that there was little loss of chloroplast integrity. These observations support the conclusion of Fraser (1969) that virus infection may directly affect chloroplast ribosomal RNA synthesis and stability which could later lead to alterations in chloroplast metabolism.

## III. The Cytoplasm

Babos (1966a,b) studied the ribonucleic acid content and the rate of $^{32}P$ incorporation into various subcellular fractions of TMV-infected leaves. He observed that the RNA content of all fractions not containing complete virus decreased with leaf expansion. The amount of ribosomal RNA did not appear to be affected by virus infection. The rate

of $^{32}P$ incorporation into ribosomal RNA during incubation periods up to 90 min was the same for virus-infected and control leaves. However, with longer periods of incubation, the specific activity of ribosomal RNA from infected leaves was 10-30% higher than from control material. He concluded that the excess $^{32}P$ was not incorporated into ribosomal RNA but into extraneous RNA bound to the ribosomes. The base composition of the ribosomal RNA from infected leaves exhibited no difference from that of control leaves. It appeared that TMV-RNA is synthesized de novo and that cytoplasmic ribosomal RNA synthesis or degradation is not affected by the multiplication of TMV in the cell.

These observations contradicted the earlier observations of Reddi (1963) that virus infection is associated with a rapid degradation of ribosomes and ribosomal RNA. Reddi had reported that the nucleosides produced by degradation of the ribosomes were incorporated into complete virus and that the amount of degradation of ribosomes was approximately equal to the amount of TMV-RNA synthesized.

A more qualitative approach to the study of nucleic acid metabolism in virus-infected cells was reported by Kubo et al. (1965). Using chromatography on methylated albumin kieselguhr columns, Kubo (1966) was able to effectively separate viral and cellular nucleic acids. The relative rate of $^{32}P$ incorporation into viral RNA was highest 48 hr after inoculation, after which time the rate of

incorporation declined. Incorporation of $^{32}P$ into cellular ribosomal RNA appeared to be stimulated shortly after infection; however, incorporation began to decline by 96 hr p.i. at a time when the rate of viral RNA synthesis was also declining.

Hirai and Wildman (1969) reported that $^{32}P$ incorporation into the 28 S and 18 S cytoplasmic ribosomal RNAs, as analyzed by acrylamide gel electrophoresis, was not affected by TMV multiplication 72 hr after inoculation; however, this was after the period of most rapid TMV synthesis (Hirai and Wildman, 1967a). Similar observations were made by Fraser (1969). Using two strains of TMV, vulgare (light/dark green mosaic symptoms) and flavum (severe yellow and green mosaic), he observed no effect on $^{32}P$ incorporation into the 28 S and 18 S cytoplasmic ribosomal RNAs as a result of infection with the vulgare strain, but there appeared to be a small reduction into the ribosomal RNAs with the flavum strain. Both strains caused severe reduction in the synthesis of chloroplast ribosomal RNA.

Fraser (1972) reported, in a detailed study, the effects of the two strains of tobacco mosaic virus on the RNA metabolism of tobacco leaves. The age of the leaf at the time of inoculation determined to a large degree what effect infection would have on nucleic acid metabolism. Leaves which were very young (1.5 cm) at the time of inoculation exhibited a decreased rate of cytoplasmic ribosomal RNA

synthesis. By the time the leaf had reached the 5 cm stage at the time of inoculation, cytoplasmic ribosomal RNA exhibited a decreased rate of degradation as compared to control leaves. Phosphorus$^{32}$ incorporation into cytoplasmic ribosomal RNA appeared to be stimulated during the first few days after inoculation, but, during the main period of TMV-RNA synthesis, incorporation of $^{32}P$ into ribosomal RNA appeared to be reduced.

Fraser's observations on the importance of the age of the leaf at the time of inoculation may explain some of the conflicting results reported by other workers. (Reddi, 1963; Kubo, 1966; Hirai and Wildman, 1969). The age of the leaf at the time of inoculation apparently greatly determines the effect virus infection will have on ribosomal RNA metabolism.

The observations of Babos (1966b) indicate that, while not directly affecting ribosomal RNA synthesis, virus infection may lead to attachment of extraneous RNA to cytoplasmic ribosomes. Babos and Shearer (1969) reported that actinomycin-D (AMD) treatment inhibited host ribonucleic acid synthesis by about 90%. Infected tissue exhibited excess AMD-resistant RNA synthesis not accountable for by RNA incorporation into complete virus. This RNA was found to be associated with the light ribosomal RNA (18 S). When cytoplasmic ribosomes were disassociated by EDTA, the

actinomycin-D resistant RNA was equally distributed between a low molecular weight component and RNA associated with the 40 S ribosomal subunit (Babos, 1969).

Jackson et al. (1972) has demonstrated a low molecular weight component (LMC) present in nucleic acid preparations extracted from TMV-infected cells. The synthesis of the LMC was resistant to AMD treatment and may represent the same low molecular weight RNA referred to by Babos (1969). This material may represent viral messenger RNA (Babos, 1971).

## Relation of Replicative Form and Replicative Intermediate to TMV-RNA Synthesis and Their Location in the Cytoplasm

In 1964, Erikson et al. demonstrated the presence of a double-stranded RNA structure in E. coli cells infected with the RNA-containing bacteriophage, R17. A similar observation was reported by Baltimore et al. (1964) in nucleic acid extracts of HeLa cells infected with poliovirus. Shipp and Haselkorn (1964), Burdon et al. (1964) and Ralph et al. (1965) reported the isolation and characterization of a double-stranded RNA structure from TMV-infected tobacco leaves. The double-stranded nature of the structure was deduced from its resistance to deoxyribonuclease and ribonuclease treatment. It was susceptible to alkaline hydrolysis. The material banded upon cesium sulfate density gradient centrifugation at a lower density than single-stranded

TMV-RNA. The presumed double-stranded RNA exhibited a sharp thermal melting curve. The annealing characteristics of the denatured double-stranded RNA, when allowed to reanneal with $^{32}P$ TMV-RNA, indicated that one of the strands was identical to TMV-RNA. Shipp and Haselkorn (1964) estimated that there was approximately one double-stranded RNA per $10^3$ complete virus particles. Burdon et al. (1964) calculated that this represents about .5% of the total viral RNA. The physical properties of the double-stranded RNA isolated from TMV-infected leaves were similar to the properties reported for the bacteriophage double-stranded RNA which is referred to as the replicative form (RF) (Burdon et al., 1964).

Fenwick et al. (1964) proposed that the active replicative form of R17-RNA existed as a double-stranded structure with single-stranded tails. More recent work has confirmed this structure (Erikson and Gordon, 1966; Baltimore and Girard, 1966) in R17 and poliovirus-infected cells respectively. The partially double-stranded RNA containing single-stranded tails has been designated the replicative intermediate (RI). Pace et al. (1967), using QB RNA polymerase, and Girard (1969), using crude poliovirus RNA polymerase, have shown that the RI is the immediate precursor to viral RNA. Shipp and Haselkorn (1964), Burdon et al. (1964), Ralph et al. (1965) and Ralph and Wojcik (1969) used ribonuclease in their procedures for isolation of double-stranded RNA from TMV-infected leaves. Such procedures

would have destroyed the "tails" of the replicative intermediate, thus producing, in effect, RF. Recently, Nilsson-Tillgren (1969, 1970) and Jackson et al. (1971) have devised procedures which do not entail ribonuclease digestion for the isolation of double-stranded RNA. They have thus been able to demonstrate the presence of both RF and RI in nucleic acid extracts from TMV-infected tobacco leaves. The RI purified in this manner is similar to the bacteriophage RI and poliovirus RI in several aspects.

Ralph and Clark (1966) attempted to determine the intercellular location of the double-stranded viral RNA in infected tobacco leaves. Double-stranded TMV-RNA could not be detected in nucleic acid extracts from the nuclear-chloroplast fraction isolated from infected leaves; however, 40% of the total double-stranded RNA could be extracted from the mitochondrial fraction. They concluded that the double-stranded RNA may be associated with the mitochondria. They did not rule out the possibility that the double-stranded RNA may have originated from nuclei and/or chloroplasts which were ruptured during the isolation process. In 1969, Ralph and Wojcik reported on studies involving the in vitro synthesis of double-stranded RNA by subcellular fractions isolated from TMV-infected leaves. As was reported earlier (Ralph and Clark, 1966), double-stranded RNA could not be detected in the nuclear fraction, but virtually all double-stranded RNA was associated with the mitochondrial

fraction. In in vitro experiments, three subcellular fractions were isolated: A nuclear fraction containing most of the DNA, a chloroplast fraction, and a post chloroplast-mitochondrial fraction which contained only a small percentage of the chloroplasts. The fractions were incubated in a standard assay mixture for cell-free RNA synthesis. The assay mixture contained UTP-$^{3}$H, incorporation of which was used to monitor RNA synthesis. Actinomycin-D was also added to inhibit DNA-dependent RNA synthesis (Sänger and Knight, 1963; Kubo, 1966). After the appropriate time of incubation, double-stranded RNA was isolated as described by Ralph and Clark (1966). The nuclear fraction synthesized little if any double-stranded RNA. The chloroplast fraction contained approximately 33% of the in vitro synthesized double-stranded RNA, the post chloroplast-mitochondrial fraction approximately 66%. Ralph and Wojcik concluded that double-stranded RNA is not synthesized in the nuclei or chloroplasts. It was observed that, while not synthesizing double-stranded viral RNA, nuclei from infected cells did incorporate radioactive-labeled RNA precursors into RNA even in the presence of actinomycin-D. AMD completely inhibited RNA synthesis in nuclei isolated from healthy tobacco leaves. This apparent AMD-resistant RNA synthesis in nuclei of TMV-infected cells may account for the observations of Smith and Schlegel (1965) and De Zoeten and

Schlegel (1967), who concluded from their autoradiographic studies of AMD-treated cells that TMV-RNA was synthesized in the nucleus.

Bradley and Zaitlin (1971) reported the in vitro synthesis of high molecular weight virus-specific RNA. The fractionation procedures used were essentially those of Ralph and Wojcik (1969), except that the mitochondrial fraction was homogenized after isolation. Tritiated uridine incorporation into TCA-insoluble material by this preparation was not affected by deoxyribonuclease or AMD treatments. The product synthesized was insensitive to DNAase and RNAase treatments but destroyed by alkaline treatment. Polyacrylamide gel electrophoresis of the product yielded two bands of uridine incorporation. One of the bands co-electrophoresed with purified TMV-RF (Jackson et al., 1971). The second band was estimated to be of higher molecular weight and, on the basis of partial ribonuclease sensitivity, was concluded to be TMV-RI. The in vitro system described by Bradley and Zaitlin (1971) did not form single-stranded RNA.

The work of Ralph and Wojcik (1969) and Bradley and Zaitlin (1971) indicated that the synthesis of double-stranded RNA occurs in a subcellular fraction which sediments with mitochondria. Further characterization of the site of synthesis of double-stranded RNA was presented by Ralph et al. (1971). The mitochondrial fraction isolated from TMV-infected, $^{32}$P-labeled plants was subjected to density

gradient centrifugation in 30-60% sucrose gradients. The gradients contained three bands or fractions. Electron microscopic examination revealed that the top fraction was composed mainly of smooth membranes. Twenty-four per cent of the double-stranded TMV-RNA was found to be associated with this fraction. The middle fraction, containing 49% of the double-stranded RNA, was composed of rough membranes, ribosomes and threadlike virus particles. The bottom fraction contained mitochondria and membrane-bound virus crystals and yielded 21% of the total extractable double-stranded RNA. Based on these observations, Ralph et al. concluded that the TMV-RNA was synthesized in association with membraneous material present in the cytoplasm. A similar association of double-stranded viral RNA and viral RNA synthesis with membranes was made by Caliguiri and Tamm (1969, 1970a,b) in poliovirus-infected HeLa cells.

Jackson et al. (1972) demonstrated that in short term labeling experiments uridine-$^{3}H$ was incorporated into the RI and RF. With longer incubation with non-radioactive uridine, the radioactivity was chased completely from the RI but only partially from the RF into TMV-RNA. The observations of Jackson et al. that label cannot be completely chased from the RF and also that, in later stages of infection, only RF can be isolated, indicate that not all of the RF is an active precursor in TMV-RNA synthesis. Nilsson-Tillgren (1969) has also reported a similar build-up of

double-stranded RNA later in infection. It may be that RF is an end product (Jackson et al., 1972; Nilsson-Tillgren, 1970; Woolum et al., 1967). McDonnell and Leventow (1970) concluded that the RF observed in nucleic acid extracts of poliovirus-infected cells may represent an end product of virus infection.

## Site of Viral Protein Synthesis

### I. The Nucleus

Using tobacco mosaic virus-specific fluorescent antibody, Hirai and Hirai (1964) demonstrated that, shortly after inoculation of tomato leaf hair cells, there was a specific fluorescence associated with the nucleus. The fluorescence disappeared within 1-3 hr after inoculation. When ultraviolet light-inactivated TMV was used as inoculum, the fluorescence associated with the nucleus after inoculation still occurred. However, when TMV-RNA was used as inoculum, no early fluorescence was observed. When active TMV or TMV-RNA was used as inoculum, a second period of fluorescence became apparent beginning 6 hr after inoculation. As time passed, the fluorescence became less pronounced in the nucleus and increasing amounts of cytoplasmic fluorescence were observed. Hirai and Hirai concluded from these results that shortly after infection there is a specific interaction between the TMV particle and the nucleus. Because fluorescence was noted in the nucleus before it was

in the cytoplasm, they concluded that TMV protein synthesis occurred in the nucleus. However, because intact cells were used for staining, it would have been difficult to distinguish between fluorescence occurring in close association with the nuclear membrane or within the nucleus itself.

Nagaraj (1965) studied TMV protein distribution in mesophyll cells of N. tabacum, 'Xanthi-nc," at various times after inoculation using fluorescein-conjugated TMV antibodies. Specific fluorescence was observed 18-24 hr after inoculation as tiny specks of stained material distributed in the cytoplasm which later formed a fluorescent ring around the chloroplasts. In thin sections, no TMV-specific fluorescence was observed in the nuclei or chloroplasts.

The ferritin-antibody method developed by Singer (1959), which provided a means of specifically localizing protein with the electron microscope, was used by Shalla and Amici (1967) to study TMV antigen distribution in cells of tomato leaves. They observed a progressive increase in viral antigen in the cytoplasm 2-4 days after inoculation. No antigen was detected in the nucleus by this method. However, nuclei isolated from leaf homogenates did exhibit intranuclear ferritin granules after in vitro conjugated antibody treatment. The inability to demonstrate the viral antigen in the nucleus in situ was attributed to inability of the conjugated antibody to penetrate the nuclear membrane. No

complete virus particles were observed in the nuclei although they appeared to contain viral antigen.

The inability of ferritin-conjugated antibody to enter the nucleus in situ was overcome by Langenberg and Schlegel (1969) with the use of iodine$^{132}$-labeled antibody and electron microscope autoradiographic techniques. During the first 12 hr p.i., the antigen concentration, as determined by autoradiography, increased slightly more rapidly in the nucleus than in the cytoplasm. Later in the infection, the amount of cytoplasmic antigen increased rapidly with relatively little increase in the amount of nuclear antigen. The authors concluded that one explanation for the observed results was that viral protein was synthesized in the nucleus and then translocated to the cytoplasm, where assembly of complete virus occurred.

Although there have been extensive electron microscopic studies of TMV-infected cells, the demonstration of complete virus in the nucleus appears to be a rare occurrence (Milne, 1966a,b; Shalla and Amici, 1967; Esau and Cronshaw, 1967; Esau, 1968).

## II. The Chloroplast

Zaitlin and Boardman (1958) reported an association of tobacco mosaic virus with chloroplast isolated from tobacco leaves. After repeated washing, TMV still remained associated with the chloroplasts. After lysis in borate

buffer, the washed chloroplasts were found to contain from 0.6 to 4.2% the amount of virus isolated from whole cells. Boardman and Zaitlin (1958) studied incorporation of $^{14}C$-aspartic acid into TMV associated with the chloroplasts and TMV found in the supernatant after sedimentation of chloroplasts. They found, with labeling periods up to 18 hr, that the specific activity of the TMV isolated from chloroplasts was 2.5 to 11 times higher than the specific activity of TMV in the post chloroplast supernatant. Based on these observations they concluded that the chloroplast may be the site of assembly of complete TMV. However, in a later paper (Zaitlin et al., 1968) concluded that the chloroplast was not the site of synthesis or of accumulation of complete virus. Singer (1972), using SDS-polyacrylamide gel electrophoresis of proteins isolated from sub-cellular fractions of infected leaves, concluded that the chloroplast is the site of assembly of complete virus.

Shalla (1964) and Milne (1966b), using electron-microscopy, observed clusters of TMV particles in chloroplasts of infected leaves; however, the clusters were membrane-bound indicating that they may have been engulfed by the chloroplasts rather than synthesized there. Esau (1968) reported the presence of TMV aggregates in chloroplasts; the aggregates were not membrane-bound. Shalla (1968) reported that the strain of the virus may to a large degree determine whether or not it will be membrane-bound

when observed in the chloroplast. If the leaf was infected with the U-1 strain of TMV, virus clusters found in the chloroplast were membrane-bound; if the leaf was infected with the U-5 strain, virus clusters in the chloroplast were not membrane-bound. From these observations, Shalla concluded that the U-5 strain of TMV may be synthesized in the chloroplast.

The chloroplast as the site of synthesis of viral coat protein is still being debated. As pointed out by Fraser (1969) and Shalla (1968), the strain of virus employed may to a large degree determine the reaction of the chloroplast to virus infection and that different strains of the virus may have different sites of synthesis. But the observations of Fraser (1972) and Hirai and Wildman (1969) on the drastic effect of TMV infection on chloroplast-ribosomal RNA metabolism, and the observations of Zaitlin et al. (1968) that chloramphenicol, a potent inhibitor of chloroplast-ribosomal protein synthesis, does not inhibit virus synthesis would appear to preclude the chloroplast as the site of viral coat protein synthesis at least for the strains of virus used in their experiments.

## III. The Cytoplasm

The cytoplasm has universally been accepted as the major site of accumulation of complete TMV particles; however, the cytoplasm as the site of viral protein synthesis remains an area of discord.

The cytoplasmic ribosomes are generally agreed upon to be the site of protein synthesis in the cytoplasm. If TMV-RNA acts as messenger for viral protein synthesis, the association of TMV-RNA with cytoplasmic ribosomes and/or polysomes would be a strong indication that viral protein synthesis does occur in the cytoplasm (Schlegel et al., 1967).

Diener (1962) reported the presence of infectious viral RNA in leaf extracts ground in sodium chloride solutions buffered at pH 9-10. The infectious viral RNA co-sedimented with cellular ribonucleic acid upon sucrose density gradient centrifugation. The infectivity present in the nucleic acid zone was destroyed by ribonuclease treatment, which demonstrated that the infectivity in this zone was indeed due to viral RNA and not complete virus. Diener demonstrated that the free viral RNA was not an artifact produced by stripping of coat protein from virus particles. The viral RNA was present in leaf extracts 2-3 days after inoculation, during the period of most rapid virus synthesis; however, leaves extracted 3 weeks after inoculation yielded little infectivity in the nucleic acid zone.

A more definitive study was reported by van Kammen (1961). Ribosomes from TMV-infected leaves were isolated and complete virus particles contaminating the ribosome preparation were removed by precipitation with virus-specific antibody. The ribosomes were then extracted with phenol and

infectivity remaining in the preparation was determined. Van Kammen concluded that there was more infectious viral RNA present in the ribosome preparations than could be accounted for by contaminating complete virus particles. An increase in infectious viral RNA present in the ribosome preparation was observed just prior to the period of most rapid virus accumulation.

A functional relationship of TMV-RNA with polysomes was demonstrated by Kiho (1968). Cytoplasmic polysome preparations isolated from TMV-infected leaves exhibited a polysome region (360-380 S) which was not present in preparations from healthy leaves. TMV coat protein specific antibody precipitation of this polysome fraction suggested that these polysomes were involved in the synthesis of viral coat protein. McCarthy et al. (1970) also demonstrated a large, apparently specific polysome region present in extracts of mung bean infected with a strain of TMV.

In 1970, Kiho demonstrated the presence of parental TMV-RNA in association with polysomes shortly after infection. In some cases, partially uncoated TMV was found in association with polysomes. He concluded that ribosomes apparently associated immediately with TMV-RNA as the RNA became exposed by the uncoating process.

Babos (1971) has shown by RNA-RNA hybridization experiments that TMV-RNA is associated with cytoplasmic ribosomes. The ribosomes were associated with a rapidly

labeled RNA which was rendered ribonuclease-resistant when annealed with TMV-specific double-stranded RNA, but not with TMV-RNA. On the basis of these experiments and earlier work (Babos and Shearer, 1969), he concluded that the rapidly labeled RNA associated with cytoplasmic ribosomes was TMV-RNA which may act as messenger.

## Materials and Methods

### Inoculation and Uridine-$^{3}$H or Leucine-$^{14}$C Labeling of Tissue Culture Cells

A pigmented cell culture derived from stem tissue of tobacco (Nicotiana tabacum L. var. Havana 38) was used throughout this study. The cells were maintained and inoculated as previously described (Murakishi et al., 1970, 1971). A common strain of TMV was used for inoculation at a concentration of 150 µg/ml of cell suspension. Cells were inoculated in 1-g batches suspended in 3 ml liquid medium. Cells were then pooled and washed with 30 ml fresh medium. Three gram aliquots of cells were transferred to a 4 cm disc of Whatman No. 4 filter paper and then placed in a petri plate containing 20 ml of medium solidified with 1% agar. The cells were incubated at approximately 25° under light provided by Gro-lux fluorescent lamps. At 12-hr intervals after inoculation the cells were transferred on the filter paper to a 5 cm petri plate containing 2 ml of fresh liquid medium

supplied with 50 μC uridine-$^{3}$H-GL (420 mC/mM) or 2 μC leucine-$^{14}$C-U (291-306 mC/mM) and incubated under light for 12 hr. During the labeling period the medium was constantly agitated. Control cells sham inoculated with phosphate buffer (0.1 M, pH 7.6) were treated in like manner. Inoculated and control cells were transferred from the agar medium to liquid medium containing uridine-$^{3}$H or leucine-$^{14}$C every 12 hr throughout the 168-hr period studied. After each labeling period the cells were washed with 20 ml cold 0.25 M sucrose and divided into two 1.5-g (wet weight) aliquots, one of which was frozen at -35° to be used for extraction of complete virus; the other aliquot was immediately extracted with phenol to isolate total cellular nucleic acid.

## Determination of Uridine-$^{3}$H and Leucine-$^{14}$C in Acid-Insoluble Material

Cells were incubated in liquid medium containing uridine-$^{3}$H or leucine-$^{14}$C for the prescribed period of time, collected on coarse filter paper and washed with cold 0.25 M sucrose containing either unlabeled uridine ($1 \times 10^{-4}$ M) or leucine ($1 \times 10^{-3}$ M). Two hundred and fifty milligram-aliquots of cells were then removed and ground in a conical glass homogenizer containing 1.75 ml 100 mM Tris-HCl, pH 8.0, 0.1% mercaptoethanol, $1 \times 10^{-3}$ M leucine, $1 \times 10^{-4}$ M uridine. The homogenate was then centrifuged at approximately 1000 x g for 5 min. One-half milliliter of the supernatant was

then precipitated with 1.5 ml cold 20% trichloroacetic acid (TCA) and held in the cold for 15 min. The precipitate was collected on Millipore glass prefilters (AP 2001900, Millipore Corp., Bedford, Mass.), washed with 10 ml 5% TCA, 10 ml 95% ethanol and 5 ml ethanol:ether (v:v). The filters were air dried and the precipitate solubilized with 0.5 ml of a solution made up of 20% NCS solubilizer, 3.75% water and 76.25% toluene for 4 to 8 hr at 37$^{o}$ (Haslam et al., 1970). Ten milliliters of toluene based POPOP-PPO (0.05 g/l-4.0 g/l) solution was then added and the preparations placed in the dark at 4$^{o}$ for 12 hr. Radioactivity was then determined in a Packard Tri-Carb liquid scintillation counter. The discriminator settings were adjusted to 30 and 250, 60% gain for counting the uridine-$^{3}$H, and to 250 and 1000, 17% gain for counting leucine-$^{14}$C. The counting efficiency was approximately 25% and 53% for uridine-$^{3}$H and leucine-$^{14}$C, respectively.

## Extraction of Total Cellular and Viral Nucleic Acid

Total nucleic acids were extracted from 1.5 g of cells by grinding (20 strokes) in a Tenbroeck glass homogenizer containing 10 ml distilled water, 0.5% sodium dodecyl sulfate (SDS) w/v and 0.1% disodium naphthalene sulfonate w/v. Immediately after homgenization 10 ml of water-saturated phenol containing 10% m-cresol v/v and 0.1%

8-hydroxyquinoline w/v was added (Kirby, 1965). The homogenate was then mixed for 10 min on a Model K-500 J Vortex mixer (Scientific Industries, New York). The phases were separated by centrifugation at 18,000 x g for 10 min. All steps in the extraction procedure were carried out at 4°. The aqueous phase was removed, made 0.3 M with respect to NaCl, and again extracted with the phenol mixture for 5 min. This procedure was repeated twice. Nucleic acids were precipitated from the aqueous phase by addition of 2.5 volumes 95% ethanol, stored at -35° for 12 hr, and collected by centrifugation at 20,000 x g for 20 min. The pellet was washed once with 75% ethanol containing 0.1% NaCl w/v and once with 3.0 M sodium acetate, pH 6.0. The final pellet was then dissolved in electrophoresis buffer (0.04 M Tris, 0.02 M sodium acetate, 0.001 M sodium EDTA brought to pH 7.8 with glacial acetic acid) containing 5% sucrose w/v. The ultraviolet absorption spectra was then determined in a Beckmann DB spectrophotometer. The 240-260-280 nm ratios were in the order of 1:2:1. The concentration of the nucleic acid was determined assuming an $E_{260nm}^{0.1\%} = 25$. The concentration of the nucleic acid was then adjusted to 40 µg/0.1 ml.

## Preparation of Polyacrylamide Gels and Electrophoretic Separation of Nucleic Acid Species

The 2.4% polyacrylamide gels were prepared essentially as described by Bishop et al. (1967). The stock acrylamide solution was prepared by dissolving 3.75 g acrylamide (Eastman Organic Chemicals) and 0.185 g bis-acrylamide in a final volume of 25 ml distilled water. The gels were prepared by mixing 4 ml of the stock acrylamide solution, 8.33 ml 3E buffer (0.12 M Tris, 0.06 sodium acetate, 0.003 M sodium EDTA brought to pH 7.8 by addition of glacial acetic acid) and 12.45 ml distilled water. Dissolved air was removed by gentle mixing under vacuum for 15 min and then 0.02 ml N, N, N', N' tetramethylethylenediamine and 0.2 ml freshly prepared aqueous 10% ammonium persulfate was added. The solution was mixed gently for 30 sec and 2 ml were transferred to Plexiglass tubes (12 cm x 1 cm, 0.7 i.d.), one end of which was covered with Parafilm. The gels were allowed to polymerize for 20 min and were transferred to 1E buffer (0.04 M Tris, 0.02 M sodium acetate, 0.001 M sodium EDTA, pH 7.8) containing 0.2% recrystallized SDS (100 ml/gel) and allowed to stand at 4° for 72 hr prior to use. The recrystallized SDS was prepared by boiling ethanol saturated with SDS for 3 hr. The hot ethanol-SDS solution was then filtered to remove undissolved SDS and cooled to -25° for 12 hr; the recrystallized SDS was collected by filtration and air dried.

The polyacrylamide gels were transferred to Plexiglass tubes, one end of which was covered with dialysis membrane and pre-run for 30 min prior to use. Twenty micrograms (0.5 O.D. units) of nucleic acid in a volume of 0.05 ml 1E buffer containing 5% sucrose was applied to the gels and electrophoresis carried out for either 90 min or 150 min at 10 V/cm, 5 mA/gel.

## Determination of Relative RNA Concentration and Label Distribution in the Gels

Immediately after electrophoresis the gels were scanned in a Gilford gel scanner (260 nm) and electropherograms recorded. The area under each optical density peak was then determined and converted to micrograms RNA assuming an $E_{260\ nm}^{0.1\%} = 25$. After scanning, the gels were frozen in dry ice and sliced into 1 nm sections. The gel sections were placed in scintillation vials and dried at $160^{\circ}$ for 12 hr and then solubilized with 0.5 ml of a solution made up of 20% solubilizer for 12 hr at $37^{\circ}$. The milliliters of toluene based POPOP-PPO solution was added and the preparations were placed in the dark at $4^{\circ}$ for 12 hr. Radioactivity was then determined in a Packard Tri-Carb liquid scintillation counter.

## Extraction and Density Gradient Centrifugation of Complete TMV

Complete TMV was extracted from infected tissue culture cells after freezing and thawing of the material. Tissue (1.5 g) was ground in a glass homogenizer containing 2.5 ml of extraction buffer (100 mM Tris-HCl, pH 8.0, 0.1% mercaptoethanol). The homogenate was centrifuged at 1000 x g for 10 min. The supernatant was then centrifuged at approximately 22,000 x g for 15 min. The supernatant was layered over a 1 ml 30% sucrose cushion and centrifuged at 134,000 x g for 90 min. The resulting pellet was suspended in 1 ml of distilled water and placed in the cold for 12 hr. This material was layered on a linear 10-40% sucrose gradient (100 mM Tris HCl, pH 8.0). The gradients were subjected to centrifugation at 80,000 x g for 90 min in a SW 25.1 rotor and fractionated with an ISCO Model D fractionater coupled to a Model VA-2 U.V. analyzer (254 nm). The material corresponding to the viral band region of the gradient was collected or 1 ml fractions were collected along the entire gradient. After addition of 0.5 mg bovine serum albumin (BSA), the material was precipitated with 20% trichloroacetic acid (TCA), collected on a glass filter (Millipore) and washed with 10 ml 5% TCA, 10 ml cold 95% ethanol and 5 ml ethanol:ether (v:v). After drying the precipitate was solubilized as described above. Radioactivity was determined

in a liquid scintillation counter. The concentration of virus in the virus band was determined assuming an $E_{254\ nm}^{0.1\%} = 3.06$.

## Preparation and Use of Uridine-$^3$H Labeled TMV for Marker RNA

Tissue culture cells were inoculated, transferred to filter paper, and incubated in 2 ml medium containing uridine-$^3$H (25 μC/ml). The medium was removed and fresh medium added at 24-hr intervals. After 120 hr incubation the cells were harvested and washed with cold 0.25 M sucrose and frozen. The cells were ground and the virus isolated as previously described by Murakishi et al. (1971). Virus to be used as a source of marker RNA was further purified by centrifugation on a 10-40% sucrose gradient; material banding in the TMV region was collected by ISCO fractionation. Virus purified in this manner had a specific activity of 288 cpm/μg.

To identify the position of viral RNA in the polyacrylamide gels, labeled virus was mixed with unlabeled tissue culture cells 60 hr post inoculation. Total nucleic acids were then extracted and subjected to electrophoresis for 90 min. The optical density profile and radioactivity was determined as described above.

# Results

## Gel Electrophoresis of Nucleic Acids Extracted from Healthy and TMV-Infected Tobacco Tissue Culture Cells and Identification of TMV-RNA

At 60 hr post inoculation (p.i.) total nucleic acids were extracted from both healthy and TMV-infected tissue culture cells. The nucleic acids were subjected to electrophoresis for 90 min in 2.4% polyacrylamide gels. Nucleic acids extracted from control tissue cultures yielded four discrete optical density peaks (Figure 1A). Based on relative migration rates, the peaks corresponded to DNA (2.5-3.7mm), 25 S cytoplasmic ribosomal RNA (16.3-22.5 mm), 18 S cytoplasmic ribosomal RNA (27.5-33.7 mm) and 4-5 S RNA (57.5-67.5 mm) (Loening and Ingle, 1967). Nucleic acids extracted from TMV-infected tissue culture exhibited the four major optical density peaks observed with the nucleic acids extracted from healthy tissue plus a fifth peak (7.5-12.5 mm) (Figure 1B). A small optical density peak which migrated slightly faster than the DNA was occasionally observed in electropherograms of nucleic acids from both healthy and infected cells.

To determine if the material migrating into the gel as a band (7.5-12.5 mm) was TMV-RNA, uridine-$^{3}H$ labeled TMV-RNA was subjected to electrophoresis with unlabeled nucleic acids isolated from infected cells 60 hr p.i. The

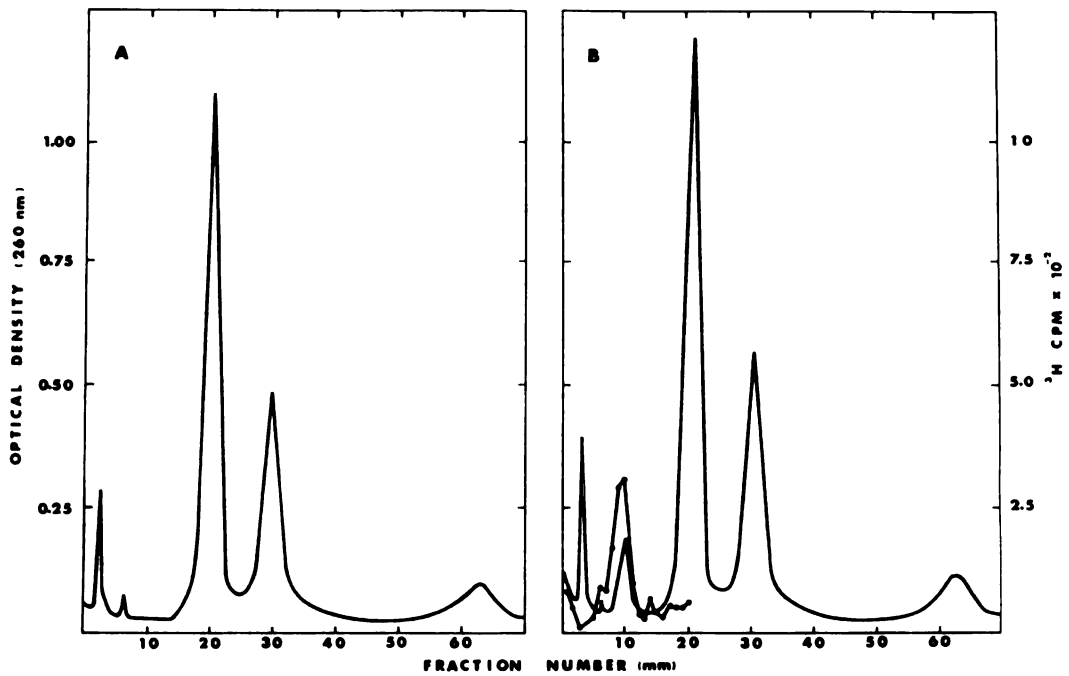


Figure 1. Polyacrylamide gel electrophoresis of nucleic acids extracted 60 hr post inoculation. Twenty micrograms of phenol-extracted nucleic acid was applied to 2.4% polyacrylamide gels and subjected to electrophoresis for 90 min at 5.0 mA/gel, 10 V/cm. (A) Unlabeled nucleic acids extracted from control cells 60 hr after inoculation with buffer only. Based on relative migration rates the major optical density peaks represent, from left to right, DNA, 25 S RNA, 18 S RNA and 4-5 S RNA (Loening and Ingle, 1967). (B) Unlabeled nucleic acids extracted from virus-infected cells 60 hr after inoculation. Immediately prior to extraction of the nucleic acids, purified uridine-$^{3}H$ labeled TMV was added to the infected cells to serve as a radioactive marker for TMV-RNA synthesized in the infected cells. After electrophoresis the gels were scanned and sliced into 1-mm sections and radioactivity determined. TMV-RNA migrated between the DNA and 25 S host RNA. (——, OD; ●—●, cpm).

source of the uridine-$^{3}$H labeled TMV-RNA was complete virus purified by sucrose density gradient centrifugation (Figure 2). Virus purified in this manner migrated as a single band of optical density absorbing material which corresponded in position to the peak radioactivity distribution in the sucrose gradient and was apparently free of contamination by cellular materials.

The optical density peak observed in electropherograms of nucleic acids extracted from the infected cells corresponded in position to the radioactivity peak resulting from uridine-$^{3}$H labeled TMV-RNA extracted from purified complete virus (Figure 1B). The coincidence of these two peaks shows that the new peak observed with nucleic acids from infected tissue culture is TMV-RNA.

Gel electrophoresis of nucleic acids isolated from tobacco leaf tissue resolved four ribosomal RNA species. Two of these RNA species, namely the 25 S and 18 S species, corresponded to the subunit RNAs of the 80 S cytoplasmic ribosomes. The 23 S and 16 S RNAs corresponded to the subunit RNA of 70 S chloroplast ribosomes (Hirai and Wildman, 1969; Fraser, 1969). In Figure 1 (A and B) it can be seen that no optical density peaks occurred in the area that would correspond to the 23 S and 16S chloroplast ribosomal subunit RNAs. To determine if the extraction technique employed was effectively extracting chloroplast ribosomal RNA, total nucleic acids were extracted from both healthy and

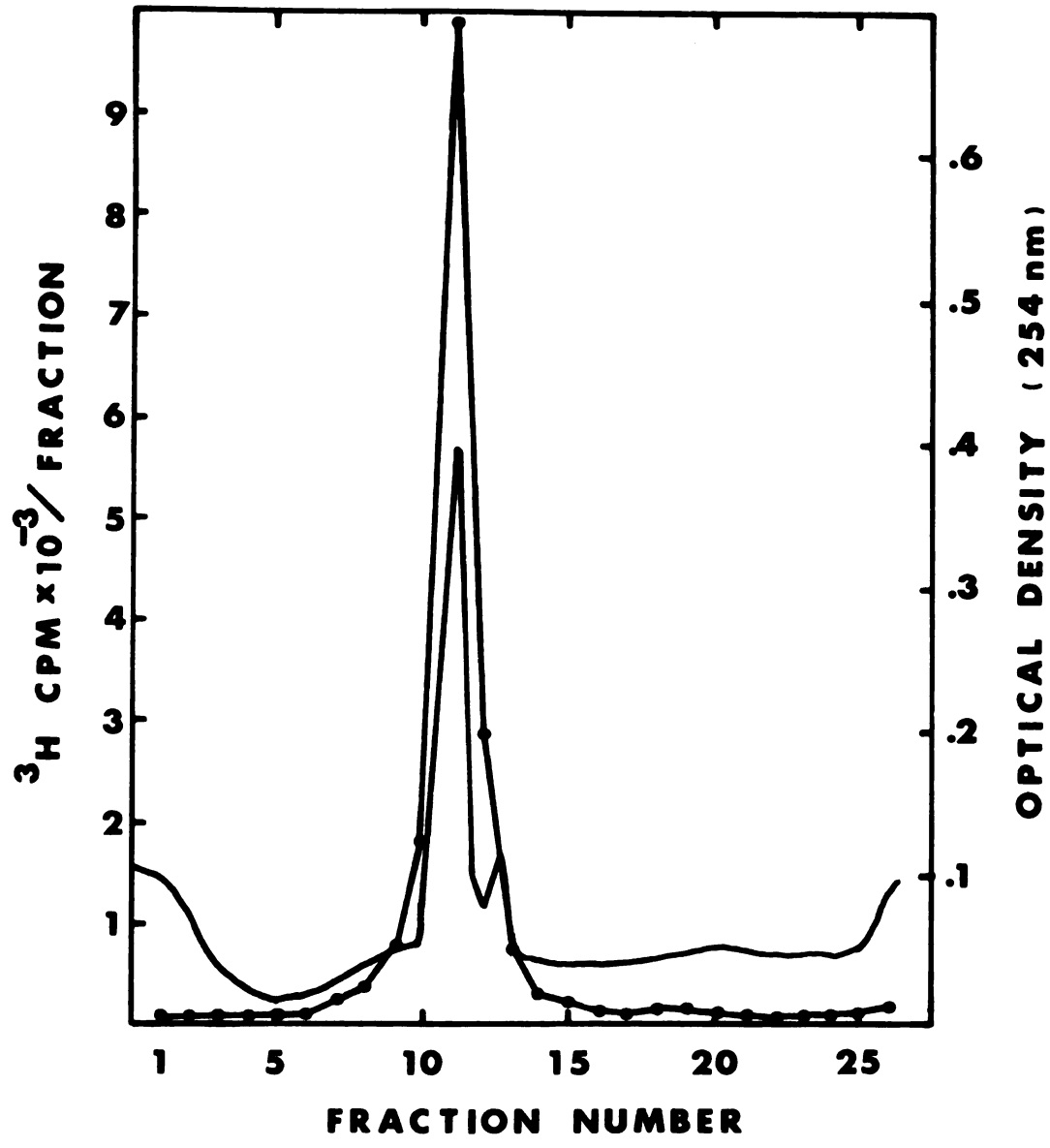


Figure 2. Density gradient centrifugation and purification of uridine-$^3$H labeled TMV used as source of marker RNA. Complete TMV was extracted from inoculated tissue culture cells exposed to uridine-$^3$H (25 μC/ml) for 120 hr. The virus was partially purified as described by Murakishi et al. (1971). The virus was then subjected to centrifugation on 10-40% sucrose gradients for 90 min at 80,000 g; material banding in the region of TMV was collected by ISCO fractionation and used as a source of marker TMV-RNA. (——, OD; ●—●, cpm).

TMV-infected leaf tissue of H-38 tobacco plants and subjected to electrophoresis. Both 80 S and 70 S ribosomal subunit RNAs were resolved and separated by gel electrophoresis. This indicated that the extraction procedure used was efficiently extracting chloroplast ribosomal RNAs.

## Incorporation of Uridine-$^{3}H$ and Leucine-$^{14}C$ into Trichloroacetic Acid-Insoluble Cellular Material

Cells incubated for 48 hr on filter paper pads which were placed on agar-solidified medium were able to incorporate either uridine-$^{3}H$ or leucine-$^{14}C$ into acid-insoluble material when transferred from agar-solidified medium to liquid medium containing either uridine-$^{3}H$ or leucine-$^{14}C$. Incorporation of either uridine-$^{3}H$ or leudine-$^{14}C$ into acid-insoluble material was monitored at 3-hr intervals during the incubation period. Incorporation of uridine appeared linear during the 12-hr incubation period; however, leucine incorporation often deviated from linear with incubation periods extending beyond 9 hr (Figure 3). The cause of this deviation was not determined but it was not due to depletion of leucine-$^{14}C$ from the incubation medium, as the rate of incorporation increased rapidly during the 9-12-hr interval. No consistant difference was observed in the ability of either virus-infected or non-infected cells to incorporate uridine or leucine into macromolecules.

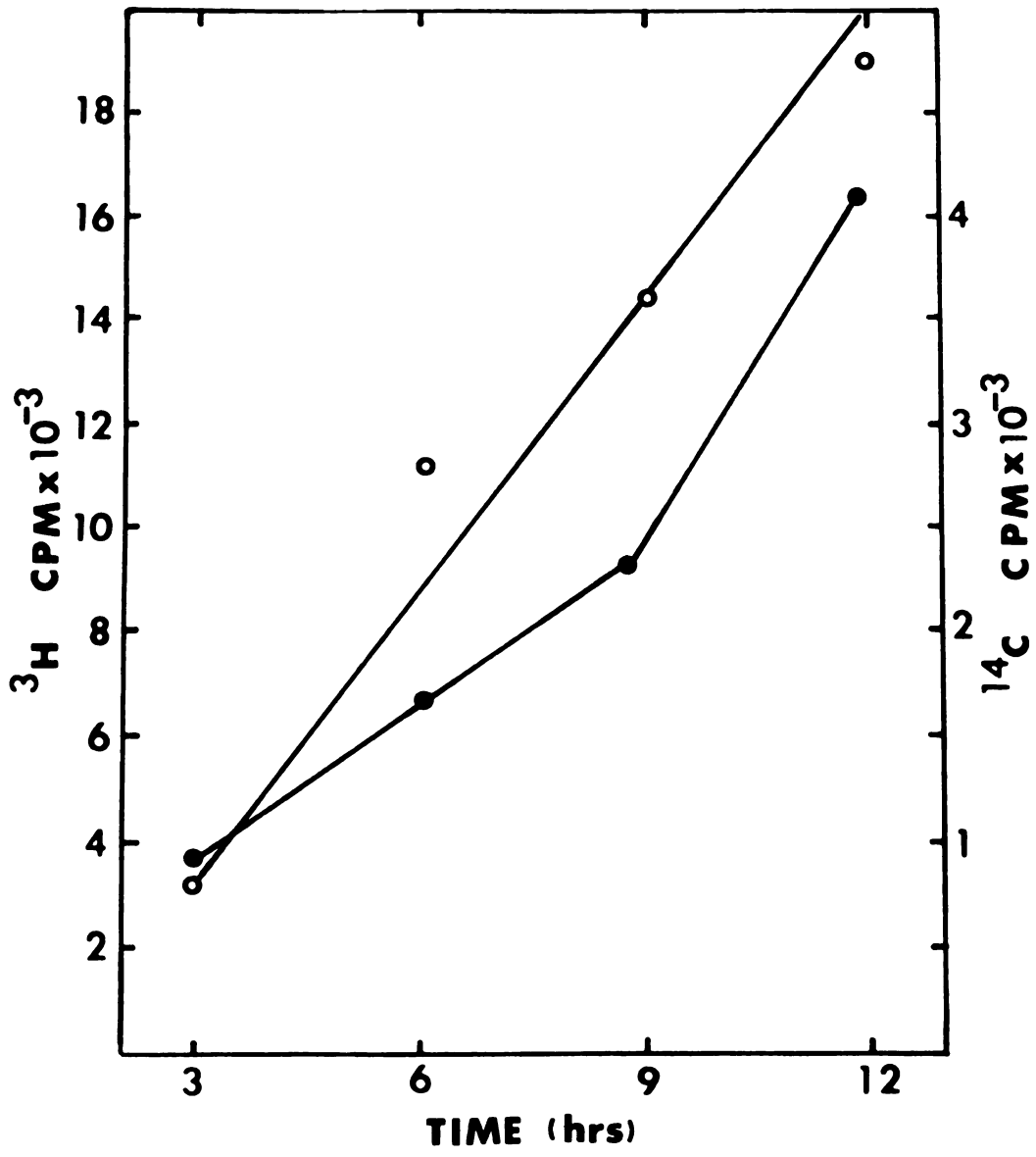


Figure 3. Incorporation of uridine-$^3H$ and leucine-$^{14}C$ into acid-insoluble material. Cells were exposed to either uridine-$^3H$ (25 µC/ml) or leucine-$^{14}C$ (1 µC/ml). Incorporation into acid-insoluble material was monitored at 3-hr intervals. Two hundred and fifty milligram samples were harvested and homogenized in 1.75 ml Tris-HCl buffer (pH 8.0). One-half milliliter was then precipitated with 1.5 ml cold 20% trichloroacetic acid, collected on a glass filter and washed with 10 ml 5% TCA, 95% ethanol and 5 ml ethanol:ether (v:v). The precipitate was then solubilized with NCS solubilizer solution and radioactivity determined. (o—o, $^3H$ cpm; ●—●, $^{14}$ C cpm).

A standard 12-hr exposure to uridine-$^{3}H$ or leucine-$^{14}C$ was selected to minimize variability of incorporation. Shorter incubation periods often led to greater variability of incorporation between duplicate samples.

Cells incubated on agar-solidified medium exhibited continual growth and increase in fresh weight over the 168-hr period studied. To determine if the ability of the cells to incorporate either uridine-$^{3}H$ or leucine-$^{14}C$ into macromolecules varied with time of incubation on agar-solidified medium, cells were transferred at 12-hr intervals after inoculation from the solid medium to liquid medium containing uridine-$^{3}H$ or leucine-$^{14}C$. Incubation in liquid medium was continued for 12 Hr; the amount of uridine-$^{3}H$ or leucine-$^{14}C$ incorporation was then determined by acid precipitation. The ability of the cells to incorporate uridine or leucine did not change drastically over the 156-hr period studied (Table 1). However, there appeared to be a slight decrease in incorporation of uridine and leucine during the 60-96-hr period. Although there was considerable variability among replicates of the same experiments, this trend was observed in duplicate experiments.

## Phenol-Extractable Nucleic Acids and Specific Activity of 25 S Cellular Ribosomal RNA

Nucleic acids were extracted from inoculated or non-inoculated cells by the phenol-SDS technique after various

Table 1. Incorporation of Uridine-$^{3}H$ and Leucine-$^{14}C$ into Acid-Insoluble Material after Incubation on Agar Medium.

After inoculation, cells were incubated on agar medium. At 12-hr intervals cells were transferred from the agar medium and incubated in liquid medium containing uridine-$^{3}H$ (25 μC/ml) and leucine-$^{14}C$ (1 μC/ml) for a 12-hr period. Two hundred and fifty milligrams of cells were harvested, ground in 1.75 ml Tris-HCl buffer, and 0.5 ml of the homogenate precipitated with 20% TCA and collected on a glass filter and then washed with 5% TCA, 95% ethanol and ethanol:ether (v:v). After solubilization of the precipitate, radioactivity was determined.

| Labeling Period (hr p.i.) | Cpm/.065 gm | |
|---|---|---|
| | Uridine-$^{3}H$ | Leucine-$^{14}C$ |
| 12-24 | 15976±701 | 4102±236 |
| 24-36 | 23149±3206 | 3242±516 |
| 36-48 | 24002±2450 | 3221±382 |
| 48-60 | 23137±1538 | 3427±628 |
| 60-72 | 18265±2197 | 3121±666 |
| 72-84 | 16025±505 | 2695±198 |
| 84-96 | 18875±1424 | 3092±127 |
| 96-108 | 21441±2121 | 3513±857 |
| 108-120 | 25422±2726 | 3798±431 |
| 132-144 | 25917±3029 | 3320±432 |

intervals of incubation on agar-solidified medium. No consistant difference in total nucleic acid content was observed between inoculated or non-inoculated cells over the 168-hr period studied. The amount of phenol-SDS extractable nucleic acid did not vary greatly when determined at 12-hr intervals over a 144-hr incubation period on agar-solidified medium (Table 2).

Polyacrylamide gel electrophoresis of nucleic acids extracted from virus-infected cells exposed to uridine-$^{3}$H during the 48-60-hr period after inoculation revealed that the uridine-$^{3}$H had been incorporated into both the 25 S and 18 S cellular ribosomal species as well as into TMV-RNA (Figure 4). The distribution of radioactivity in the gel corresponded closely to the distribution of ultraviolet light absorbing material in the gel. This correspondence did not change throughout the 168-hr period studied.

To demonstrate in a more precise manner that the ability of the cells to incorporate uridine-$^{3}$H into cellular RNA did not change with time of incubation on agar-solidified medium, the specific activity (cpm/μg) of the 25 S cellular RNA was determined at various intervals after inoculation by computing the amount of 25 S RNA (μg) per gel and uridine-$^{3}$H (cpm) incorporated into this RNA species. The specific activity of the 25 S cellular ribosomal RNA did not change drastically throughout the 168-hr period studied (Table 3).

Table 2. Total Phenol-Extractable Nucleic Acids.

After inoculation, cells were incubated on agar medium. At 12-hr intervals, 1.5 g of cells were harvested and nucleic acids extracted by the phenol-SDS technique. The nucleic acids were then precipitated with 2.5 volumes 95% ethanol. After washing, the nucleic acids were dissolved in 0.2 ml buffer and the nucleic acid concentration determined by optical density measurement assuming an $E^{0.1\%}_{260\ nm} = 25$.

| Hr p.i. | µg RNA/1.5 g Cells |
|---|---|
| 24 | 490 |
| 36 | 370 |
| 48 | 480 |
| 60 | 530 |
| 72 | 530 |
| 84 | 590 |
| 96 | 600 |
| 120 | 495 |
| 144 | 500 |

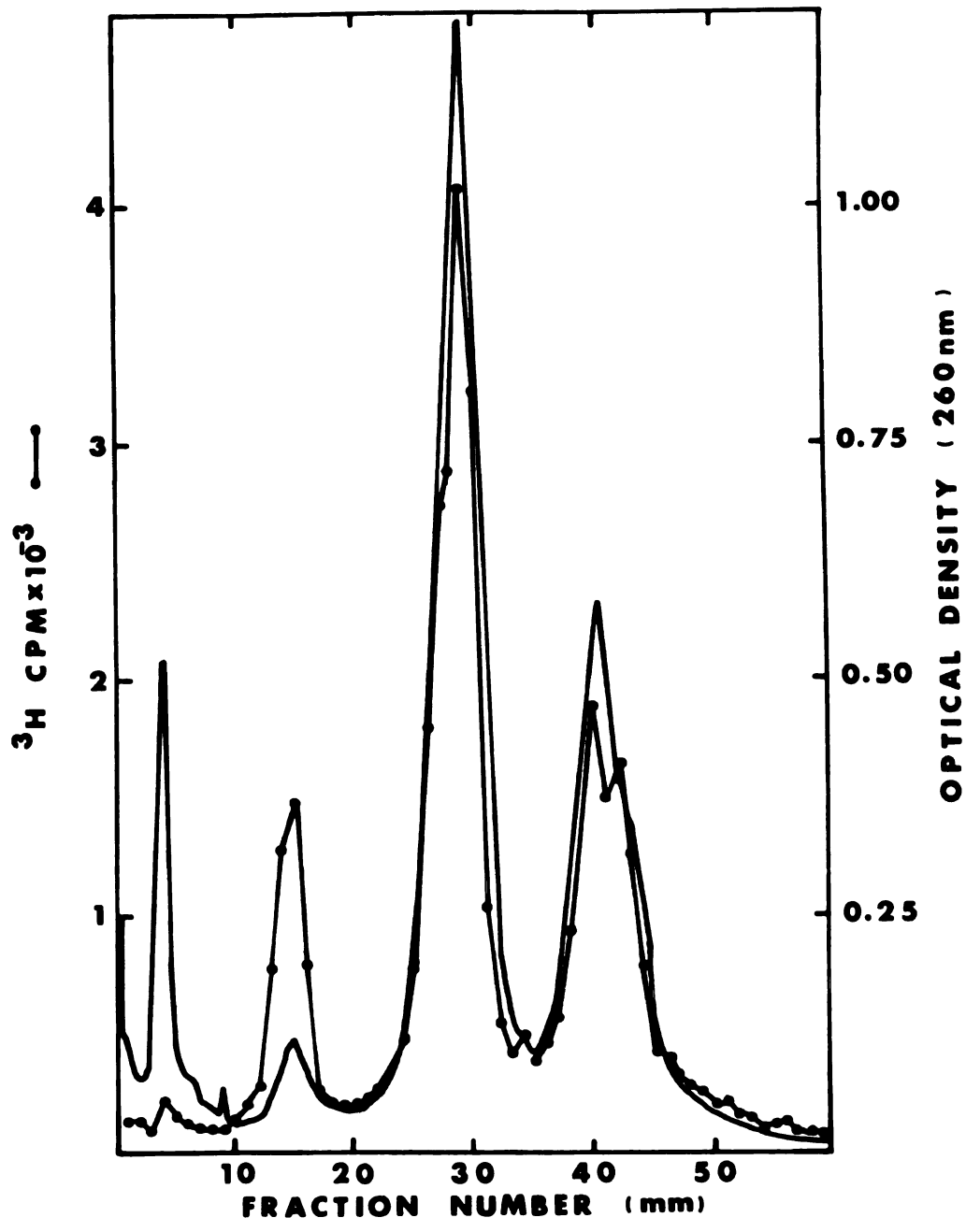


Figure 4. Polyacrylamide gel electrophoresis of uridine-$^3$H nucleic acids extracted from virus-infected cells. Cells were exposed to uridine-$^3$H (25μC/ml) 48-60 hr p.i. Twenty micrograms of phenol-extracted nucleic acid was applied to 2.4% polyacrylamide gels and subjected to electrophoresis for 150 min at 5.0 mA/gel, 90 V/gel. The gels were then scanned at 260 nm and radioactivity determinations made throughout the gel. Based on relative migration rates the major optical density and radioactivity peaks represent, from left to right, DNA, TMV-RNA, 25 S RNA, 18 S RNA. (——, OD; ●—●, cpm).

Table 3. Specific Activity of Cellular RNA.

Specific activity of 25 S host ribosomal RNA was determined at 24-hr intervals after inoculation. Labeling, extraction and electrophoresis was carried out as in Figure 4. The area under the 25 S RNA optical density peak was converted to micrograms RNA. Total radioactivity corresponding to the 25 S RNA optical density peak was divided by the total amount of 25 S RNA to give the specific activity (cpm/μg 25 S RNA).

| Labeling Period (hr p.i.) | Cpm/μg RNA Cellular 25 S |
|---|---|
| 12-24 | 3379 |
| 24-36 | -- |
| 36-48 | 4171 |
| 48-60 | -- |
| 60-72 | 2976 |
| 72-84 | -- |
| 84-96 | 3770 |
| 108-120 | -- |
| 132-144 | 4779 |
| 156-168 | 3507 |

However, a slight but reproducible decrease in specific activity was observed during the 60-72-hr period. The decrease in specific activity of the 25 S RNA (60-72 hr p.i.) corresponded in time to the decreased ability of the cells to incorporate either uridine-$^{3}H$ or leucine-$^{14}C$ into acid-insoluble material.

## Rate of Viral Nucleic Acid Synthesis in Tissue Culture Cells

At 12-hr intervals after inoculation, cells were transferred to liquid medium containing uridine-$^{3}H$ (25 μC/ml) and incubated for 12 hr. At the end of each labeling period, total nucleic acids were extracted from the cells and subjected to electrophoresis for 2.5 hr. Figures 5A and 5B are optical density profiles of nucleic acids extracted from uninfected cells and from cells infected for 24 hr, respectively. A small optical density peak corresponding in position to TMV-RNA was present in the electropherogram of nucleic acids extracted from infected cells 24 hr p.i. During the 12-24-hr labeling period, only a small amount of uridine-$^{3}H$ was incorporated into TMV-RNA (Figures 5B and 6). Between 36-48 hr p.i., the rate of viral RNA synthesis, as expressed by uridine-$^{3}H$ incorporation, increased to approximately 4 times that of the 12-24-hr period (Figures 5C and 6). The amount of viral RNA present in the infected cells doubled during the second 24-hr period following inoculation

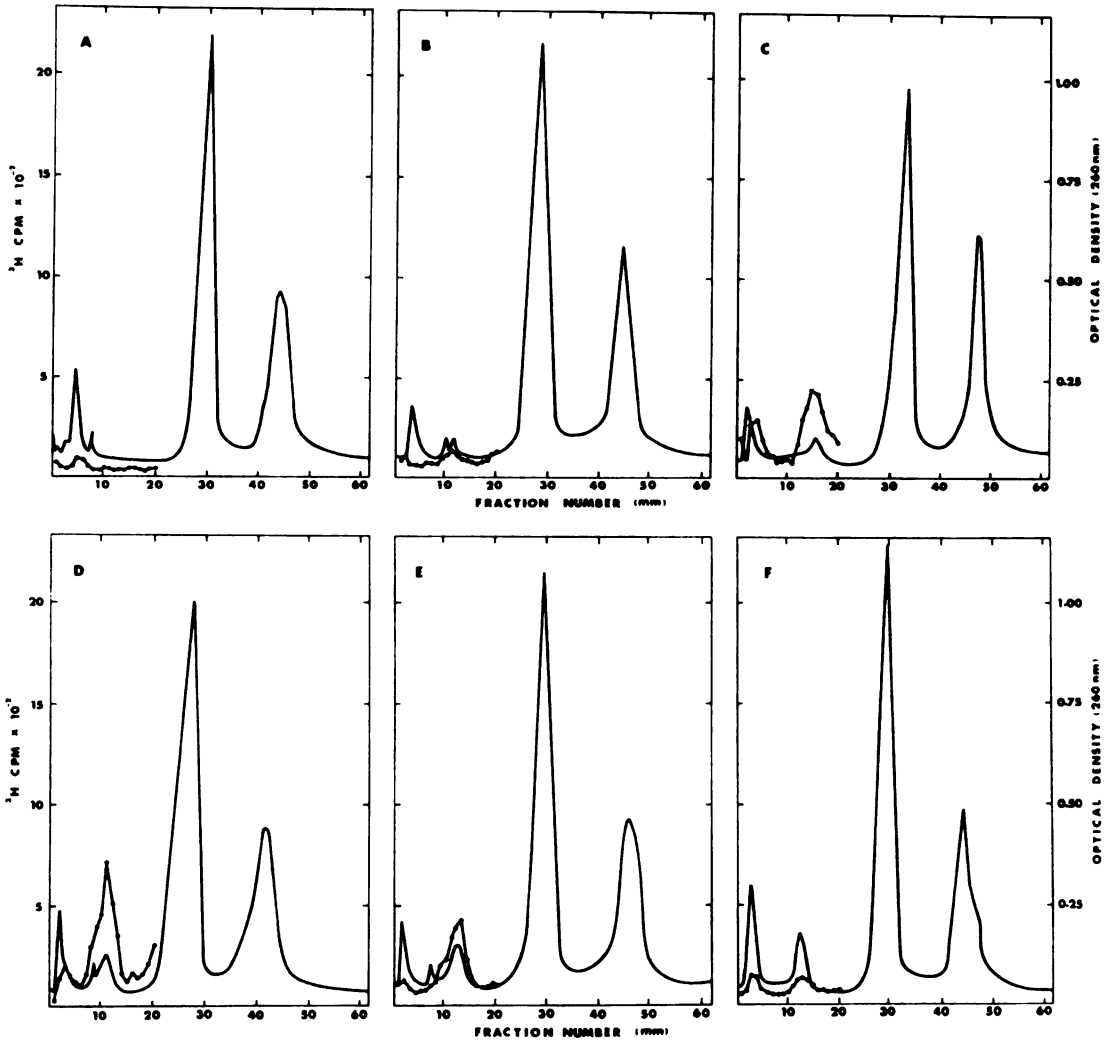


Figure 5. Optical density profile and radioactivity of nucleic acids extracted from control and virus-infected cells. The cells were exposed to uridine-$^{3}H$ (25 μC/ml) for 12 hr prior to nucleic acid extraction. Electrophoresis was carried out on 2.4% gels for 150 min at 5.0 mA/gel. Radioactivity determinations were confined to the first 20 mm of the gels, that portion of the gel known to contain TMV-RNA. (A) Nucleic acids from control cells labeled for 12 hr; (B) nucleic acids from infected cells labeled 12-24 hr post inoculation (p.i.); (C) nucleic acids from infected cells labeled 36-48 hr p.i.; (D) nucleic acids from infected cells labeled 48-60 hr p.i.; (E) nucleic acids from infected cells labeled 72-84 hr p.i.; (F) nucleic acids from infected cells labeled 132-144 hr p.i. (——, OD; ●—●, cpm).

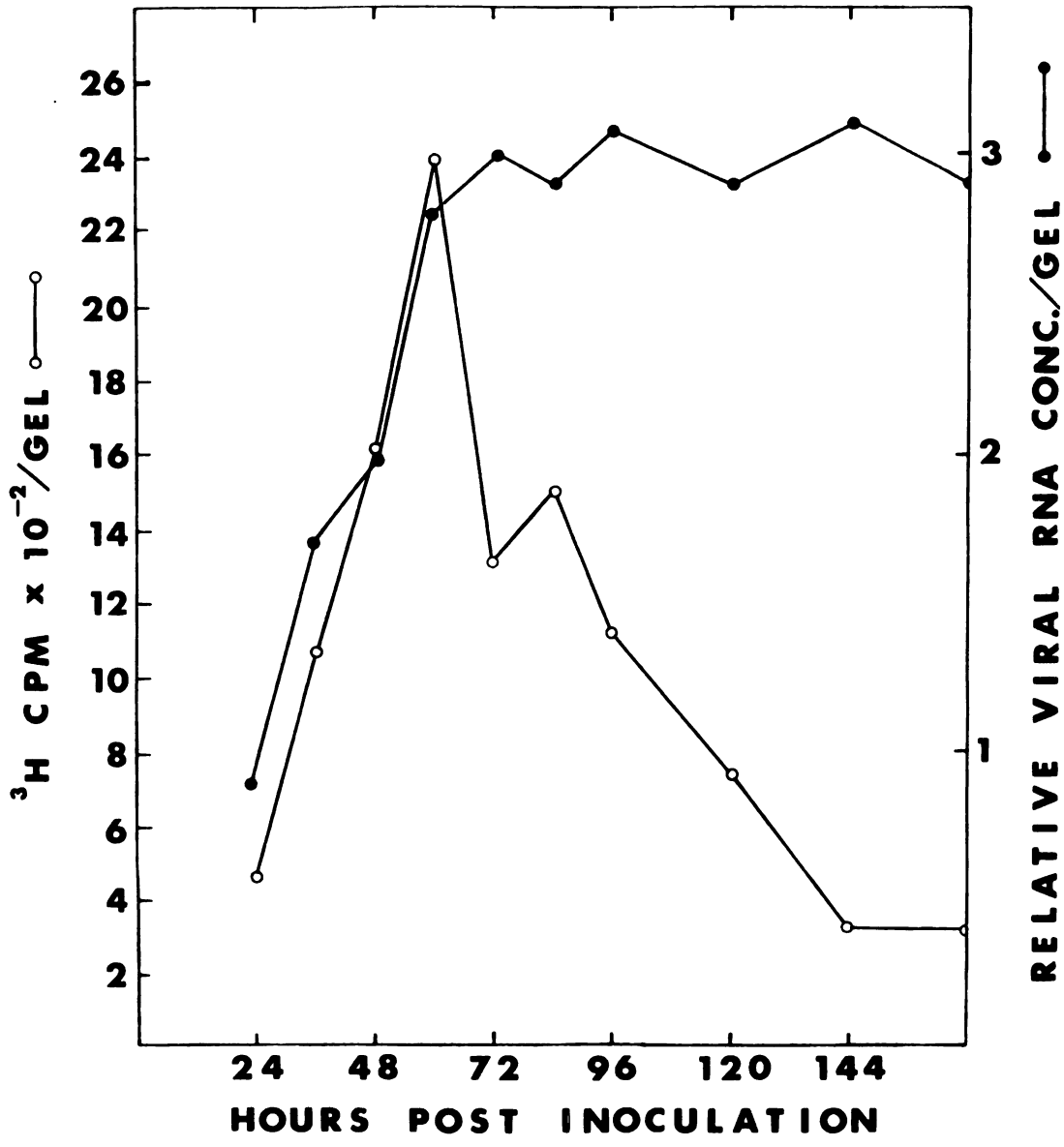


Figure 6. Rate of incorporation of uridine-$^3$H into viral RNA and accumulation of viral RNA. Labeling, extraction and electrophoresis of the nucleic acids was carried out as described in Figure 5. Rate calculations were made by determining cpm/min/gel corresponding in position to the TMV-RNA optical density peak. All calculations were corrected for background radioactivity observed with nucleic acids from control cells. Total viral RNA determinations were made by converting the area under the viral RNA optical density peak to micrograms RNA assuming an $E^{0.1\%}_{260\ nm} = 25$. Both rate and total viral RNA determinations represent the average of two experiments. (●—●, relative viral RNA conc.; o—o, cpm).

(Figure 6). During the 48-60-hr period, the rate of incorporation of uridine-$^3$H into viral RNA reached its peak, approximately 6 times that of the 12-24-hr period (Figures 5D and 6). During the first 60 hr p.i., the rate of uridine-$^3$H incorporation into viral RNA and the rate of accumulation of viral RNA was nearly linear (Figure 6). By 60 hr p.i., approximately 10 µg of TMV-RNA could be recovered from 1.5 g of inoculated cells by phenol-SDS extraction. This amount of TMV-RNA represented approximately 2% of the total phenol-SDS extractable nucleic acid from 1.5 g of cells (Table 2).

After reaching a peak between 48 and 60 hr p.i., the rate of uridine-$^3$H incorporation into viral RNA began to decline. By the 60-72-hr period, the rate of uridine-$^3$H incorporation into viral RNA had decreased to approximately 60% that of the 48-60-hr period (Figure 6). This sharp decrease in the rate of uridine-$^3$H incorporation was observed over a single 12-hr labeling period (60-72 hr p.i.) but was reproducible. During the 72-84-hr period, the rate of uridine-$^3$H incorporation into TMV-RNA increased over the 60-72-hr period (Figure 6). The apparent increase in rate over the 60-72-hr period was observed in duplicate experiments. The total amount of phenol-extractable TMV-RNA had increased by 3 µg during the 60-84-hr period. After the increased rate observed during the 72-84-hr period, the rate of uridine-$^3$H incorporation into TMV-RNA declined during the

remainder of the incubation period (96-168 hr p.i.) (Figures 5E, 5F and 6). During the 132-144-hr period, incorporation of uridine-$^{3}$H into viral RNA was only 13% that of the peak rate (Figures 5F and 6). While the rate of uridine-$^{3}$H incorporation was declining, the relative viral RNA concentration per gel remained constant (Figure 6); however, a net loss of approximately 2 µg in phenol-extractable TMV-RNA was observed over the 60-168-hr period in duplicate experiments.

At 12-hr intervals after inoculation, the specific activity of the viral RNA was determined. During the first 60 hr p.i., the specific activity of the viral RNA increased, reaching approximately 8.5 x $10^{3}$ cpm/µg during the 48-60-hr labeling period (Table 4). This was followed by a sharp drop in specific activity during the 60-72-hr period which corresponded to the period of decreased rate of uridine-$^{3}$H incorporation into viral RNA (Figure 6). The specific activity of the viral RNA increased during the 72-84-hr period over that observed during the 60-72-hr period. After this time, the specific activity of the viral RNA decreased (84-168 hr p.i.) and by 168 hr p.i. had declined to approximately 1 x $10^{3}$ cpm/µg.

To determine whether the decline in the rate of incorporation of uridine-$^{3}$H into viral RNA after 60 hr p.i. was due to a drastic change in the cellular pool of nucleic acid precursors, the specific activity (cpm/µg) of the 25 S

Table 4. Specific Activity of Cellular and Viral RNA.

Specific activity of 25 S host ribosomal RNA was determined at 24-hr intervals after inoculation. Labeling, extraction and electrophoresis was carried out as in Figure 4. The area under the 25 S RNA optical density peak was converted to micrograms of RNA. Total radioactivity corresponding to the 25 S optical density peak was divided by the total amount of 25 S RNA to give the specific activity (cpm/μg 25 S RNA). Specific activity of the viral RNA was determined at 12-hr intervals after inoculation. The specific activity of the viral RNA was determined as described for the 25 S host ribosomal RNA. Values represent the average of two experiments.

| Labeling Period (hr p.i.) | Cpm/μg RNA | |
|---|---|---|
| | Cellular 25 S | Viral |
| 12-24 | 3379 | 4966 |
| 24-36 | -- | 6305 |
| 36-48 | 4171 | 8095 |
| 48-60 | -- | 8539 |
| 60-72 | 2976 | 4393 |
| 72-84 | -- | 5206 |
| 84-96 | 3770 | 3611 |
| 108-120 | -- | 2548 |
| 132-144 | 4779 | 1028 |
| 156-168 | 3507 | 1111 |

cellular ribosomal RNA was determined at 24-hr intervals following inoculation. A comparison of the specific activity of the 25 S cellular RNA and the specific activity of the viral RNA is depicted in Table 4. The specific activity of the cellular RNA remained relatively constant during the 168-hr period although there appeared to be a slight decrease during the 60-72-hr period; whereas, the specific activity of the viral RNA increased rapidly during the first 60 hr p.i. The specific activity of the viral RNA then declined throughout the remainder of the 168-hr period with the exception of a slight increase during the 72-84-hr period (Table 4).

## Rate of Uridine-$^{3}$H and Leucine-$^{14}$C Incorporation into Complete Virus and Rate of Accumulation of Complete Virus

The relationship between viral RNA synthesis and synthesis of viral coat protein was studied by following the incorporation of uridine-$^{3}$H labeled viral RNA and leucine-$^{14}$C labeled viral coat protein into complete virus particles.

To study the rate of *in vivo* synthesized uridine-$^{3}$H-TMV-RNA incorporation into complete virus, infected cells were incubated in liquid medium containing uridine-$^{3}$H (25 μC/ml) at 12-hr intervals after inoculation. Incubation was continued for 12 hr and complete virus was extracted and partially purified by differential centrifugation and

then subjected to density gradient centrifugation. The gradients were fractionated and the amount of uridine-$^{3}$H labeled virus was determined.

The incorporation of *in vivo* synthesized uridine-$^{3}$H labeled TMV-RNA into complete virus is depicted in Figure 7. The amount of uridine-$^{3}$H-TMV-RNA incorporated into complete virus during the 12-hr labeling periods increased rapidly from 24-60 hr p.i. This was paralleled by a rapid increase in the amount of extractable complete virus. After the first 60 hr, the rate of incorporation of uridine-$^{3}$H labeled TMV-RNA decreased sharply between 72-96 hr after inoculation. The period during which there was a decreased rate of TMV-RNA incorporation into complete virus varied from experiment to experiment but never extended beyond 96 hr p.i. The rate of incorporation of uridine-$^{3}$H labeled TMV-RNA increased during the 84-108-hr period. Again, the period during which an increased rate of incorporation was observed varied with the experiment but never exceeded 120 hr p.i. The period during which the increased rate of uridine-$^{3}$H labeled TMV-RNA incorporation into complete virus was observed did not extend beyond a 12-hr labeling period; that is, the increased rate observed during the 84-108-hr period in the various experiments did not continue for more than 12 hr. After this time the rate of uridine-$^{3}$H *in vivo* labeled TMV-RNA incorporation into complete virus decreased for the remainder of the period studied (120-156 hr p.i.).

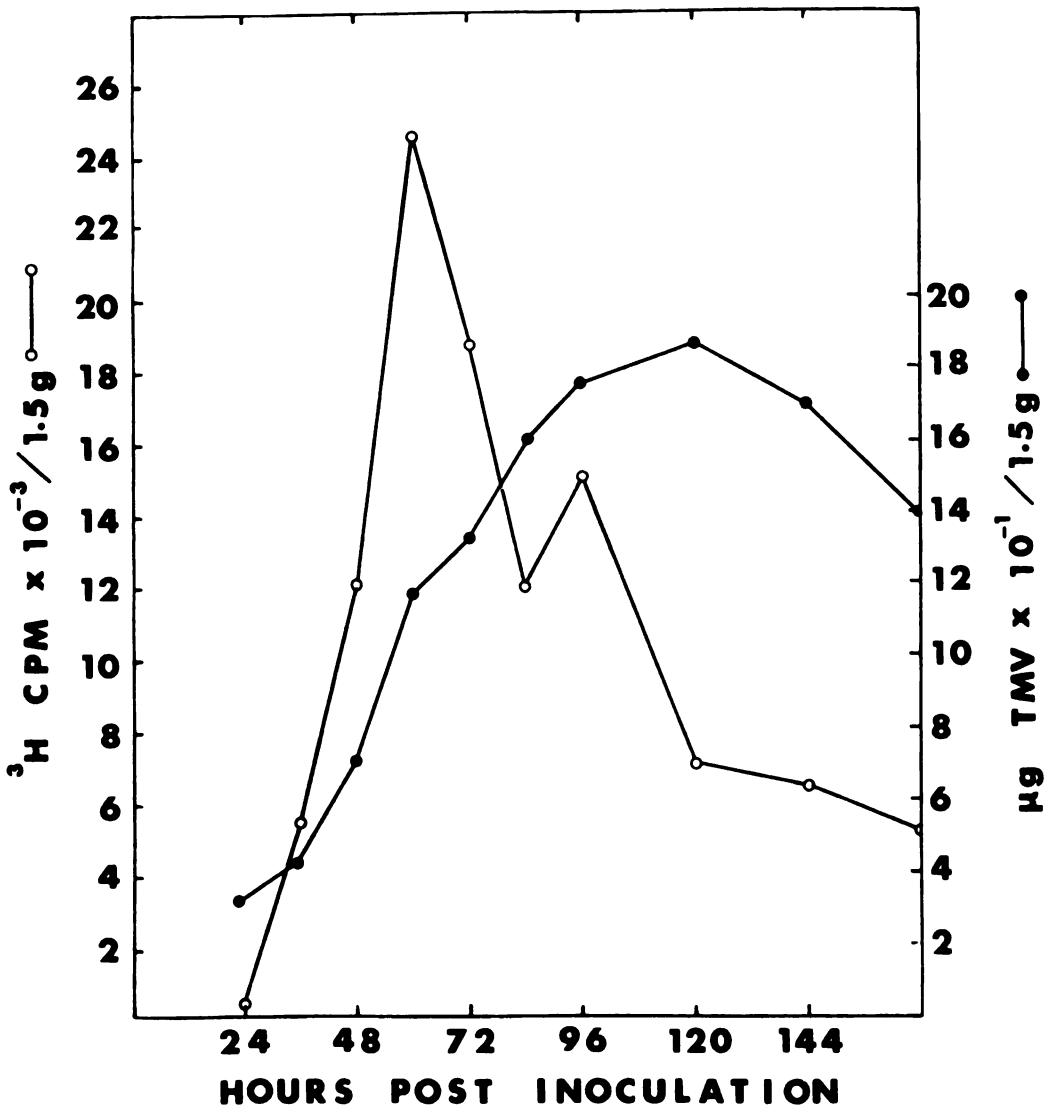


Figure 7. Rate of incorporation of *in vivo* synthesized uridine-$^3$H-TMV-RNA into complete virus and accumulation of complete virus. At 12-hr intervals after inoculation, cells were exposed to uridine-$^3$H (25 µCi/ml) for a 12-hr period. Complete virus was extracted and subjected to centrifugation on 10-40% sucrose gradients at 80,000 g for 90 min. Gradients were fractionated with an ISCO fractionator coupled to a U.V. analyzer (254 nm). The viral band region was collected, precipitated with 10% TCA, and washed with 5% TCA, 95% ETOH, ethanol: ether (v:v), then radioactivity was determined. The TMV concentration was calculated by converting the area under the virus optical density peak to micrograms complete virus assuming an $E^{0.1\%}_{254\ nm} = 3.06$.
(o—o, incorporation of uridine-$^3$H-TMV-RNA into complete virus; ●—●, accumulation of complete virus).

Although complete virus continued to accumulate in inoculated cells after the first 60 hr p.i. (Figure 7), the rate of accumulation declined sharply after this period (Figure 8). The decreased rate of accumulation of complete virus continued from 72-96 hr p.i. depending on the particular experiment. During this period the rate of accumulation of complete virus often approached zero or in some cases an apparent loss of complete virus was observed although incorporation of uridine-$^{3}$H-TMV-RNA still continued at a low level (Figures 7 and 8). After this period (72-96 hr p.i.) a sharp increase in the rate of accumulation of complete virus occurred from 84-120 hr p.i., depending on the experiment. This period of increased rate of accumulation of complete virus appeared to last for only 12 hr and corresponded to the period during which there was an increased rate of incorporation of uridine-$^{3}$H labeled TMV-RNA into complete virus. The rate of accumulation of complete virus then declined (120-156 hr p.i.) and after this often fell to zero although there was continued incorporation of uridine-$^{3}$H labeled TMV-RNA into complete virus (Figures 7 and 8).

To study the relationship of viral coat protein synthesis to incorporation of uridine-$^{3}$H labeled TMV-RNA into virus and the accumulation of complete virus, inoculated cells were transferred at 12-hr intervals to liquid

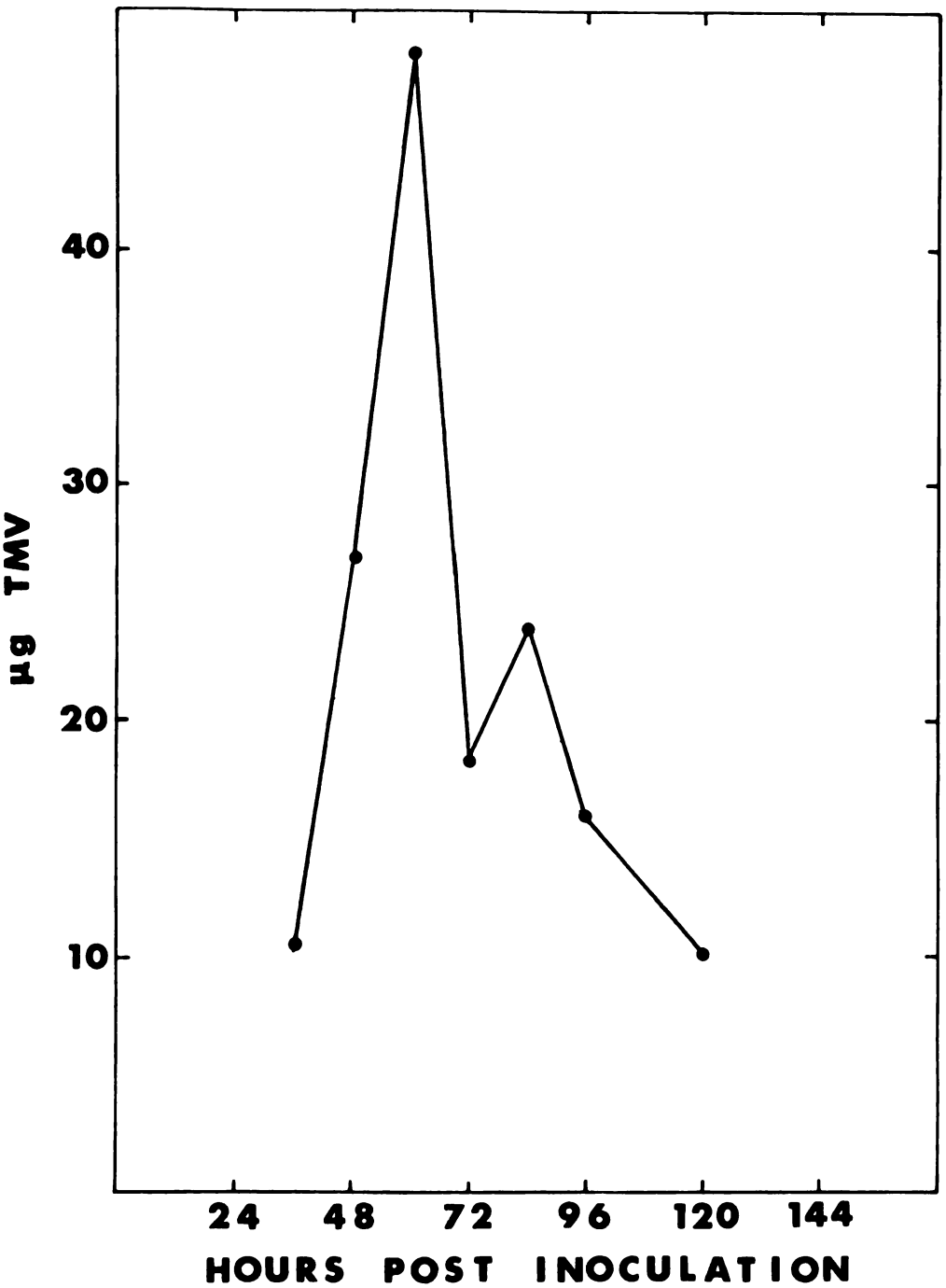


Figure 8. Rate of accumulation of complete virus. Complete virus was extracted and the concentration determined as in Figure 7. The rate of accumulation of complete virus was determined by calculating the actual microgram increase during each successive 12-hr period. Each point represents the average of two experiments.

medium containing leucine-$^{14}C$ (1 μC/ml) and uridine-$^{3}H$ (25 μC/ml) and incubated for 12 hr. Complete virus was then isolated and purified by density gradient centrifugation and both uridine-$^{3}H$ and leucine-$^{14}C$ incorporation determined (Figure 9).

The rate at which in vivo synthesized leucine-$^{14}C$ labeled coat protein is incorporated into complete virus is depicted in Figure 10. The rate at which leucine-$^{14}C$ labeled coat protein, synthesized during a given 12-hr period, was incorporated into complete virus increased rapidly during the first 72 hr post inoculation. However, incorporation of uridine-$^{3}H$ labeled TMV-RNA began to decline after the first 60 hr p.i. This relationship of leucine-$^{14}C$ labeled coat protein incorporation into complete virus to the incorporation of uridine-$^{3}H$ labeled TMV-RNA into complete virus was observed in three experiments. The rate of incorporation of leucine-$^{14}C$ labeled coat protein into complete virus extended for a 12-hr period beyond the first 60 hr or the rate remained relatively constant for a 24-hr period after reaching a maximum at 60-84 hr p.i. (Figure 10 and Table 5). Incorporation of uridine-$^{3}H$ labeled TMV-RNA into complete virus as well as the rate of accumulation was observed to be decreasing during this period (Figure 10 and Table 5). During the 84-96-hr period, rate of incorporation of leucine-$^{14}C$ labeled coat protein decreased and paralleled the decrease in the rate of incorporation of uridine-$^{3}H$ labeled TMV-RNA into complete virus.

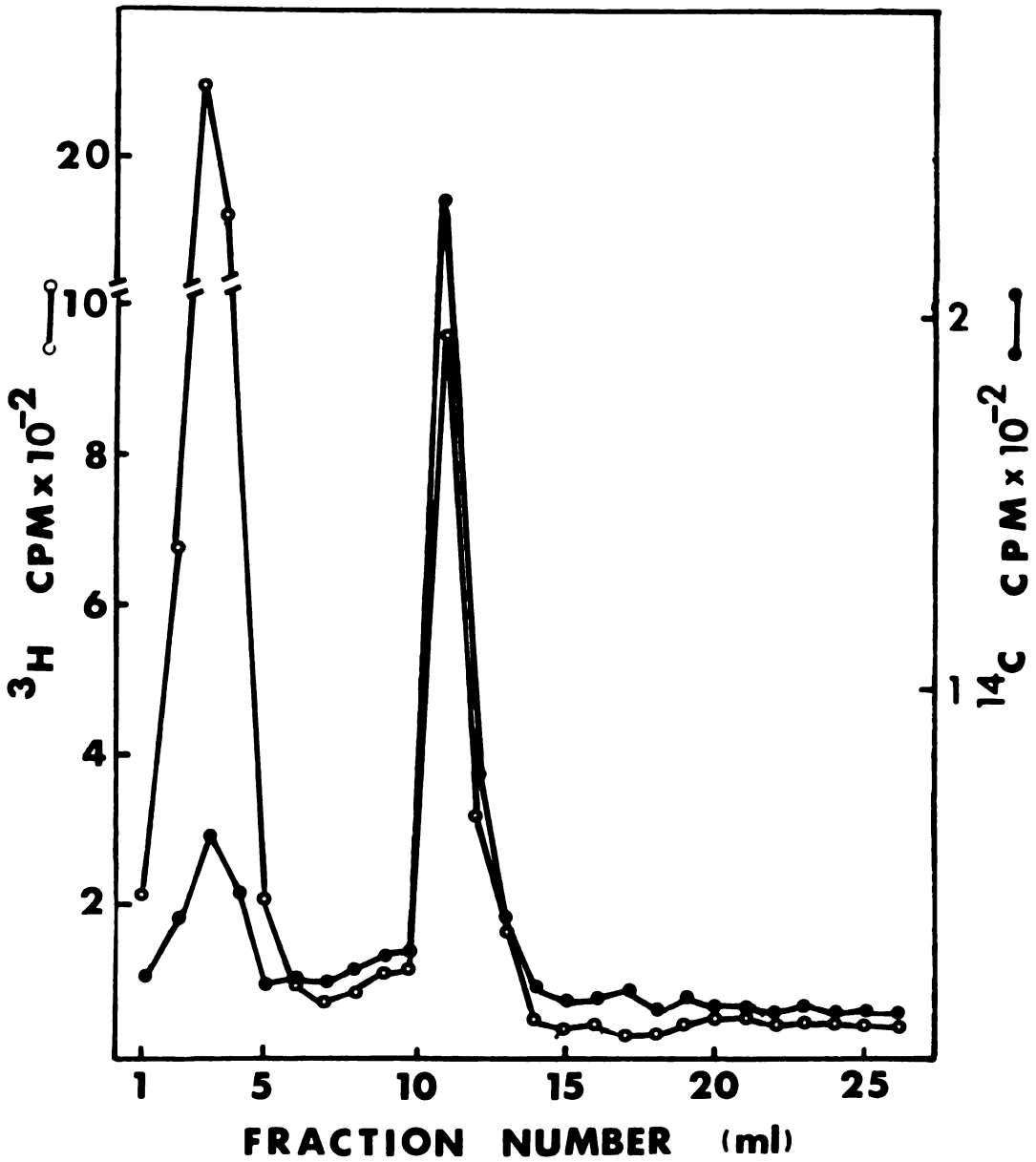


Figure 9. Density gradient centrifugation of complete virus extracted from cells exposed to uridine-$^3$H and leucine-$^{14}$C 48-60 hr post inoculation. Fractions (1 ml) were collected along the gradient and processed as described in Figure 7. TMV peak, fractions 10-14. (o—o, $^3$H-cpm; ●—●, $^{14}$C-cpm).

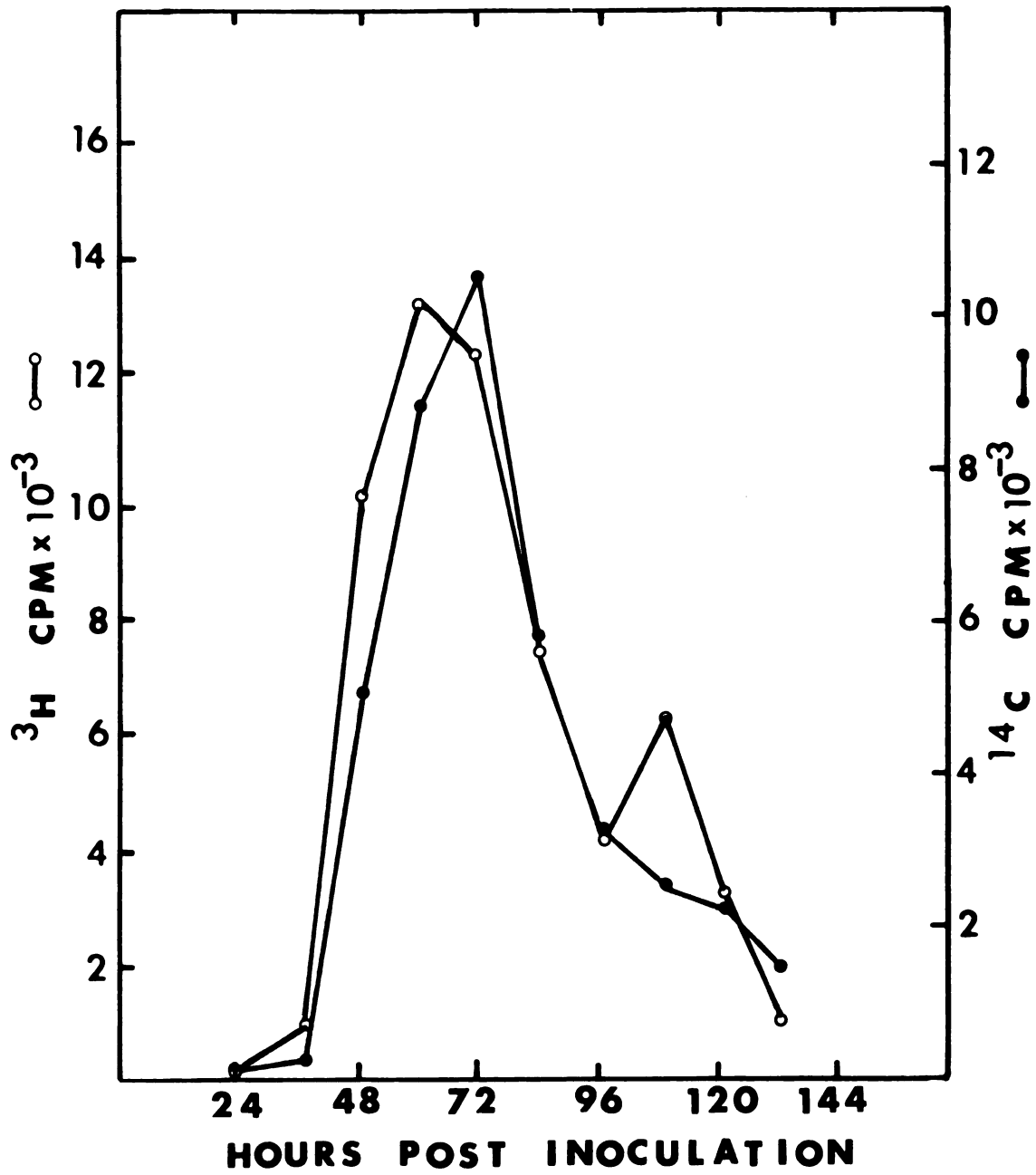


Figure 10. Rate of incorporation of uridine-$^3$H and leucine-$^{14}$C into complete virus. Cells were co-labeled with uridine-$^3$H (25 µC/ml) and leucine-$^{14}$C (2 µC/ml) at 12-hr intervals after inoculation. Complete virus was isolated as described in Figure 7. (o—o, $^3$H-cpm; ●—●, $^{14}$C-cpm).

Table 5. Incorporation of Uridine-$^{3}H$ and Leucine-$^{14}C$ into Complete Virus and Rate of Accumulation of Complete Virus.

Cells were co-labeled with uridine-$^{3}H$ (25 μC/ml) and leucine-$^{14}C$ (2 μC/ml) for 12 hr at 12-hr intervals after inoculation. Complete virus was isolated as described for Figure 7.

| Labeling Period (hr p.i.) | Complete Virus | | |
|---|---|---|---|
| | Δμg | Uridine-$^{3}H$ cpm | Leucine-$^{14}C$ cpm |
| 12-24 | -- | 98 | 131 |
| 24-36 | 3 | 345 | 134 |
| 36-48 | 16 | 1683 | 438 |
| 48-60 | 24 | 1924 | 563 |
| 60-72 | 19 | 1417 | 466 |
| 72-84 | 11 | 1344 | 586 |
| 84-96 | -- | 817 | 361 |
| 96-108 | 10 | 1360 | 363 |
| 108-120 | -- | 482 | 193 |
| 120-132 | -- | 360 | 178 |
| 132-144 | -- | 427 | 171 |

The rate of incorporation of leucine-$^{14}C$ labeled coat protein did not increase but remained relatively constant during the period (84-120 hr p.i.) when there was a sharp increase in both the incorporation of uridine-$^{3}H$ labeled TMV-RNA into complete virus and accumulation of complete virus (Table 5). After this period, the rate of incorporation of leucine-$^{14}C$ labeled coat protein into complete virus decreased slowly. During the period (120-144 hr p.i.) when there appeared to be a net loss of virus, incorporation of leucine-$^{14}C$ labeled coat protein into complete virus was observed (Figure 10 and Table 5).

## Observations on Crystalline Viral Inclusion Formation in Inoculated Tissue Culture

Tobacco tissue culture cells which were inoculated with TMV and incubated on agar-solidified medium produced crystalline viral inclusions. The number of crystalline inclusions present in the cells varied from one to as many as 10-14 crystals per cell. There is a direct correlation between the number of crystal-bearing cells of cell aggregates and the concentration of virus in inoculated cells (Murakishi et al., 1970). Inoculated cultures containing few crystals invariably had a low concentration of virus, whereas cultures containing many crystals yielded a high virus titer.

During the course of the experiments reported, an attempt was made to correlate the appearance of crystalline viral inclusions with viral RNA synthesis and the accumulation of complete virus. At 12-hr intervals after inoculation, random clumps of cells were observed microscopically at 100 and 430 x magnification to detect viral inclusions. The earliest that crystals were observed was 44 hr p.i. In such cases, a single cell or a few cells of a given clump contained crystals. In any given experiment, very few aggregates contained crystal-bearing cells at this time. By 72 hr p.i., virtually all of the cells of a given aggregate were observed to contain crystals. The number of cells in individual aggregates varied from 5 to 25. The number of such inclusion-bearing cell aggregates varied widely from experiment to experiment.

Crystalline inclusions were observed in both larger, older cells and in younger cells. In older cells, the crystals were often appressed against the cell wall by the large central vacuole. Both large and small crystals were observed to be transported around the cell by cyclosis. Cells containing crystalline inclusions were observed to undergo division, with the crystals being distributed to both daughter cells during cytokinesis.

Observation of older cells of crystal-bearing aggregates beyond 84 hr p.i. suggested that considerable degradation of the viral crystalline inclusions had occurred.

Older crystals appeared to have ragged edges and often contained large fractures. Crystalline inclusions lost their angular dimensions and appeared rounded and fluid in nature. Younger cells of the same aggregate often contained crystalline inclusions which did not exhibit degenerate changes.

Many cell aggregates remained free of crystalline viral inclusions even at 156 hr p.i. Presumably, the cells of these aggregates were not initially infected and, due to the static incubation on agar-solidfied medium, the virus was not able to spread from cell aggregate to cell aggregate.

## Discussion

During the first 60 hr after inoculation of tissue culture cells, the rate of viral RNA synthesis increased and reached a maximum during the 48-60-hr period (Figure 6). This conclusion is based on the observation that both the rate of uridine-$^{3}H$ incorporation into viral RNA and the specific activity of the viral RNA increased during each successive 12-hr labeling period up to 60 hr p.i. The increasing specific activity of the viral RNA (Table 4) indicates that during each successive 12-hr period, increasing amounts of viral RNA were synthesized, the largest amount being synthesized during the 48-60-hr period. At this time the specific activity of the viral RNA reached $8.5 \times 10^{3}$ cpm/μg. Figure 6 indicates that the relative amount of viral RNA increased rapidly during the first 60 hr p.i.

Kubo (1966) reported that the rate of $^{32}P$ incorporation into viral RNA synthesized in inoculated tobacco leaves increased rapidly during the first 48 hr after inoculation; however, rate determinations made during the 48-96-hr period were not reported. It is therefore possible that the actual peak rate of viral RNA synthesis was reached during the 48-96-hr period. The specific activity of the viral RNA (cpm/O.D.) increased during the first 48 hr p.i., reaching a peak 48 hr after inoculation. Jackson et al. (1972) studied viral RNA synthesis in cell suspensions prepared from tobacco mosaic virus-infected tobacco leaves and found that the rate of uridine-$^{3}H$ incorporation increased rapidly during the first 72 hr after inoculation. The kinetics of viral RNA synthesis in inoculated tissue culture cells therefore appears to be similar to that observed in inoculated leaf tissue in that the rate of viral RNA synthesis increases very rapidly during the first 48-72 hr after inoculation.

In inoculated tissue culture, the rate of viral RNA synthesis declines sharply after the first 60 hr p.i. (Figure 6). This is paralleled by a decrease in specific activity of the viral RNA (Table 4). Kubo (1966) reported that the rate of viral RNA synthesis in inoculated tobacco leaves, as measured by $^{32}P$ incorporation, declined between 48-96 hr p.i. A decrease in specific activity of the viral RNA was also recorded during this time. Data of Jackson et al. (1972) also indicates that the rate of viral RNA

synthesis in cells isolated from virus-infected tobacco leaves, as measured by uridine-$^{3}$H incorporation into viral RNA, declines sharply after 72 hr p.i. However, in the whole leaf system and in the isolated leaf cell system, the decline in the rate of viral RNA synthesis, as measured by radioactive precursor incorporation, is accompanied by a decrease in the rate of precursor incorporation into cellular ribosomal RNA. Babos (1966b), Fraser (1969, 1972) and Hirai and Wildman (1969) have demonstrated that virus infection does not directly alter the metabolism of cellular ribosomal RNA; therefore, the alteration in incorporation of radioactive precursors into cellular RNA observed by Kubo (1966) and Jackson et al. (1972) may have been due to conditions under which their experiments were performed. Due to the impairment of incorporation of radioactive precursors in cellular ribosomal RNA occurring in their systems after the 48-72-hr period, it would be difficult to accurately access viral RNA synthesis using incorporation of radioactive precursors into viral RNA as a parameter.

In inoculated tissue culture cells, the decrease in the rate of viral RNA synthesis, as measured by uridine-$^{3}$H incorporation into viral RNA after 60 hr p.i., is not paralleled by a drastic decrease in incorporation of uridine-$^{3}$H into cellular ribosomal RNA. This is demonstrated by the observation that neither the ability of the cells to incorporate uridine-$^{3}$H into acid-insoluble material (Table 1)

nor the specific activity (cpm/μg) of the cellular ribosomal RNA (Table 3) changes greatly during the 168-hr period studied. If there was a trend toward decreased ability of the cells to incorporate uridine-$^{3}$H into cellular RNA, it would have been reflected in these measurements. Comparison of the specific activity of the 25 S cellular ribosomal RNA and the specific activity of the viral RNA (Table 4) clearly demonstrates that the specific activity of the cellular RNA remains relatively constant while the specific activity of the viral RNA decreases during the 60-168-hr period. The observation that the specific activity of the cellular ribosomal RNA does not change drastically during the course of viral replication is consistant with the reports of Babos (1966b), Fraser (1969, 1972) and Hirai and Wildman (1969) that virus infection does not directly alter matabolism of cellular ribosomal RNA and is also consistant with the observations of Nakata and Hildebrandt (1967) that no gross morphological changes in cellular organelles could be observed in callus cells derived from TMV-infected tobacco plants.

Although there appears to be a slight decrease in the ability of the cells to incorporate uridine-$^{3}$H into acid-insoluble material (72-96 hr p.i.) and a slight decrease in the specific activity of the 25 S cellular RNA (60-72 hr p.i.), these changes were observed to be transitory in nature (Tables 1 and 4). It has not been possible to determine if

this transitory change in the cells is directly related to the initiation of the decreased rate of viral RNA synthesis. The decrease in the rate of viral RNA synthesis observed during the 60-72-hr period is followed by a brief period (72-84 hr p.i.) during which the rate of viral RNA synthesis increased, after which time it continued to decline (84-168 hr p.i.).

During the first 60 hr p.i., the amount of phenol-extractable viral RNA increased in a near linear manner (Figure 6). After this time there is little increase in the amount of viral RNA. This observation appears to be consistant with the conclusion that the rate of viral RNA synthesis declined after the first 60 hr p.i. Fraser (1972) has reported a similar observation in inoculated tobacco leaves. The amount of phenol-extractable viral RNA increased rapidly during the first 120-240 hr after inoculation and thereafter remained relatively constant.

The peak rate of incorporation of _in vivo_ synthesized uridine-$^{3}$H-TMV-RNA into complete virus occurred during the 48-60-hr period (Figure 7), the period of most rapid viral RNA synthesis. The peak rate of accumulation of complete virus also occurred during this period (Figure 8). The close correlation between the rate of viral RNA synthesis, the rate of incorporation of viral RNA into complete virus and the rate of accumulation of complete virus suggests that viral RNA is incorporated into complete virus rather rapidly

after its synthesis. Engler and Schramm (1959), Diener (1962) and Sakar (1965) have reported that, in the leaf, a large excess of free viral RNA is synthesized prior to formation of complete virus. In our experiments the possibility that an excess of viral RNA, over the amount incorporated into complete virus, existed in the cells during the first 60 hr p.i. could not be ruled out. A direct comparison between the total phenol-extractable viral RNA--which would include free viral RNA--and RNA present in complete virus only could not be made because different extraction techniques were employed. However, the observation that the sharp decline in the rate of viral RNA synthesis observed during the 60-72-hr period (Figure 6) is paralleled by a decline in the rate of incorporation of viral RNA into complete virus (Figure 7) and a decline in the rate of accumulation of complete virus (Figure 8) would argue against there being a large excess of viral RNA in the cells at this point (60-72 hr p.i.). If there were a large excess of viral RNA during the 60-72-hr period, a decline in the rate of viral RNA synthesis would not immediately affect the rate of accumulation of complete virus.

The decline in the rate of incorporation of uridine-$^{3}$H-TMV-RNA and in the rate of accumulation of complete virus observed during the 60-84-hr period is followed by a brief period during which the rate of incorporation of TMV-RNA into complete virus and the accumulation of complete virus

increases. After this time these rates declined throughout the remainder of the period studied. Complete virus, however, continued to accumulate until approximately 92-132 hr p.i. This observation is consistant with that reported by Murakishi et al. (1971) that, based on infectivity, the amount of infectious virus continues to accumulate in virus-infected tissue culture cells after the period of most rapid synthesis and is also consistant with the observation of Takebe et al. (1971) that virus continues to accumulate in virus-infected protoplasts after the period of most rapid synthesis. Similar observations have been reported in virus-infected tobacco leaves (Hirai and Wildman, 1967a; Oxelfelt, 1970; Singer, 1972).

To determine if there was a correlation between incorporation of viral RNA into complete virus and the synthesis and incorporation of viral coat protein into complete virus, inoculated cells were exposed to uridine-$^{3}H$ and leucine-$^{14}C$ at various intervals after inoculation. The increase in uridine-$^{3}H$-TMV-RNA incorporation into complete virus observed during the first 60 hr p.i. was paralleled by a rapid increase in incorporation of leucine-$^{14}C$-coat protein into complete virus (Figure 10). However, during the 60-72-hr period the rate of uridine-$^{3}H$ incorporation into complete virus declined while the incorporation of leucine-$^{14}C$ continued to increase or remained relatively constant during the 60-84-hr period (Figure 10 and Table 5). This difference

in rate of incorporation was observed in three experiments in which the cells were co-labeled with uridine-$^{3}H$ and leucine-$^{14}C$ and therefore appears to be consistant.

Interpretation of these results is difficult because of the interacting events which are occurring: synthesis of viral RNA, synthesis of viral coat protein, incorporation of viral RNA into complete virus and incorporation of viral coat protein into complete virus. One explanation for the continued high rate of incorporation of leucine-$^{14}C$-coat protein into complete virus while the rate of viral RNA synthesis is declining may be that an excess of viral coat protein is synthesized during the first 60 hr p.i.; however, the maximum rate of coat protein synthesis may not be reached until after the first 60 hr p.i. Boardman and Zaitlin (1958) reported excess soluble antigen, presumably TMV coat protein, in extracts from infected leaves. In $^{14}C$-aspartic acid labeling experiments up to 18 hr, the specific activity of the soluble antigen was much greater than antigen associated with complete virus. They concluded that a pool of excess viral coat protein existed and that excess coat protein was used in the assembly of complete virus. Experiments employing polyacrylamide gel electrophoresis of soluble proteins from virus-infected tissue culture cells indicate that excess free viral coat protein may be present at later stages (>72 hr p.i.) of infection. Coat protein apparently in excess of that accountable for in complete virus has been

reported by van Loon and van Kammen (1970) and Singer (1972). Polyacrylamide gel electrophoresis of soluble proteins extracted from inoculated 'Xanthi nc' tissue culture cells also indicates that excess viral coat protein may be synthesized (Beachy and Murakishi, 1971b).

If excess coat protein is present in TMV-infected tissue culture cells and the rate of synthesis of coat protein is increasing or remaining constant while the rate of viral RNA synthesis is decreasing, the pool of excess viral coat protein would be diluted by freshly synthesized protein (leucine-$^{14}$C-coat protein); therefore, the relative amount of leucine-$^{14}$C-coat protein incorporated into complete virus could be increasing or remaining constant. The data in Figure 10 and Table 5 is consistant with this interpretation; while the rate of viral RNA synthesis and the rate of accumulation of complete virus is decreasing, the amount of leucine-$^{14}$C incorporated into complete virus is remaining relatively constant. After reaching a peak rate of incorporation during the 60-84-hr period, incorporation of leucine-$^{14}$C into complete virus declined throughout the remainder of the period studied.

Zech (1952) and Nilsson-Tillgren et al. (1969) have studied the formation of crystalline viral inclusions in tobacco leaves following inoculation with TMV. These workers suggest that an apparent synchronous formation of viral inclusions in certain areas of the leaf indicates that

all of the cells in these areas are in the same stage of infection. Nilsson-Tillgren (1969) reported that large amounts of viral RNA are synthesized just prior to and during the apparent synchronous appearance of crystalline inclusions. In inoculated tissue culture a similar situation occurs. Concurrent with the rapid increase in the rate of viral RNA synthesis, the rapid increase in incorporation of uridine-$^{3}H$ and leucine-$^{14}C$, and the rapid accumulation of complete virus during the first 60-72 hr p.i., is the formation of crystalline viral inclusions. The tissue culture used in these experiments is composed of numerous cell aggregates. During the first 44 hr after inoculation, crystals were rarely observed and then only in one or two cells of a given aggregate. However, by 72 hr p.i. large aggregates of crystal-bearing cells were present. Although the number of crystal-bearing aggregates varied from experiment to experiment (Murakishi et al., 1970), virtually every cell of such aggregates contained one or more crystalline inclusions by 72 hr p.i. Observations of older cells of crystal-bearing aggregates beyond 84 hr p.i. suggested that considerable degradation of the viral inclusions had occurred. Older crystals appeared to have ragged edges and often contained large fractures. The observation that the crystals appear over a relatively short period (24 hr) indicates that either all of the cells of such aggregates were infected at the

time of inoculation or, more likely, that the virus was able to spread rapidly through the cells of an aggregate after the initial infection of one or a few cells.

Recent electron microscopic observations indicate that plasmodesmata connect the cells of a given aggregate and that no external opening in the cells could be found (Hartmann, 1971). Plasmodesmata could allow for rapid spread of the virus throughout the cells of an aggregate. The time required for the spread of virus throughout the cell aggregate may be relatively short compared to the time required for a given cell to synthesize its maximum amount of virus. Zech, as reviewed by Mundry (1963), reported that, based on changes in nuclear and cytoplasmic RNA content, virus synthesis was initiated in the five lower cells of a leaf hair within 9 hr after inoculation of the apical hair cell. Matthews(1970) reported that the time required for the movement of infectious material from the epidermis to the mesophyll in inoculated tobacco leaves was estimated to be from 4-10 hr (Uppal, 1934; Dijkstra, 1962). The nature of this infectious material was not determined but could have come from the initial inoculum or have been TMV-RNA or complete virus synthesized in the initially inoculated cell.

The time required for a given cell to synthesize its maximum amount of virus has been estimated in isolated tobacco protoplasts. In the isolated protoplast system, virus spread does not occur and therefore an estimation of a single

step growth curve is possible (Aoki and Takebe, 1969). The time required for a single step growth curve in inoculated protoplasts ranges from 22-72 hr depending on culture conditions (Aoki and Takebe, 1969; Takebe and Otsuki, 1969; Takebe et al., 1971).

The observations on the time required for virus to spread some distance from the point of inoculation (4-10 hr) and the time required for a single step growth curve of TMV in inoculated protoplasts (22-72 hr) indicate that our conclusion that virus must spread very rapidly throughout the cells of an aggregate and that these cells synthesize their maximum amount of virus within the first 60-72 hr appears reasonable. Once all of the cell aggregates synthesize their maximum complement, the rate of virus synthesis drops off rapidly (60-72 hr p.i.). If no further spread of the virus occurred, virus synthesis in the inoculated tissue culture would cease; however, virus synthesis does continue as measured by uridine-$^{3}H$ incorporation into viral RNA and incorporation of uridine-$^{3}H$ and leucine-$^{14}C$ into complete virus (Figures 6, 7 and 10).

The static incubation employed in these experiments allowed for minimum mechanical cell damage during incubation which could have allowed for virus spread from aggregate to aggregate. The electron microscopic study reported by Hartmenn (1971) indicates that there are no natural external openings in the walls of tissue culture cells grown on agar medium.

Light microscopic observations indicate that inoculated tissue culture incubated on agar medium are composed of infected crystal-bearing aggregates and aggregates which are free of virus. Aggregates lying in close contact with crystal-bearing aggregates were observed to remain free of viral inclusions as late as 168 hr after inoculation. Hildebrandt and Riker (1958), Chandra and Hildebrandt (1966) and Hirth and Lebeurier (1965) also reported that callus produced from virus-infected tobacco plants consists of aggregates of infected cells enmeshed in a network of apparently healthy cells. These observations are consistant with the idea that virus spread does not occur between cell aggregates.

Esau and Gill (1969) reported that leaf mesophyll cells of Nicotiana tabacum, containing large aggregates of TMV, exhibited normal cytokinesis and were observed to undergo division with the aggregates of virus being distributed between the daughter cells. Hansen and Hildebrandt (1966), Chandra and Hildebrandt (1966), Goldin et al. (1967) and Nakata and Hildebrandt (1967) have reported that virus-infected callus cells were capable of undergoing cell division. In inoculated tissue culture, we have also observed division of virus-infected cells. Cells containing viral crystalline inclusions were observed in the act of division and the inclusions were often distributed between the

daughter cells. These observations indicate that virus spread in inoculated tissue culture after the first 60 hr p.i. occurs through division of virus-infected cells.

Kassanis et al. (1958) demonstrated that virus spread in inoculated tissue cultures was very slow. Slow spread of virus through division of virus-infected cells could account for the slight increase in the rate of virus synthesis observed during the 84-120-hr period and also for the low level of virus synthesis observed to continue during the 120-168-hr period.

Chandra and Hildebrandt (1966) studied micro-culture of single tobacco cells infected with TMV. They observed that virus-infected cells were able to divide, but that division was more frequent with inclusion-free cells. Over the time their study was conducted, 50% of the inclusion-free cells divided, whereas only 10% of the inclusion-bearing cells divided. The reduced division of virus-infected cells could account for the frequent loss of virus from culture derived from virus-infected plants (Hildebrandt, 1958; Hansen and Hildebrandt, 1966). In effect, the virus-containing cells could have been diluted out by the more rapid growth of healthy cells. Although no difference in growth rates of inoculated or uninoculated cultures was observed, the decreased rate of virus synthesis observed after the first 60 hr could be accounted for by the fact that virus-infected cells divide at a slower rate than uninfected cells.

It appears likely that the rapid burst of virus synthesis--as measured by uridine-$^{3}H$ incorporation into viral RNA, uridine-$^{3}H$ and leucine-$^{14}C$ incorporation into complete virus, and accumulation of complete virus--observed during the first 60 hr after inoculation of tobacco tissue culture is due to rapid spread of the virus throughout initially inoculated cell aggregates and that after this time virus spread and synthesis is limited to division of virus-infected cells.

## Summary

Tobacco mosaic virus ribonucleic acid synthesis, incorporation of TMV-RNA, and TMV coat protein were studied at 12-hr intervals after inoculation of tobacco (Nicotiana tabacum L. var. Havana 38) tissue culture. The rate of TMV-RNA synthesis, as measured by the incorporation of uridine-$^{3}H$ into viral nucleic acid, increased in a linear manner during the first 60 hr after inoculation. At this time the rate of viral RNA synthesis was approximately six times that of the 12-24-hr period. During the 60-72-hr post inoculation period, the rate of viral RNA synthesis declined sharply; however, during the 72-84-hr period the rate increased. After this time, the rate of viral RNA synthesis declined throughout the remainder of the 168-hr period studied. During the first 60-72 hr, the relative viral RNA concentration increased in a near linear manner; after this

time the concentration of viral RNA remained constant. The peak rate of incorporation of *in vivo* synthesized uridine-$^{3}$H-TMV-RNA into complete virus occurred during the 48-60-hr period, the period of most rapid viral RNA synthesis. The rate of incorporation of viral RNA into complete virus then declined until the 84-128-hr period when the rate of incorporation increased slightly. After this time, the rate of incorporation of TMV-RNA declined slowly.

The rate of incorporation of coat protein into complete virus was measured by following incorporation of leucine-$^{14}$C into complete virus. Leucine-$^{14}$C incorporation increased rapidly during the first 60-72 hr after inoculation, reaching a maximum rate during the 72-84-hr period depending upon the experiment. In all experiments, the maximum rate of leucine-$^{14}$C-coat protein incorporation into complete virus was reached during the period when the rate of uridine-$^{3}$H-TMV-RNA incorporation was declining. The incorporation of leucine-$^{14}$C into complete virus declined after this time (84-168 hr p.i.). Leucine-$^{14}$C incorporation did not appear to increase during the 84-120-hr period, the time during which the rate of incorporation of uridine-$^{3}$H into complete virus was observed.

The rate of accumulation of complete virus increased rapidly during the first 60 hr p.i. and then declined sharply; however, the rate of accumulation increased for a brief period during the 84-120-hr period depending upon the

experiment. Accumulation of complete virus continued until approximately 96-132 hr p.i. After this time, an apparent loss of complete virus was occasionally observed.

Crystalline viral inclusions were rarely observed before 48 hr p.i., but at 72 hr p.i. large aggregates of crystal-bearing cells were present. After this time no increase in the number of crystal-bearing aggregates was observed. Observation of older cells of crystal-bearing aggregates beyond 84 hr post inoculation suggested that degradation of viral crystalline inclusions had occurred. Crystal-bearing cells were observed to undergo cell division with viral crystalline inclusions being distributed between the daughter cells. Many cell aggregates remained free of inclusions even at 156 hr p.i.

The ability of the cultures to incorporate uridine-$^{3}H$ or leucine-$^{14}C$ did not change drastically throughout the 156-hr period studied. The amount of nucleic acid extractable from the cells by the phenol-SDS technique remained relatively constant during this period. The specific activity (cpm/μg) of the 25 S cellular ribosomal RNA, determined after a 12-hr exposure to uridine-$^{3}H$ at 24-hr intervals after inoculation, remained relatively constant.

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PART TWO

# THE EFFECT OF KINETIN ON VIRUS SYNTHESIS

## Introduction and Literature Review

In 1955, Miller et al. demonstrated that kinetin (6-furfurylaminopurine) stimulated division and growth of tobacco cells in culture. Since this time, there has been extensive research aimed at determining the precise mode of action of kinetin. Many of the biological effects of kinetin or kinetin-like compounds on cellular metabolism have been reviewed by Miller (1961). Kinetin treatment retards protein and chlorophyll loss from detached tobacco leaves (Richmond and Lang, 1957). Osborne (1962) reported that senescence of attached tobacco leaves was possibly related to an impairment of nucleic acid synthesis which led to a net loss of proteins, and that a similar process occurred in detached leaves or leaf discs. Kinetin treatment of detached leaves or leaf discs temporarily arrested the aging process by maintaining RNA synthesis at a high rate. Precursor incorporation studies indicated that both RNA and protein synthesis were maintained longer in leaves treated with kinetin. Parthier et al. (1964) reported that kinetin stimulated uptake and incorporation of some $^{14}C$ amino

acids of tobacco leaf discs at kinetin concentrations ranging from $10^{-5}$ to $10^{-6}$ M. Shibaoka and Thimann (1970) have concluded that the primary action of kinetin may be to inhibit the action or synthesis of ribonuclease or protease thus preventing breakdown of ribonucleic acids and proteins rather than kinetin having a direct stimulatory effect on RNA or protein synthesis.

Kinetin concentrations greater than $10^{-5}$ M appeared to severely inhibit uptake and incorporation of amino acids into protein of tobacco leaf discs (Parthier et al., 1964). Incorporation of $^{32}P$ into cellular RNA of peanut cotyledons was stimulated by benzyladenine, a compound with cytokinin activity, at low concentrations ($0.4 \times 10^{-3}$M); however, at higher concentrations, incorporation of $^{32}P$ into RNA was inhibited by as much as 90% (Carpenter and Cherry, 1966). A similar situation has been demonstrated with tobacco tissue culture. Low concentrations of kinetin ($10^{-8}$ to $10^{-6}$ M) stimulated growth (Nudel and Bamberger, 1971) and incorporation of $^{32}P$ into RNA (Srivastava, 1967) of tobacco tissue culture cells. At higher concentrations (>$10^{-6}$ M) kinetin inhibited incorporation of $^{3}H$-uracil and $^{14}C$-leucine into cellular RNA and protein respectively (Nudel and Bamberger, 1971).

The early work of Richmond and Lang and Osborne on the effect of kinetin on RNA and protein synthesis stimulated interest in the possible effects kinetin may have on

plant virus replication (Goodman et al., 1967). Daft (1963) reported that spraying of kinetin ($1.5 \times 10^{-4}$ M) on detached leaves of Nicotiana glutinosa 4 days before and 5 days after inoculation with TMV increased lesion production. Under the same spraying regimen, virus increase was recorded in detached leaves of N. tabacum. He concluded that the increased virus production was due to delaying of senescence of the detached leaves by kinetin treatment. Contrary to Daft's report on increased local lesion production with kinetin treatment, Milo and Srivastava (1969b) found that floating N. glutinosa leaves on kinetin (approx. $10^{-4}$ M) immediately after inoculation inhibited lesion production; however, virus production was stimulated in both N. glutinosa and N. tabacum by kinetin treatment.

Király and Szirmai (1964) reported that kinetin ($2 \times 10^{-4}$ M) treatment of N. glutinosa leaf discs reduced local lesion number and diameter and that the appearance of lesions was delayed 30-35 hr. TMV production based on local lesion assay was inhibited 77% by kinetin treatment. When whole leaves were used, kinetin treatment was more effective in inhibiting virus production (80-90%). Application of kinetin to intact plants did not affect virus replication in the plant. They concluded that in the leaf discs or detached leaves kinetin treatment stimulated cellular RNA and protein synthesis and that this may have

interfered with virus synthesis. But they did not demonstrate that kinetin stimulated cellular RNA and protein synthesis under their conditions.

Selman (1964) studied the effect of kinetin on infection of petunia and tomato leaves with tomato spotted-wilt virus (TSWV). Kinetin reduced the size and number of local lesions when supplied to the lower surface of petunia leaf strips; however, kinetin inhibited TSWV multiplication in tomato leaflets only when supplied at the site of inoculation. The effect of kinetin treatment was dependent on kinetin concentration; at approximately $2 \times 10^{-4}$ M, inhibition of local lesions was most pronounced. Goodman et al. (1967) reported that Pozár and Király (1964) found that susceptibility to infection with TMV was inversely related to the ability of tobacco leaves to incorporate amino acids into leaf protein. Kinetin treatment increased amino acid incorporation into leaf protein and therefore reduced susceptibility to virus infection.

Loss of virus from TMV-infected tobacco tissue culture has been observed (Hildebrandt, 1958; Hansen and Hildebrandt, 1966). Media used to culture tobacco cells commonly contains kinetin. Milo and Srivastava (1969a) studied the effect of various cytokinins on maintenance of virus titer in TMV-infected pith tissue grown in culture. Based on infectivity assay, they found that many cytokinins stimulated cell growth but appeared to substantially inhibit

virus replication. $N^6$-isopentenyladenosine both stimulated cell growth and appeared to allow continuous virus synthesis. They concluded that many cytokinins may directly inhibit virus replication in cultured tobacco cells.

In all of the above reports on the effects of kinetin or related cytokinins on virus synthesis in detached leaves, leaf discs or tobacco tissue culture infectivity assays were used. In many of the experiments, determinations were not made to ascertain the effects of kinetin on cellular metabolism in the particular system studied. Although inhibition of virus synthesis was observed in many cases, it was not determined whether kinetin treatment affected viral RNA synthesis or whether it affected viral protein synthesis.

In the study reported herein, an attempt was made to study the effect of a high concentration of kinetin on viral RNA synthesis and viral protein synthesis in inoculated tobacco tissue culture. A concentration of kinetin that clearly inhibited cellular RNA synthesis was selected in order that a direct comparison could be made between the effect of kinetin on cellular RNA synthesis and its effect on viral RNA synthesis.

## Materials and Methods

### Inoculation and Incubation of Tissue Culture Cells

A cell culture derived from stem tissue of tobacco (Nicotiana tabacum L. var. Havana 38) was used during this study and maintained and inoculated as described by Murakishi et al. (1971). Tobacco mosaic virus was used for inoculation at a concentration of 150 µg/ml of cell suspension. Cells were inoculated in 1-g batches in 3 ml liquid medium. Cells were then pooled and washed with 30 ml fresh medium. Three-gram aliquots of cells were transferred to a 4 cm disc of Whatman No. 4 filter paper placed on medium solidified with 1% agar. Incubation was carried out at approximately 25$^{o}$ under light provided by Gro-lux fluorescent lamps.

### Preparation of and Treatment with Kinetin

Kinetin--6-furfurylaminopurine (Sigma Biochem.)--was prepared by making a suspension of kinetin in liquid medium (27 mg/50 ml). At this concentration kinetin is not soluble in aqueous solution; therefore, the suspension was autoclaved for 15 min to dissolve the kinetin. Immediately after autoclaving, the solution was diluted with fresh medium, 10 parts to 1 part kinetin solution, giving a final kinetin concentration of $2.5 \times 10^{-4}$ M.

At 28 hr post inoculation, cells were transferred to small glass petri plates containing either 2 ml fresh liquid medium or kinetin medium with either uridine-$^{3}H$ (25 μC/ml) and/or leucine-$^{14}C$ (1 μC/ml) added and incubated for either 3, 6, 9 or 12 hr.

Determination of Uridine-$^{3}H$ or Leucine-$^{14}C$ Incorporation into Cellular RNA, Viral RNA and Complete Virus

For determinations of the rate of uridine-$^{3}H$ or leucine-$^{14}C$ incorporation into acid-insoluble material, 250-mg aliquots of cells were removed from the incubation medium and ground in 1.75 ml (Tris-HCl, pH 8.0, 0.1% mercaptoethanol, 1 x $10^{-3}$ M unlabeled uridine, 1 x $10^{-4}$ M unlabeled leucine). The homogenate was centrifuged at 1000 x g for 5 min. After addition of 500 μg bovine serum albumin (BSA), 0.5 ml of the homogenate was precipitated with 20% trichloroacetic acid (TCA) and collected on a glass filter pad, washed with 10 ml 5% TCA, 10 ml 95% ethanol and 5 ml ethanol:ether (v:v). The precipitate was then dissolved in 0.5 ml NCS solubilizer solution, 10 ml POPOP-PPO toluene solution added, and the preparations held in the dark for 12 hr at 4°. Radioactivity was then determined in a Packard Tri-Carb liquid scintillation counter.

For determinations of uridine-$^{3}H$ incorporation into cellular and viral RNA after kinetin treatment, total nucleic acids were extracted from 1.5 g cells by the phenol-SDS technique. The nucleic acids were precipitated by addition of 2.5 volumes 95% ethanol and stored at -35°. After washing with 95% ethanol, the precipitate was dissolved in electrophoresis buffer and subjected to polyacrylamide gel electrophoresis as described by Bishop et al. (1967). The gels were then scanned in a Gilford gel scanner (260 nm) and frozen and sliced into 1 mm sections. The gel sections were dissolved and radioactivity determinations made as described above.

Determination of uridine-$^{3}H$ and leucine-$^{14}C$ incorporation into complete virus was made by extraction of complete virus from 1.5 g cells by homogenization in buffer (Tris-HCl, pH 8.0, 0.1% mercaptoethanol, 1 x $10^{-4}$ M unlabeled uridine and 1 x $10^{-3}$ M unlabeled leucine). The homogenate was centrifuged at 22,000 x g for 15 min, the supernatant layered over a 30% sucrose cushion and the virus pelleted by centrifugation at 134,000 x g for 90 min. The virus pellet was dissolved in 1 ml distilled water (12 hr at 4°) and subjected to centrifugation on a 10-40% sucrose gradient at 23,000 x g for 90 min. The gradient was then scanned and fractionated on an ISCO Model D fractionator coupled to a Model VA-2 U.V. analyzer (254 nm). One-milliliter fractions along the gradient were collected and precipitated with trichloroacetic acid and radioactivity determinations made as described above.

## Results

### Incorporation of Uridine-$^{3}H$ and Leucine-$^{14}C$ into Acid-Insoluble Material During Treatment with Various Concentrations of Kinetin

After inoculation, cells were incubated on agar-solidified medium containing approximately $1.25 \times 10^{-6}$ M kinetin for 48 hr. The cells were then transferred to liquid medium containing uridine-$^{3}H$ and/or leucine-$^{14}C$ and increasing concentrations of kinetin ($1.25, 2.50, 5.00 \times 10^{-6}$ M; $1.25, 2.50, 5.00 \times 10^{-5}$ M; $1.25, 2.50, 5.00 \times 10^{-4}$ M), and were incubated for 12 hr (Figure 1). Incorporation of uridine-$^{3}H$ appeared to be slightly stimulated over control levels ($1.25 \times 10^{-6}$ M) by concentrations of kinetin ranging from $2.5 \times 10^{-6}$ to $2.5 \times 10^{-5}$ M. However, considerable variation occurred between experiments. Concentrations of kinetin greater than $2.5 \times 10^{-5}$ M produced a marked inhibition of uridine-$^{3}H$ incorporation. Kinetin concentrations of $5 \times 10^{-5}$ M, $1.25 \times 10^{-4}$ M and $2.5 \times 10^{-4}$ M resulted in inhibition of uridine-$^{3}H$ incorporation of 26%, 56% and 70% respectively. Increasing the kinetin concentration beyond $2.5 \times 10^{-4}$ M did not result in greater inhibition of uridine-$^{3}H$ into acid-insoluble material (Figure 1). Kinetin concentrations ranging from $1.25 \times 10^{-6}$ to $5 \times 10^{-4}$ M did not appear to significantly affect incorporation of leucine-$^{14}C$ into acid-insoluble material.

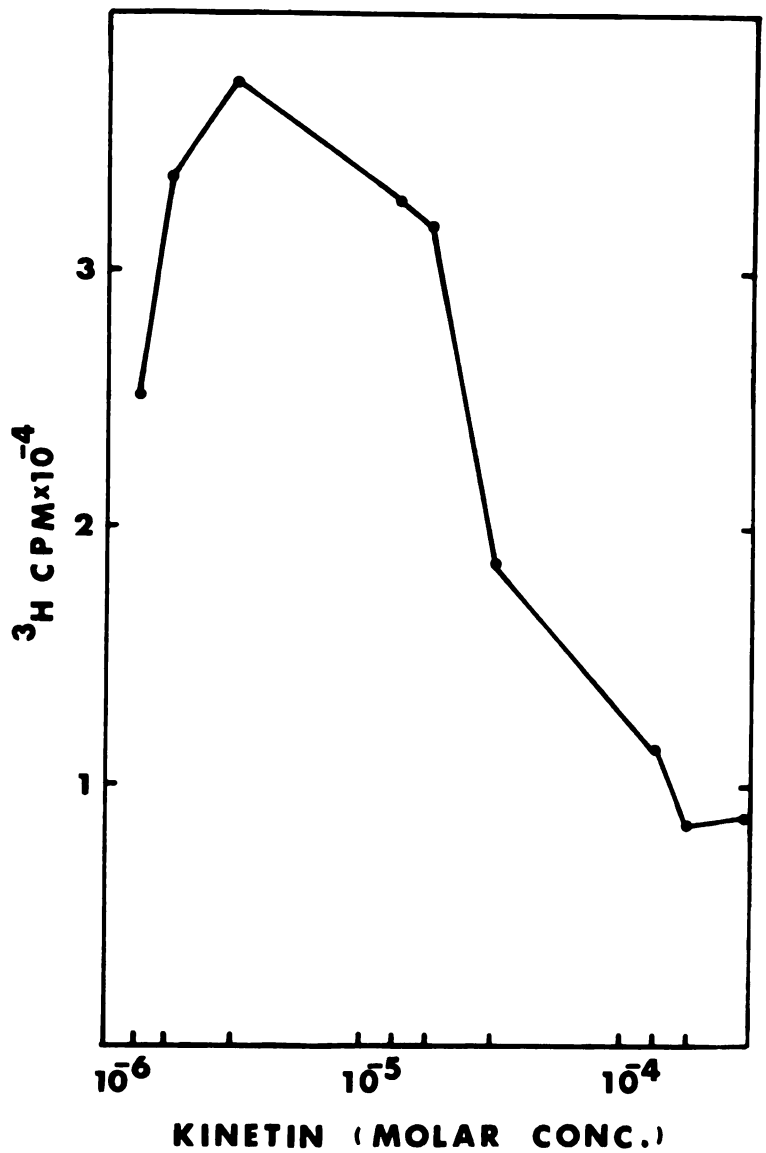


Figure 1. Effect of increasing kinetin concentration on incorporation of uridine-$^3$H into acid-insoluble material. Cells were exposed to 1.25, 2.5 and 5 x $10^{-6}$ M, 1.25, 2.5 and 5 x $10^{-5}$ M, and 1.25, 2.5 and 5 x $10^{-4}$ M kinetin during a 12-hr incubation period in the presence of uridine-$^3$H (25 µC/ml). Two hundred and fifty milligram-aliquots of cells were harvested, washed (.25 M sucrose, 1 x $10^{-3}$ M unlabeled uridine), and ground in 1.75 ml buffer (Tris-HCl, pH 8.0). One-half milliliter of the homogenate was precipitated with 20% TCA, collected on a glass filter and washed with 10 ml 5% TCA, 10 ml 95% ethanol and 5 ml ethanol:ether (v:v). After drying, the precipitate was solubilized with NCS solubilizer and radioactivity determined.

Incorporation of uridine-$^{3}H$ into acid-insoluble material over a 12-hr treatment period was studied (Figure 2). Incorporation of uridine-$^{3}H$ by non-treated cells increased in a linear manner over the 12-hr period; however, incorporation by kinetin-treated cells ($2.5 \times 10^{-4}$ M) did not increase beyond the third hour of treatment. By the end of the 12-hr treatment period, incorporation of uridine-$^{3}H$ by kinetin-treated cells had been inhibited by approximately 79%.

## Effect of $2.5 \times 10^{-4}$ M Kinetin on Incorporation of Uridine-$^{3}H$ into Viral and Cellular Ribosomal RNAs

Polyacrylamide gel electrophoresis of nucleic acids extracted from virus-inoculated cells which were exposed to uridine-$^{3}H$ during the 48-60-hr p.i. period revealed that there was considerable incorporation of uridine into the 25 S and 18 S cellular ribosomal RNA species as well as incorporation into viral RNA (Figure 3A). Kinetin treatment severely suppressed uridine incorporation into both cellular ribosomal RNA species; however, uridine incorporation into viral RNA remained relatively unaffected (Figure 3B).

To determine in a more precise manner the effect of kinetin treatment on cellular ribosomal and viral RNA synthesis, total uridine incorporation into and the specific activity of the cellular ribosomal RNA and viral RNA was determined (Tables 1 and 2). Total uridine-$^{3}H$ incorporation

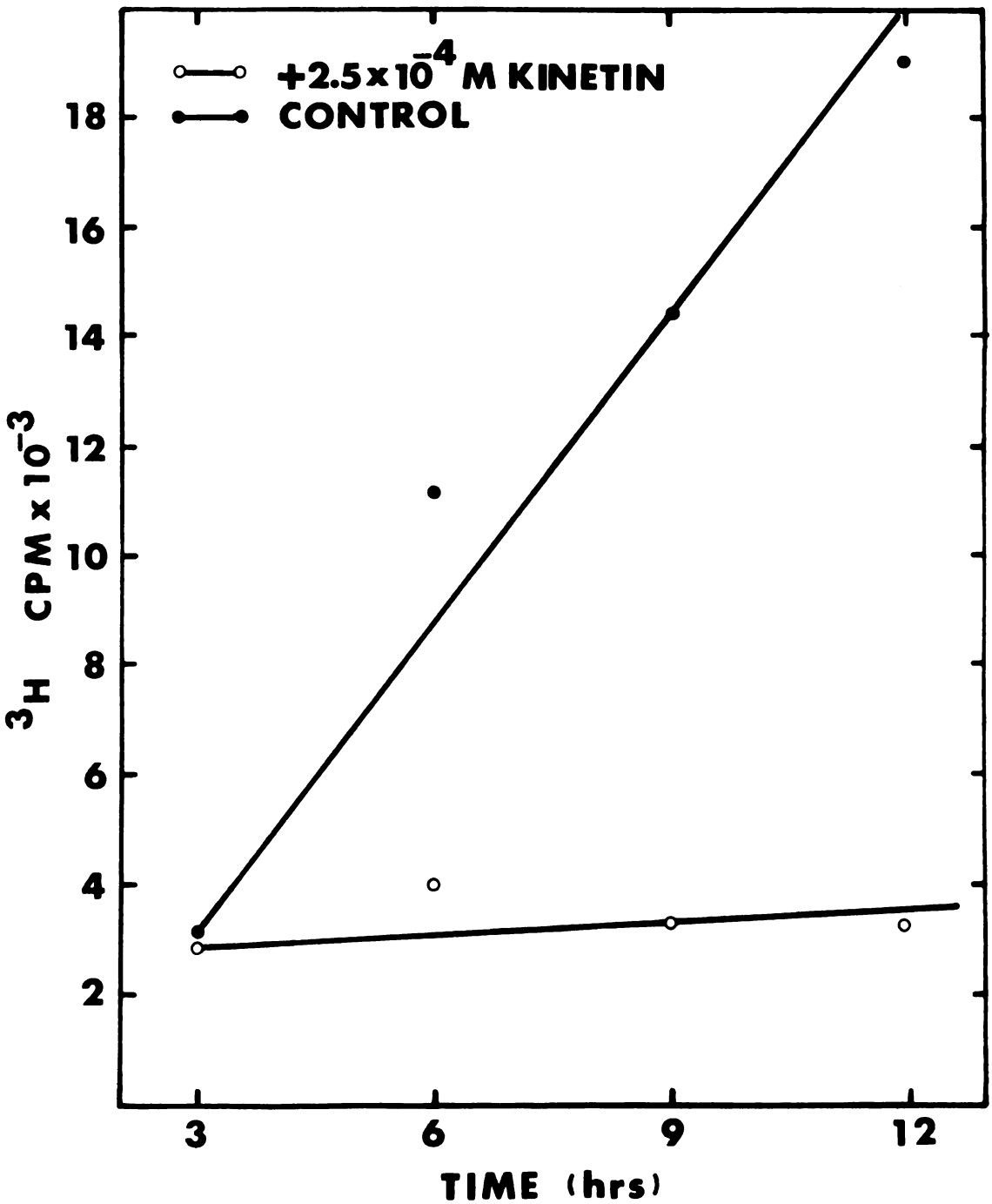


Figure 2. Effect of kinetin on incorporation of uridine-$^{3}H$ into acid-insoluble material over a 12-hr period. Cells were exposed to 2.5 x $10^{-4}$ M kinetin in the presence of uridine-$^{3}H$ (25 µC/ml) and incorporation into acid-insoluble material determined at 3-hr intervals. Radioactivity determinations were made as in Figure 1.

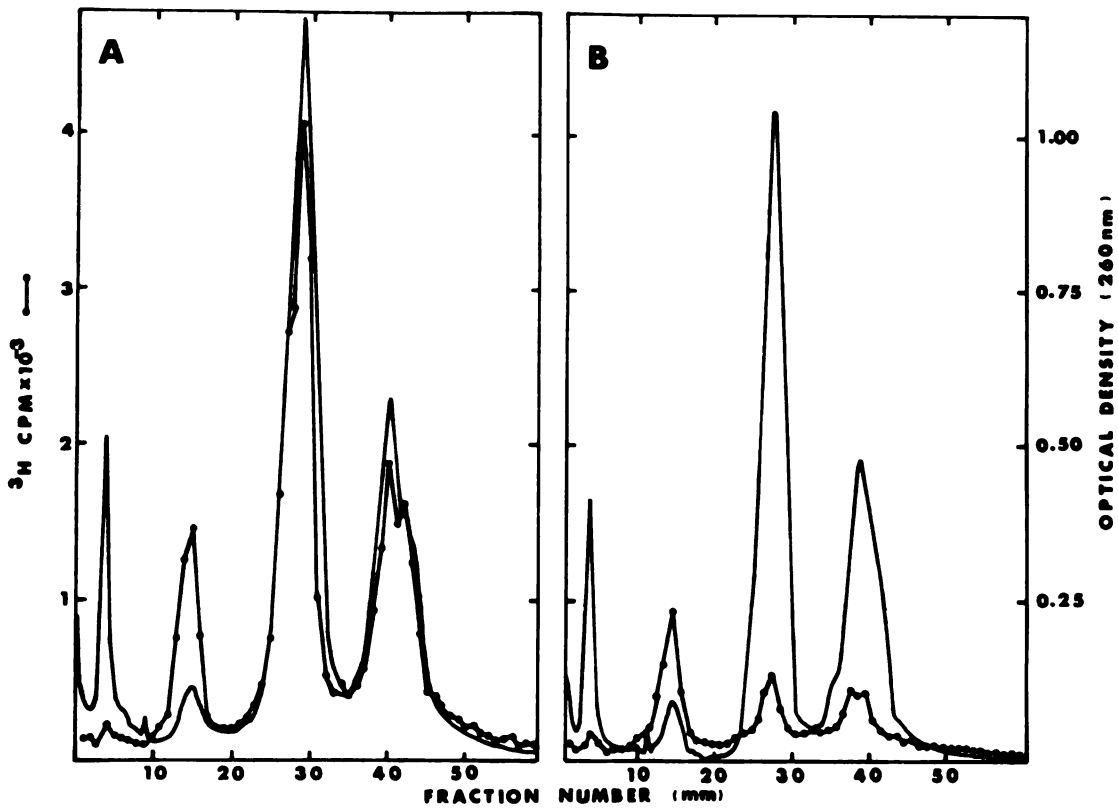


Figure 3. Effect of kinetin on incorporation of uridine-$^3$H into cellular ribosomal RNS and into viral RNA. TMV-infected cells were exposed to uridine-$^3$H during the 48-60-hr period after inoculation. (A) Liquid medium; (B) liquid medium containing 2.5 x 10$^{-4}$ M kinetin. Nucleic acids were extracted by the phenol-SDS technique and subjected to electrophoresis on 2.4% polyacrylamide gels (Bishop et al., 1967). The gels were then scanned at 260 nm, frozen and sliced into 1 mm sections. After solubilization in NCS solubilizer, radioactivity determinations were made. DNA, 4-7 mm; TMV-RNA, 12-17 mm; 28 S RNA, 24-33 mm; 18 S RNA, 34-45 mm. (——, O.D.; ●—●, cpm).

Table 1. Effect of Kinetin on the Specific Activity of Cellular RNA.

The specific activity of the 25 S and 18 S cellular ribosomal RNAs was determined after a 12-hr exposure to uridine-$^3$H in liquid medium containing $2.5 \times 10^{-4}$ M kinetin. The nucleic acids were extracted and processed as in Figure 3. The areas under the 25 S and 18 S RNA optical density peaks were converted to micrograms assuming an $E^{0.1\%}_{260\ nm} = 25$.

Total radioactivity corresponding to each peak was divided by the total amount of RNA in each peak to give the specific activity (cpm/µg RNA).

| | Total Uridine Incorporation into 25 S RNA cpm/gel | Specific Activity of 25S RNA cpm/µg | % Inhibition | Total Uridine Incorporation into 18 S RNA cpm/gel | Specific Activity of 18S RNA cpm/µg | % Inhibition |
|---|---|---|---|---|---|---|
| Control | 9350 | 1703 | | 7750 | 2017 | |
| | | | 72 | | | 71 |
| Plus Kinetin | 2701 | 380 | | 2256 | 577 | |
| Control | 19141 | 2857 | | 10731 | 2190 | |
| | | | 73 | | | 67 |
| Plus Kinetin | 3577 | 760 | | 2926 | 732 | |
| Average Inhibition | | | 72.5±.5% | | | 69.0±2% |

Table 2. Effect of Kinetin on the Specific Activity of TMV-RNA

The cells were treated and nucleic acids processed as in Table 1. Specific activity was determined assuming an $E^{0.1\%}_{260\ nm} = 25$ for TMV-RNA.

| | Total Uridine Incorporation into Viral RNA cpm/gel | Specific Activity of Viral RNA cpm/μg | % Inhibition |
|---|---|---|---|
| Control | 4599 | 11,492 | |
| | | | 1.5 |
| Plus Kinetin | 2484 | 11,290 | |
| Control | 1315 | 3968 | |
| | | | $^{+}3.5$ |
| Plus Kinetin | 1220 | 4109 | |

into both the 25 S and 18 S ribosomal RNA species was inhibited by kinetin treatment; however, no quantitative difference in phenol-extractable cellular RNA was observed between kinetin-treated and non-treated cells. The average specific activity of the 25 S RNA extracted from non-treated cells was 2280 cpm/μg, whereas the specific activity of the 25 S RNA from kinetin-treated cells was 570 cpm/μg, an inhibition of approximately 73% by kinetin treatment. A similar inhibition in specific activity of the 18 S cellular RNA (70%) was produced by kinetin treatment (Table 1). The inhibition in specific activity of the ribosomal RNA (71%) as determined by polyacrylamide gel electrophoresis and the inhibition in incorporation of uridine-$^{3}$H into acid-insoluble material (75%) is in close agreement.

While kinetin treatment produced a drastic inhibition in the specific activity of the cellular ribosomal RNAs (70%), the specific activity of the viral RNA appeared unaffected by kinetin treatment (Table 2). Although there appears to be less total uridine incorporation into viral RNA with nucleic acids extracted from kinetin-treated cells, this may have been due to different amounts of nucleic acids being applied to the gels. Specific activity determinations would have taken this into account and indicate that uridine-$^{3}$H incorporation into viral RNA was relatively unaffected by kinetin treatment when compared to incorporation into cellular RNA (Tables 1 and 2).

Effect of $2.5 \times 10^{-4}$ M Kinetin on Incorporation of Uridine-$^{3}$H into Complete Virus

Density gradient analysis of complete virus extracted from kinetin treated and non-treated cells indicates that uridine-$^{3}$H incorporation into complete virus is inhibited by kinetin-treatment (Figure 4). Incorporation into what appears to be cellular ribosomes, based on relative sedimentation rates, is also clearly inhibited by kinetin treatment. Incorporation of leucine-$^{14}$C into complete virus did not appear to be significantly affected by kinetin treatment. Incorporation of leucine-$^{14}$C into the ribosomal particles was approximately equal in both kinetin-treated and non-treated cells (Figure 5). Total uridine-$^{3}$H incorporation into complete virus was inhibited an average of 34% by kinetin treatment (Table 3). The actual yield of complete virus (µg/1.5 g cells) isolated from kinetin-treated cells was inhibited an average of 33%. Because incorporation of uridine-$^{3}$H into complete virus and the yield of complete virus was reduced to the same extent by kinetin treatment, the specific activity (cpm/µg) of the virus remained approximately the same as that of the virus extracted from non-treated cells (Table 3). Total leucine-$^{14}$C incorporation into complete virus from kinetin-treated cells was not significantly lower than that incorporated into virus extracted from non-treated cells (Table 3); however, as mentioned earlier, the total yield of complete virus from

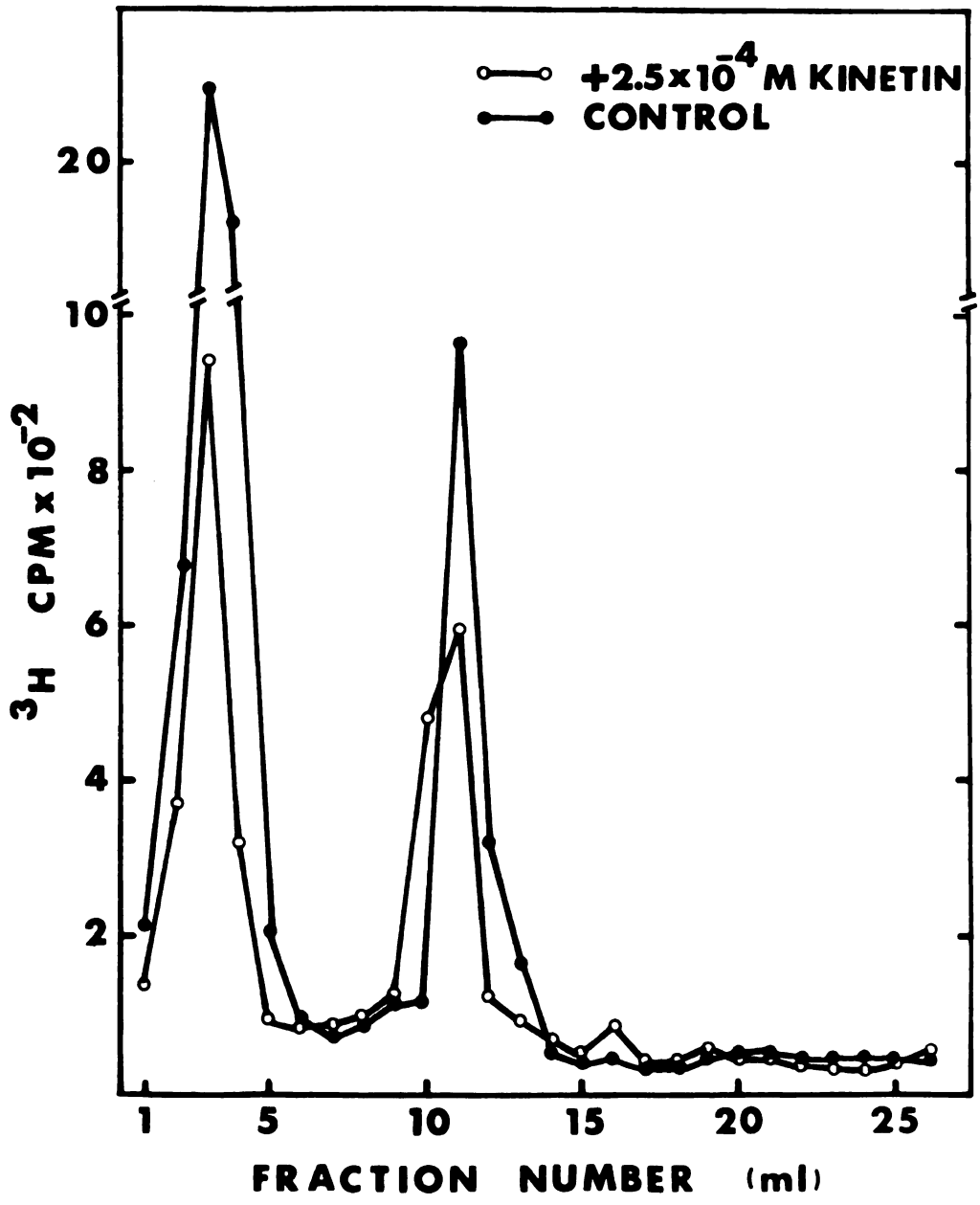


Figure 4. Effect of kinetin on uridine-$^{3}$H incorporation into complete TMV. TMV-infected cells were exposed to uridine-$^{3}$H during the 48-60-hr period after inoculation (●—●, liquid medium; o—o, liquid medium containing 2.5 x $10^{-4}$ M kinetin). Complete virus was extracted and purified by density gradient centrifugation on 10-40% sucrose gradients. The gradients were scanned (254 nm) and 1 ml fractions collected. After addition of 500 µg BSA, radioactivity was determined as in Figure 1. TMV, fractions 10-13; ribosomal particles, fractions 2-5.

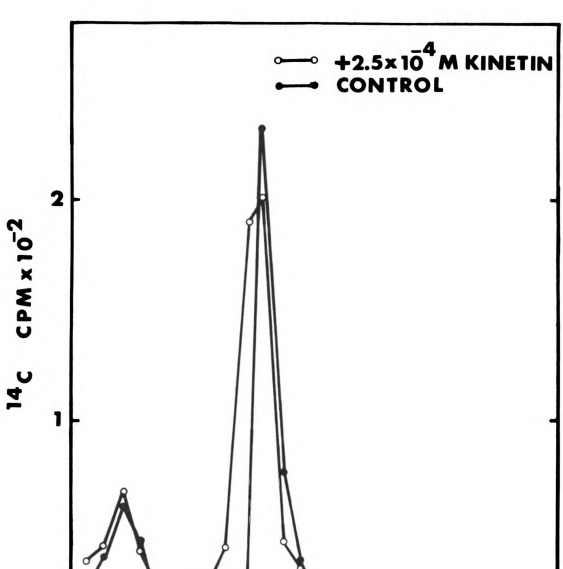


Figure 5. Effect of kinetin on leucine-$^{14}$C incorporation into complete virus. TMV-infected cells were exposed to leucine-$^{14}$C (1 µg/ml) during the 48-60 hr period after inoculation (●—●, liquid medium; o—o, liquid medium containing 2.5 x $10^{-4}$ M kinetin). Complete virus was extracted as in Figure 4. TMV, fractions 10-13; ribosomal particles, fractions 2-5.

Table 3. Effect of Kinetin on the Yield and Specific Activity of Complete TMV.

Cells were treated and complete virus processed as described in Figure 4. Micrograms complete virus was determined from optical density profiles of virus subjected to density gradient centrifugation assuming an $E^{0.1\%}_{254\ nm} = 3.06$.

| | Complete Virus | | | | | | |
|---|---|---|---|---|---|---|---|
| | $^{3}$H-cpm | % Inhibition $^{3}$H-cpm | Specific Activity $^{3}$H-cpm/µg | % Inhibition Complete Virus | $^{14}$C-cpm | Specific Activity $^{14}$C-cpm/µg | % Increase Specific Activity $^{14}$C-cpm/µg |
| Control | 6113 | 34 | 306 | 33 | 3544 | 175 | +51 |
| Plus Kinetin | 4036 | | 294 | | 4563 | 355 | |
| Control | 1290 | 36 | 44 | 32 | 527 | 18 | +32 |
| Plus Kinetin | 828 | | 42 | | 513 | 26 | |
| Control | 1605 | 31 | 185 | 34 | 400 | 46 | +42 |
| Plus Kinetin | 1105 | | 184 | | 478 | 80 | |

kinetin-treated cells was approximately 33% less than from non-treated cells. Therefore, the leucine-$^{14}C$ specific activity of the complete virus, as measured by cpm-leucine-$^{14}C$ incorporated per µg complete virus, is higher in the kinetin-treated than in non-treated cells (Table 3).

## Discussion

The purpose of this series of experiments was to determine the effect of a high concentration of kinetin on virus synthesis in inoculated tobacco tissue culture. A concentration of kinetin which clearly affected cellular RNA synthesis was selected because this effect could be used as an internal standard during the experiments. That is, the effect of kinetin on cellular RNA synthesis could directly be compared to the effect kinetin had on viral RNA synthesis. To select a concentration of kinetin which clearly affected cellular RNA metabolism, cells were exposed to concentrations of kinetin ranging from $1.25 \times 10^{-6}$ to $5 \times 10^{-4}$ M. Cellular RNA synthesis, as measured by uridine-$^{3}H$ incorporation, was severely inhibited at concentrations ranging from $2.5 \times 10^{-5}$ to $2.5 \times 10^{-4}$ M kinetin (Figures 1 and 2). Incorporation of uridine-$^{3}H$ into both the 25 S and 18 S cellular ribosomal RNAs, as measured by specific activity, was inhibited by approximately 70% (Table 1). This observation is in general agreement with those of Carpenter and Cherry (1966), who used peanut cotyledons, and

Nudel and Bamberger (1971), who used tobacco tissue culture to study the effect of kinetin on cellular RNA metabolism. Also in agreement with Nudel and Bamberger, treatment with $2.5 \times 10^{-4}$ M kinetin inhibited growth of the tobacco cell culture. However, the inhibition of incorporation of leucine-$^{14}$C reported by Nudel and Bamberger was not observed in our system over kinetin concentrations ranging from $1.25 \times 10^{-6}$ to $5 \times 10^{-4}$ M.

To study the effect of $2.5 \times 10^{-4}$ M kinetin on viral RNA synthesis, inoculated cells were exposed to kinetin 48-60 hr after inoculation. During this period viral RNA synthesis is proceeding at maximum rate. If there was an inhibitory effect on viral RNA synthesis, it could be easily observed during this period. Kinetin did not appear to drastically affect incorporation of uridine-$^{3}$H into viral RNA over the 12-hr treatment period (Figure 3). The specific activity of viral RNA also did not appear to be affected by kinetin treatment (Table 2). As pointed out earlier, under the same conditions and in the same cultures, cellular ribosomal synthesis was inhibited by 70% by kinetin treatment (Table 1). Nudel and Bamberger (1971) postulated that the activity of kinetin as an inhibitor of cellular RNA synthesis is closely related to its stimulatory activity as a growth regulator rather than to a general toxicity effect. Our observations are in agreement with this postulate. If the inhibitory action of kinetin on cellular ribosomal

synthesis was due to a general toxicity effect, then this effect would have inhibited viral RNA synthesis to the same degree. Sänger and Knight (1963) reported that actinomycin-D inhibited cellular RNA synthesis while not affecting viral RNA synthesis. The specificity was reportedly due to the fact that actinomycin-D inhibited DNA-directed RNA synthesis (Hurwitz et al., 1962); viral RNA synthesis, being RNA-directed RNA synthesis, was not affected. Our results indicate that kinetin may exhibit a similar specificity, affecting cellular RNA synthesis while not affecting viral RNA synthesis. Milo and Srivastava (1969a) implied that even low concentrations of kinetin directly inhibited virus synthesis in tobacco tissue culture. Based on our observations, it would appear that if kinetin does directly inhibit virus synthesis, its effects must be on something other than viral RNA synthesis.

While kinetin did not appear to affect viral RNA synthesis over the 12-hr period studied, it did inhibit incorporation of viral RNA into complete virus and reduce the accumulation of complete virus (Figure 4 and Table 3). The fact that different lots of uridine-$^{3}$H, which differed in specific activity, were used may account for the wide variation in uridine-$^{3}$H specific activity of the complete virus. The inhibition of accumulation of complete virus remained constant over the three experiments reported (Table 3) and suggests that high kinetin concentration may

affect viral protein synthesis. This is in conflict with the observation that kinetin treatment did not appear to affect leucine-$^{14}C$ incorporation into acid-insoluble material. But this observation alone does not rule out the possibility that protein synthesis is inhibited by kinetin treatment. An indication that protein synthesis is affected by kinetin treatment is that, while accumulation of complete virus is inhibited by approximately 35%, incorporation of leucine-$^{14}C$ into complete virus appears to be unaffected (Figure 5 and Table 3). This being the case, the leucine-$^{14}C$ specific activity of the viral coat protein incorporated into complete virus in kinetin-treated cells must be higher than in non-treated cells (Table 3). A similar observation can be made on a specific cellular protein, namely ribosomal protein. While ribosomal RNA synthesis is inhibited by approximately 70% (Figure 4 and Table 1) as measured by uridine-$^{3}H$ incorporation, the amount of leucine-$^{14}C$ incorporation into ribosomes appears unaffected (Figure 5). Because of lack of ribosomal RNA, ribosome synthesis (union of ribosomal RNA and ribosomal protein) would be inhibited, yet incorporation of leucine into ribosomes appears unaffected; therefore, the specific activity of the ribosomal protein must be higher in kinetin-treated cells than in non-treated cells. These observations indicate that kinetin treatment may inhibit protein synthesis, but that the protein being

synthesized has a higher specific activity (leucine-$^{14}$C-cpm/μg). Osborne (1962) reported that kinetin treatment increased the leucine-$^{14}$C specific activity of proteins in tobacco leaf discs. The fact that kinetin inhibits ribosomal synthesis would in itself suggest that protein synthesis is going to be adversely affected due to lack of newly synthesized ribosomes.

Due to the fact that both cellular and viral protein synthesis may be affected by kinetin treatment, it is not possible to determine if kinetin treatment specifically affects virus protein synthesis. It may be that the prolonged treatment adversely affected cellular metabolism. In view of this observation and those of Nudel and Bamberger (1971) and Parthier et al. (1964) that high concentrations of kinetin affected protein metabolism in tobacco tissue culture cells and tobacco leaf discs, the early work on the effect of kinetin on virus synthesis in isolated leaves and leaf discs (Daft, 1963; Selman, 1964; Király and Szirmai, 1964; Aldwinckle and Selman, 1967; Milo and Srivastava, 1969a,b) has to be reappraised because of high concentrations of kinetin used. The conflicting results reported by these workers may be due to differences in the effective kinetin concentration within the cells and, as pointed out by Osborne (1962), there may be considerable variation in the biochemical response of different plant species to kinetin.

However, based on the observations reported in this paper, it may be concluded that, in the system studied, kinetin does not directly adversely affect TMV-RNA synthesis.

## Summary

Kinetin concentrations greater than $2.5 \times 10^{-5}$ M severely inhibited uridine-$^{3}H$ incorporation into tobacco tissue culture cells. At a concentration of $2.5 \times 10^{-4}$ M, kinetin inhibited the specific activity of cellular RNA by approximately 70% when compared to the specific activity of RNA from non-kinetin-treated cells. Incorporation of leucine-$^{14}C$ did not appear to be affected by high kinetin concentrations.

Viral RNA synthesis in inoculated tobacco tissue culture cells, as measured by uridine incorporation, did not appear to be significantly affected by kinetin treatment; however, incorporation of viral RNA into and accumulation of complete virus was inhibited by approximately 33%. Incorporation of leucine-$^{14}C$ into complete virus appeared unaffected by kinetin treatment, even though accumulation of complete virus was inhibited.

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