Biosynthesis of bromegrass mosaic virus ribonucleic acid
by Dean Russell Branson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in BOTANY
Montana State University

Abstract:
Both de novo synthesis of nucleotides and rearrangement of ribosomal breakdown products appear to be precursor sources for bromegrass mosaic virus (BMV)-RNA. The incorporation of C^14-orotic acid and C^14-uridine, intermediates of the de novo synthesis and breakdown pathways respectively, were compared in BMV-infected barley during a period of virus synthesis.

Analyses of the C^14-BMV-RNA specific activities using the dilution factor method showed that the carbon-14 from C^14-uridine was incorporated into BMV-RNA two to three times more efficiently than the carbon-14 from C^14-orotic acid.

The essential enzymes for both pathways were demonstrated by using crude enzyme preparations. Those enzymes considered essential were: 1) uridine 5'kinase, 2) orotidine 5'phosphate pyrophosphorylase, and 3) orotidine 5'phosphate decarboxylase.

Ribosomal content was lower in BMV-infected tissue than in non-infected tissue suggesting that BMV incorporated the products of host-RNA breakdown, in a manner similar to that reported for tobacco mosaic virus infections. Continuation of ribosomal synthesis in virus infected tissue was demonstrated by the incorporation of C^14-orotic acid into ribosomal RNA.

Pyrimidine metabolism, involved in the formation of RNA precursors in BMV-infected and non-infected barley, appeared unchanged except for the additional function of BMV-RNA synthesis. Evidence for this unchanged metabolism was: 1) radioactive ribosomal RNA from C^14-orotic acid-fed barley, infected and non-infected, contained C^14-uridylic acid 3' and C^14-cytidylic acid 3' when acid hydrolysed, and 2) ribosome breakdown was not a prerequisite for BMV-RNA precursor formation. Direct incorporation, without delay, of C^14-orotic acid into BMV-RNA was observed.

These findings were consistent with the following relationship between host-RNA metabolism and BMV-RNA biosynthesis: Pyrimidine Metabolism in BMV-infected Barley
5-Phosphorylribose-1-pyrophosphate Orotic acid PRPP Uridine Uridylic acid 5' Orotidine 5' phosphate Uridylic acid 3' UDP UTP CTP Ribosomal - RNA BMV-RNA Breakdown De Novo

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

"BIOSYNTHESIS OF BROMEGRASS MOSAIC VIRUS RNA"

Dean Branson - PhD Thesis

Abstract

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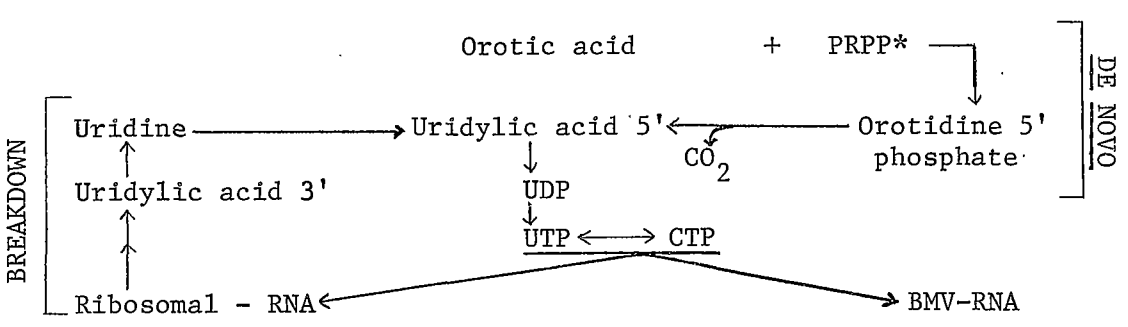
Pyrimidine Metabolism in BMV-infected Barley



* 5-Phosphorylribose-1-pyrophosphate

INTRODUCTION

The general scheme of virus synthesis by converted cells is considered to be 1) the synthesis of virus protein and virus nucleic acid, and 2) the assembly of these virus components to form viral nucleoproteins (78).

Experiments reported below are concerned with the synthesis of viral RNA, origin of the precursors, and the metabolism of host-RNA during virus synthesis.

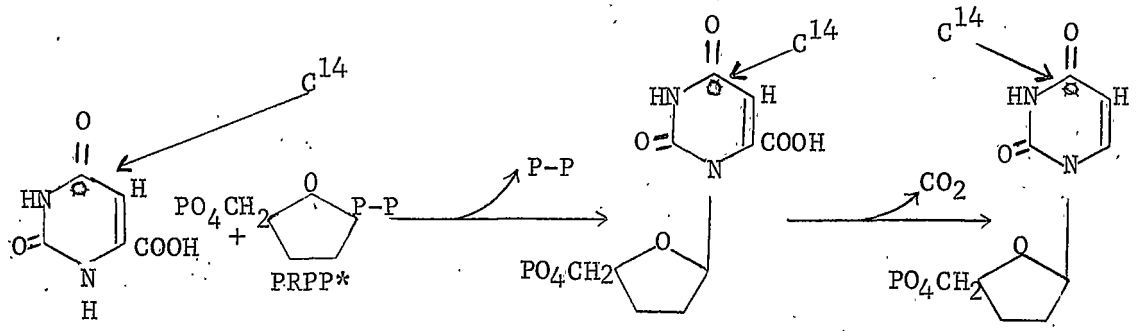
Precursors of viral RNA originate from host-RNA breakdown products and/or from de novo synthesis. The host-virus relationship governing the origin of viral RNA precursors is apparently not the same for all systems studied. Within the animal, bacterial and plant systems there are variations which prevent generalizations about the activity of a de novo synthesis pathway or a breakdown pathway during virus synthesis.

The basic questions of host-RNA metabolism during virus synthesis are: 1) continuation or cessation of host-RNA synthesis, and 2) stability or rapid decay of host-RNA content. Since a majority of the host-RNA is ribosomal and ribosomes have been demonstrated to be necessary for protein synthesis, investigations concerning the metabolism of ribosomes during virus synthesis seem important.

Precursor sources and ribosome metabolism are the subjects of this thesis. A literature review of these subjects follows.

Pyrimidine metabolism of the de novo synthesis pathway in plants has been studied only recently and it tentatively appears to be similar to that found in microbial and animal cells (16, 17, 44, 66, 67, 80, 81, 82). The proposed sequence of reactions includes a step leading to the formation of uridine-5'-phosphate (UMP5') from orotic acid (29).

PRECURSOR SOURCE: DE NOVO SYNTHESIS PATHWAY



Orotic acid-6-C^{14} Orotidine 5'PO_4 Uridine 5'PO_4

*PRPP = 5'phosphoribosyl-1-pyrophosphate

Orotic acid formation in plants appears to be similar to other systems. The initial reaction is the formation of the pyrimidine precursor, L-ureidosuccinic acid, from carbamyl phosphate and L-aspartic acid (66).

Shigematsu (74) reported that H^3-aspartic acid introduced to tobacco mosaic virus (TMV)-infected tobacco could be recovered in the nucleic acid fraction, implying that the proposed de novo synthesis pathway is active during TMV biosynthesis.

Pyrimidine metabolism of the breakdown pathway in TMV-infected plants as proposed by Reddi and Mauser (62) directly contributes to the formation of TMV-RNA. Similarly, Gilliland et. al. (38) suggest that

tobacco ringspot virus-RNA is formed by the rearrangement of host breakdown products.

PRECURSOR SOURCE: BREAKDOWN PATHWAY

$$\text{Host-RNA} \xrightarrow[\text{RNase II}]{\text{RNase I}} \text{ribonucleoside } 3'PO_4 \xrightarrow{\text{Phosphatase}} \text{ribonucleoside}$$

$$\text{ribonucleoside} + \text{ATP} \xrightarrow[\text{phosphokinase}]{\text{nucleoside}} \text{ribonucleoside } 5'PO_4 + \text{ADP}$$

Leaf ribonucleases attack all the internucleotide links giving at first nucleoside 2'3'phosphates and later completing the hydrolysis of the pyrimidine and purine cyclic phosphates to give 3'phosphates (53). Reddi and Mauser (62) demonstrated these nucleases in ribosome preparations from TMV-infected tobacco and called them RNase I and RNase II. Nucleotide phosphatase and nucleoside 5'phosphokinase activity which catalyze the formation of UMP5' from ribosomal breakdown products could be demonstrated in the same preparations.

Other pyrimidine metabolic pathways contributing to the formation of UMP5' that may play a significant role in viral RNA biosynthesis have been investigated in non-plant systems. Hurlbert and Kammen (43) reported the deamination of cytidine to uridine and its phosphorylation to UMP5'. Crawford et.al.(28) and Reichard and Sköld (63) and Reichard (64) described the direct formation of UMP5' from uracil, PRPP and UMP5' pyrophosphorylase activity. The metabolic activity of orotidine has been suggested (20, 82) but no direct evidence has been supplied. According to White et. al. (79), the only known pathway for formation of a cytidine nucleotide involves amination of uridine triphosphate to yield the

corresponding cytidine triphosphate (16, 29). Uridine triphosphate formation involves consecutive transfers of phosphate from ATP to uridine 5'phosphate with intermediate formation of uridine diphosphate (79). The hypothesized viral RNA polymerase substrate "pool" consists of UTP, CTP, ATP and GTP. The formation of UTP and CTP is by the above described pathways.

The de novo synthesis and breakdown pathways leading to the formation of viral RNA precursors are the major interests of this thesis. Direct evidence is lacking for the activity of the de novo synthesis pathway during virus synthesis. Acceptable evidence is the incorporation of an intermediate, like orotic acid, into viral RNA.

Evidence for the activity of the breakdown pathway during virus synthesis is available in the animal virus, bacteriophage and plant virus systems (18, 48). This evidence is, in some cases, the incorporation of an intermediate of the breakdown pathway into viral RNA. In other cases it is an evaluation of host-RNA metabolism during virus synthesis.

Animal virus-RNA biosynthesis has been extensively investigated using poliovirus-infected cells. So sophisticated have been the experiments that one must identify the type of host-RNA and its subcellular location when discussing host-RNA metabolism in virus-infected cells. Holland and Peterson (42) have shown that synthesis of nuclear RNA is inhibited and cytoplasmic RNA synthesis is stimulated in poliovirus-infected human cells. If the synthesis of cytoplasmic RNA is

stimulated and the cytoplasm is the site of poliovirus-RNA replication (3, 4, 5) then the most likely source of viral RNA precursors would be de novo synthesis. Consistent with de novo synthesis would be reports on the stability of ribosomal RNA in poliovirus-infected cells by Holland (41), Zimmerman et. al. (84) and Fenwick (32). Evidence supporting the alternate source of viral RNA precursors (host-RNA breakdown products) as reported by Salzman et. al. (70) and Salzman and Sebring (71), is that poliovirus incorporated radioactive uridine, adenine and cytidine in infected S 3 cells. These compounds are intermediates of the proposed breadkown pathway. Diamond and Balis (30) suggests both de novo synthesis and host-RNA breakdown as sources for poliovirus-RNA. Perhaps nuclear ribosome breakdown products cross the nuclear membrane and become available for viral RNA synthesis.

Myxoviruses such as fowl plague virus and Newcastle disease virus do not induce synthesis of inhibitors of cellular RNA synthesis (68). Inhibition of nuclear RNA appears to be characteristic of picornaviruses (31, 35, 42, 54, 70, 89). In the early stages of myxovirus-RNA biosynthesis the process appears to be an additional performance of the host cells (68), meaning that the viral RNA precursors originate from both sources; de novo and breakdown products.

Bacteriophages have been used in studies which have contributed to a model for viral RNA replication (50). Recent studies on viral RNA replication are reviewed by Burke (18), Levintow (48), Ochoa et. al. (58), and Zinder (85). The aspects of viral RNA replication of much interest

are the demonstration and characterization of a viral induced DNA-independent-RNA polymerase and a double stranded replicative form of viral RNA, which is a parental viral RNA (plus strand) complementarily paired with a template strand (minus strand) (59).

Friesen (36) reported evidence suggesting that the control of phage-RNA synthesis is subject to those factors described for the control of ribosomal and transfer RNA synthesis. This indirectly means both de novo synthesis and breakdown pathways are available to supply phage-RNA precursors. The fact that many in vivo phage labeling studies use radioactive uridine (33), an intermediate in the proposed breakdown pathway, indicates that at least host-RNA breakdown products could be virus precursors. Ellis and Paranchych (31) and Cooper and Zinder (27) conclude that viral RNA and protein are synthesized from soluble precursors. This notion is compatible with precursors arising from any source including breakdown products and de novo synthesis. The cessation of host-RNA synthesis and/or a variation in the host-RNA content in infected cells as well as the source of viral RNA precursors appear to be aspects of bacteriophage-RNA biosynthesis that have been overlooked.

RNA biosynthesis of plant viruses has been studied primarily in TMV-infected tobacco and to a lesser degree in turnip yellow mosaic virus (TYMV)-infected cabbage. Precursor sources and ribosome metabolism, aspects of viral RNA biosynthesis, are the subjects of several recent reports (1, 25, 60,61, 62). Reddi (60) has suggested that TMV induces the rapid breakdown of tobacco microsomal (ribosomal) RNA and that the breakdown products are

enzymically assimilated into TMV-RNA. The amount of TMV-RNA synthesized is approximately equal to the amount of ribosomal RNA degraded. The use of uracil -H^3 and uridine -H^3 for studies on TMV synthesis indirectly assumes a precursor source (40, 75, 76, 83).

Continuation of ribosome synthesis after TMV infection was observed by Reddi (60). He found large amounts of radioactivity in the ribosome fraction several days after inoculation and after introduction of C^{14}-precursors of ribosomes. Babos (1) and Sänger and Knight (72) were also able to demonstrate the continuation of ribosome synthesis after TMV infection.

Disagreement with Reddi on the source of viral RNA precursors was reported by Commoner et.al. (25) who indicated that TMV-RNA was synthesized largely de novo. This finding was confirmed by Babos (1) and Kubo (47) on the basis that the rate of host-RNA synthesis and host-RNA degradation was unaffected by TMV synthesis in the cells. Babos did not show, however, that de novo or breakdown products were specifically incorporated into TMV-RNA. One possible explanation suggested by Babos (1) for the disagreement was that Reddi used much younger tissue.

Stability of ribosome content in TMV-infected tissue was also supported by evidence reported by Basler and Commoner (6), Röttger (69), Commoner et.al. (24), Balandin et.al. (2), Fry and Matthews (37), and Babos (1). Generally, the ribosome content of TMV-infected samples taken at intervals after inoculation increases at first and then gradually decreases at a rate equal to or slightly greater than that of non-infected tissue samples.

The effects of virus infection on the distribution of the various classes of ribosomes have not been adequately studied except in the case of a turnip yellow mosaic virus infection in cabbage (55, 56). The 68 S ribosomes of Chinese cabbage leaves appear to be confined to chloroplasts of healthy leaves (23) in a concentration of 0.2 mg/g fresh weight (65). No 68 S ribosomes could be detected in symptomatic tissue of TYMV-infected cabbage. The concentration of 83 S cytoplasmic ribosomes (22), decrease during leaf expansion in both infected and non-infected tissue. This decrease in chloroplast ribosomes along with other indirect evidence suggests not only that TYMV-RNA is synthesized in the chloroplast but also that biosynthesis takes place by the ribosomal breakdown mechanism proposed by Reddi and Mauser (62). The decrease of ribosome content in beans infected with southern bean mosaic virus, as demonstrated by Meister (57), is also evidence for a ribosome breakdown mechanism. It is obvious that future studies on viral RNA biosynthesis should be at the subcellular level.

In light of the above review there appear to be conflicting views on the origin of plant virus RNA precursors as well as the synthesis of host-RNA during virus synthesis. The stability of ribosome content during virus synthesis is also questionable.

The main purpose of the following investigation was to determine whether orotic acid, an intermediate of the <u>de novo</u> pathway, and/or uridine, an intermediate of the breakdown pathway, could be incorporated into BMV-RNA. The relative activity of these two pathways was determined

by introducing radioactive orotic acid and uridine into similar lots of BMV-infected barley and comparing the amount of assimilated radioactivity. Parallel studies dealt with two other aspects of viral RNA biosynthesis; 1) ribosomal content, and 2) ribosomal synthesis.

Bromegrass mosaic virus infected barley is a favorable system for investigating the plant-virus relationship during biosynthesis of viral ribonucleic acid. The advantages of this system are: 1) several milligrams of virus are easily purified from a gram of infected tissue (11,21); 2) the RNA content (21%) is relatively high (10); 3) rapidly translocated radioactive viral precursors can be introduced to infected tissue with ease (7); and 4) BMV is structurally similar to other small plant, animal and bacterial RNA viruses (45, 46, 85).

MATERIALS AND METHODS

Virus Inoculation and Purification The type strain of bromegrass mosaic virus (BMV) AC-66 American Type Culture Collection, 1958 [1/] was used in this study. The virus was cultured in barley (*Hordeum vulgare* L. var. Blackhulless) in a greenhouse at an air temperature of 20-30°C unless otherwise stated.

Plants in the two-to three-leaf stage were inoculated by rubbing the leaves with a sterile cheesecloth pad soaked in crude sap from BMV-infected plants diluted 1:10 with tap water containing Celite.[2/] This method of inoculation was used for all time-sequence studies of BMV infections, virus purifications and maintainance of the stock culture.

Infected leaves were harvested two weeks after inoculation, or, in the case of replication studies, the inoculated leaves were harvested at designated times after inoculation. Leaf samples of less than fifty grams were ground with a mortar and pestle and larger samples were ground with a meat grinder. Ground samples were diluted with equal volumes for tissue weight (w/v) of pH 7.0, 0.01M potassium phosphate buffer and then frozen. Frozen samples were thawed, heated for one hour at 40°C and clarified by centrifugation for 15 min at 20,000 g in a refrigerated centrifuge. The supernatant fluid containing the crude virus preparation

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[1/] BMV-infected tissue was obtained from Dr. R. I. Hamilton of the Botany and Microbiology Department, Montana State University, Bozeman.

[2/] Diatomaceous silica, prepared by Johns-Manville Co.

was either analysed for virus mass by the sucrose density gradient centrifugation method or was further purified by differential centrifugation. The method of differential centrifugation was similar to that used by Chiu and Sill (21), Brakke (14) and Bockstahler and Kaesberg (11), except the low speed centrifugations were 15 min at 20,000 g, the high speed centrifugations were 3 hr at 100,000 g or 2 hr at 130,000 g, and the virus pellets were resuspended in 0.01M phosphate buffer pH 6.5.

Time Course of BMV Increase: Analytical Procedures

BMV replication rates were estimated by sucrose density gradient centrifugation analysis for virus mass at intervals after inoculation. Two gram samples of inoculated leaves were used to obtain crude virus preparations for sucrose density gradient centrifugation analysis. Crude virus was prepared from fresh tissue by the grind-freeze-thaw-heat method previously described. A 0.2 ml sample was overlayed with 0.1 ml buffer and floated on a gradient column prepared by layering 1.1 ml of buffered solutions (0.01M phosphate, pH 6.5) of 100, 200, 300 and 400 mg sucrose per milliliter. Gradient columns were centrifuged for 1.5 hr at 38,000 rpm in the SW39L rotor and fractionated using an ISCO Density-Gradient Fractionator. Virus zones were quantitated by their UV[3/] absorption at 254 mμ as recorded on a Nesco graphic recorder. Several runs using known concentrations of highly purified BMV established a relationship between virus mass and the area under the absorption curves. This

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3/ UV = ultraviolet