A STUDY OF CERTAIN ASPECTS OF SULFUR METABOLISM OF TOBACCO MOSAIC VIRUS INFECTED NICOTIANA TABACUM L.

by

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INTRODUCTION

In recent years considerable work has been done to elucidate physiological and metabolic aspects of plant host-virus systems. Much of this has been concerned with the effect of Tobacco Mosaic Virus on Nicotiana species, especially the systemically-invaded host N. tabacum L. Pound (1961) summarized much of this work on plant virus host physiology, and noted that many aspects of this study are still not well understood. He did point out that there is agreement among many workers on the respiratory changes associated with virus infection.

Owen (1955a, 1955b, 1956, 1957, 1958) in particular found changes in the rate of respiration of inoculated host leaves (N. tabacum) at early periods after inoculation. Since respiration provides much of the energy for other metabolic processes, respiratory changes should be reflected in various energy requiring processes such as sulfate reduction. Commoner and Dietz (1952), Commoner and Nehari (1953a), and Commoner et al. (1953b) have observed changes in nitrogen metabolism of TMV infected N. tabacum at early periods after inoculation, but it is not known what energy relations are suggested here.

Sulfur metabolism has not been examined in N. tabacum with respect to virus infection at early periods after inoculation, although the effect of sulfur nutrition on virus multiplication has been studied by Ling and Pound (1962). Thomas (1958), Gibbs and Schiff (1960), Schiff (1962), and Wilson (1962) have reviewed the literature concerning sulfur metabolism in higher plants, and each

notes that the various processes of sulfur metabolism, especially sulfate reduction, are not well understood energetically and metabolically. Little is known about sulfur metabolism in virus infected plants.

The present study was undertaken to investigate some aspects of sulfur metabolism in N. tabacum at 48 and 72 hours after inoculation with TMV. Further, only the leaves from nodes and apical portions of infected plants which had not been inoculated with the virus were studied in an attempt to observe changes in sulfur metabolism in tissues where the virus had not been initially introduced. The problem has been studied as follows:

1. Quantitative determinations were made for total sulfur, inorganic sulfur, and organic sulfur contents of the apical portions and various lower leaves of both healthy and TMV infected N. tabacum at 48 hours and 72 hours after inoculation.
2. Uptake and accumulation of S^{35} at the apical portions and at each of the leaves at five lower nodes of healthy and infected plants was determined at 48 hours and 72 hours after inoculation.
3. Free and bound S^{35}-labeled amino acids were isolated from the apices and leaves of each of five lower nodes of healthy and infected plants. A comparative quantitative estimate

of labeled amino acids at each node was made utilizing radioactive sulfur. This study was conducted only on the plants at 48 hours after inoculation.

REVIEW OF THE LITERATURE

The literature will be reviewed as follows:

I. Sulfur metabolism in higher plants.

II. Sulfur nutrition in Nicotiana tabacum with respect to the multiplication of TMV.

III. The effect of TMV on the respiration rate of the tobacco host.

I. Sulfur Metabolism in the Higher Plants

UPTAKE and DISTRIBUTION in HIGHER PLANTS: The early studies of Thomas et al. (1944) using radioactive tracters, established that sulfur enters the higher plant in at least two inorganic forms. Most of the sulfur enters the plant through the roots in the form of sulfate ($SO_4^=$) ions. However, these workers first showed that sulfur may also enter the plant through the leaves and stems in the form of sulfur dioxide (SO_2) gas, but is thereafter converted to sulfate before distribution and utilization. Wood and Barrien (1939) and Miller (1921) have shown that the roots may absorb such S-amino acids as cysteine, cystine, and methionine, but sulfur bound in these compounds is oxidized immediately to sulfate before distribution in the plant.

It is known that $SO_4^=$ uptake by plants proceeds more rapidly in the presence of sunlight than in the dark. This phenomenon is thought to be related directly to transpiration, but other processes must also be involved (Thomas et al. 1958).

After entrance and distribution of inorganic sulfur in the

plant it may be reduced to organic forms such as the amino acid cysteine. This may occur in the roots or in the leaves (Wilson 1962). Wilson (1962) and Gibbs and Schiff (1960) note that sulfate sulfur is probably first converted to organic sulfur as the -SH group of cysteine. Thereafter cysteine may be converted to cystine and methionine, bound into proteins, or serve as the precursor of other sulfur compounds in the plant such as Coenzyme A.

Harrison, Thomas, and Hill (1944) demonstrated that radioactive sulfur is more concentrated in actively growing sites such as meristematic tissues in the plant and in the embryos of germinating seeds. In these areas cell division and protein synthesis occur rapidly. Thomas (1958) reported that sulfhydyl groups are intimately associated with cell division and sulfur amino acids are also found in chloroplast protein.

SULFUR CONTAINING COMPOUNDS in the HIGHER PLANTS: In the last few years at least four extensive reviews by Thomas (1958), Young and Maw (1958), Schiff (1962) and Robinson (1963) have considered sulfur containing compounds in higher plants.

These reviews note that sulfur is found mainly incorporated in the amino acids, cysteine, cystine, and methionine and the tripepide glutathione. The essentiality of these compounds is involved primarily with protein chemistry. However, glutathione has been found (as have other sulfhydryl compounds) to be involved in many metabolic reactions in both plants and animals due to the oxidized reduced states of the disulfide linkage and the sulfhydryl group, respectively.

One very interesting point which should be noted here is that methionine in higher plants is difficult to detect in the "free amino acid pool" or "soluble nitrogen pool" because it seems to either be absent from the "pool" or to be present in amounts too small to be detected. Thomas et al. (1950) and Steward et al. (1951) have reported this for alfalfa and Burroughs et al. (unpublished data) has observed this in barley. Steward et al. (1951) considered that methionine was never present in the "soluble nitrogen pool" or was incorporated into protein to quickly to ever be present in more than minute amounts in the free form. Kylin (1953) reported methionine in the free form in deseeded wheat plants, but his fractionation technique differed from those of Steward et al. (1951) and Burroughs et al. (Unpublished). Methionine has been reported to be protein bound by all the above cited workers.

Thomas et al. (1950a), Steward et al. (1951), Kylin (1953), and Burroughs et al. (Unpublished) have found cysteine, cystine, and/or their derivatives in both free and protein bound forms in alfalfa, wheat, and barley. Glutathione has been reported in the free form, but there is some uncertainity as to its actual presence. These workers have utilized both paper chromatography and autoradiography to separate and identify these amino acids and glutathione.

Other essential sulfur compounds reported by Thomas (1958), Young and Maw (1958), Schiff (1962), and Robinson (1963), in higher plants are as follows:

1. Coenzyme A: thought to participate in the transfer

of acyl groups to pyruvate prior to pyruvate participation in the Krebs Cycle.

2. Cocarboxylase which is the pyrophosphate of thiamine and appears to participate in the Krebs Cycle.
3. Lipoic acid or 6;8-dithioctic acid which functions in several enzyme systems and now appears to be associated with photosynthesis. According to Robinson (1963) it may play a role in photosynthesis where it has been suggested that light energy is used to reduce the disulfide form to dithiol which can transfer hydrogens to pyridine nucleotides.
4. Biotin serves as a coenzyme in several carbon dioxide fixation reactions such as the formation of oxalacetate from phosphoenol pyruvate.

In addition, there are many sulfur containing compounds reported in higher plants whose essentiality has not been established. These compounds include the glycosides sinigrin and sinalbin and their hydrolytic products; the mustard oils; vinyl and allyl isothiocyanate; and other methyl, allyl, and vinyl sulfides, disulfides and mercaptans. The essentiality of most of these sulfur compounds has not been established.

SULFUR FRACTIONS in HIGHER PLANTS: The various fractions of sulfur measured in plants give an indication of changes in sulfur metabolism of healthy or diseased plants. Since part of this study

has involved measuring sulfur fractions in plant (tobacco) tissue, a general review should be given of sulfur fractions studied by other workers. The earlier reports by workers such as Nightingale, et al. (1932); Eaton (1941, 1942, and 1951); and Ergle and Eaton (1951) indicated measurement of at least four sulfur fractions in such plant species as tomato, soybean, black mustard, sunflower, and cotton. These fractions included inorganic sulfur (mostly as sulfate-sulfur), free organic sulfur (alcohol soluble), bound organic or protein sulfur (alcohol insoluble and/or heat coaguble), and total sulfur. Thomas et al. (1944a,b) and (1950b) chose to measure total sulfur, inorganic sulfur, and organic sulfur. Organic sulfur was split into three fractions to measure what they called labile sulfur or cystine sulfur, because at that time it was believed that sulfur in organic form was largely present in cystine. Therefore organic sulfur was measured as: (1) a labile sulfur (all sulfhydryl sulfur) fraction, (2) an acid soluble fraction (thought to be methionine), and (3) an acid insoluble organic fraction (all sulfur that had been missed in the first two fractions). Thomas (1958) later showed that these fractions could be combined to form the organic sulfur fraction.

SULFATE REDUCTION: Gibbs and Schiff (1960) and Wilson (1962) in recent reviews on sulfate reduction have pointed out that the reduction of sulfate sulfur to organically bound sulfur, such as sulfhydryl sulfur, is a highly endergonic or energy-requiring process involving three or four steps. They note that one worker using yeast and others using various micro-organisms have proposed

that sulfate is activated with a nucleotide such as adenosine triphosphate (ATP). After sulfate activation the reaction may proceed through a series of steps to sulfhydryl sulfur. Wilson (1962) states that experimental evidence (using micro-organisms) supports the following pathway for sulfate reduction:

$$SO_4^{=} \xrightarrow[\text{(sulfate)}]{} SO_3^{=} \xrightarrow[\text{(sulfite)}]{} S_2O_3^{=} \xrightarrow[\text{(thiosulfate)}]{\text{serine}} \text{cysteine-SH}$$

He also states that experimental evidence for this sulfate reduction pathway is lacking in higher plants though there have been reports of sulfite and thiosulfate in higher plant tissues.

Initially, this reaction may be endergonic, but may thereafter proceed exergonically, with energy being required to trigger the reaction sequence. (Gibbs and Schiff 1960). Merritt (1962b) suggests that adenosine triphosphate may be needed as an energy source for virus multiplication, but it is not known how changes in energy relations caused by plant viruses at early periods after inoculation effect energy requiring processes as sulfate reduction in both inoculated and non-inoculated leaves (of infected plants). Owen (1955a) thinks perhaps there are no initial changes in energy requiring metabolic processes at early periods of virus infection.

THE EFFECT of SULFUR on NITROGEN METABOLISM: Sulfur has an essential role in nitrogen metabolism and the effects of sulfur deficiency on nitrogen metabolism have been studied by Nightingale et al. (1932), Eaton (1935, 1941, 1942, and 1951) and Eagle and Eaton (1951) using tomato, soybean, sunflower, black mustard and cotton. Thomas (1958) has summarized the work concerning the role of sulfur in nitrate reduction. All these workers note that when sulfur

is withheld from plants in nutrient solution, an increase in ammonia and the amino acids, amides, and water soluble nitrogen compounds can be observed. Reduction of nitrate and also protein synthesis appear to be inhibited. It has been suggested that sulfhydryl groups on the surface of the enzyme may participate in the process of nitrate reduction, but little has been shown in evidence of this. Reduction of protein synthesis in sulfur deficient plants may be due to absence of sulfur-containing amino acids needed for peptide chain constituents, but sulfhydryl groups may be involved in other processes of protein synthesis in plants.

So far as can be determined, inter-relationships between sulfur metabolism, nitrogen metabolism, and TMV establishment and multiplication in tobacco host plants have not been studied but Ling and Pound (1962) have suggested that sulfur indirectly affects TMV multiplication in tobacco, because of its direct participation in nitrate reduction. However sulfhydryl groups may also be involved in some way with virus multiplication.

II. Sulfur Nutrition in N. Tabacum in Relation to the Multiplication of TMV

To this author's knowledge the only reported study concerning sulfur nutrition in virus infected plants was conducted by Ling and Pound (1962) from the point of view of the tobacco mosaic virus multiplication rather than from the point of view of the N. tabacum host. There were no measurements made of sulfur fractions. This study was made beginning the 7th day after inoculation and continuing until the 21st day after inoculation. No studies were conducted

at early hours after inoculation, but still the implications here were interesting. Briefly Ling and Pound made these observations:

1. Generally the accumulation of TMV in plants grown without sulfur or with suboptimal sulfur was markedly and consistently less than in plants receiving optimal sulfur levels.
2. In most of their experiments only extreme sulfur deficiency markedly reduced host growth and virus synthesis. Apparently with slight or moderate deficiency there is adequate sulfur for virus synthesis. In experiments lasting several weeks, however, the virus concentration reached a maximum in 3-4 weeks and remained quite constant even though host growth steadily increased. Thus under extreme deficiency (of sulfur), the available sulfur may limit virus synthesis more than host growth.
3. They concluded that since sulfur deficiency affects nitrogen metabolism of the host, the effect on virus multiplication by sulfur was an indirect effect.
4. Finally they made some interesting studies concerning the source of sulfur supplied to the plant and hence to the virus. Separate studies were conducted supplying Na_2SO_4, $Na_2S \cdot 9H_2O$, Na_2SO_3, or $Na_2S_2O_3 \cdot 5H_2O$ to N. tabacum plants in nutrient solution. All forms of inorganic sulfur were apparently taken up by the roots, but $SO_4^=$ produced slightly more growth than

$SO_3^=$ or $S_2O_3^=$, and $Na_2S \cdot 9H_2O$ produced some toxicity and less growth than $SO_3^=$ or $S_2O_3^=$. All forms of sulfur (at optimal levels) produced good virus multiplication except $Na_2S \cdot 9H_2O$, although virus multiplication was still apparent.

Markam (1959) reported that only about 1-2% of the TMV protein contained sulfur. This would account for the fact that only complete deficiency would hinder virus multiplication in N. tabacum leaves, because the virus requires only minute amounts of sulfur for multiplication.

III. The Effect of TMV on the Respiration Rate of Nicotiana Tabacum

Since cellular respiration provides most of the energy required for metabolic processes in the green plant, it is possible that interruptions in respiration might result in interruptions in energy requiring reactions.

The work of Owen (1955a,b, 1956, 1957, and 1958) has indicated that definite changes can be observed in the rate of respiration of virus infected tobacco when the work was reported on a dry weight basis (CO_2 evolved per mg. dry weight of leaf tissue per hour). Owen (1955a,b) has shown why previous studies of virus-host respiration are in disagreement.

Using TMV inoculated on N. tabacum L. leaves Owen (1955a) studied the respiration rates during the first 20 hours after infection. The respiration rate of the inoculated leaves increased (as compared with healthy leaves) almost immediately after inocu-

lation. Owen postulated that this increase might be the result of the entrance of the virus into the cell rather than early multiplication. He did not study uninoculated leaves of the infected plant.

In a second work Owen (1955b) studied respiration rates of TMV infected N. tabacum at three weeks after inoculation on leaves showing symptons. He found that the respiration of lower infected leaves showed no changes in rate when compared to healthy leaves, but the upper infected leaves showed a decrease in respiration as compared to healthy leaves. He concluded that the changes were centered mainly in the more actively growing part of the plant.

In a third study Owen (1956) observed that:

1. The respiration of inoculated leaves was increased by a constant until three weeks after inoculation when it decreased (as compared to healthy plants).
2. The respiration rate of younger non-inoculated leaves present at the time of inoculation was not affected at any time. (As compared to healthy plants.)
3. The respiration rates of leaves formed since inoculation was decreased by 10% when they showed symptoms (as compared to healthy plants). He again postulated that the rise in the rate of respiration of inoculated leaves might be caused by entrance of TMV into the epidermal cells and also might

reflect some reaction preparatory to virus synthesis.

In a fourth study Owen (1957) inoculated N. tabacum with tobacco etch virus and observed that the rate of respiration of the inoculated leaves was almost immediately increased, but no study was conducted on non-inoculated leaves at early hours after inoculation. However non-inoculated leaves were studied at the time symptoms developed and were found to have increased respiration rates (as opposed to the TMV studies which showed decreases in respiration rates in this instance). Evidently different viruses cause different effects on respiration.

Owen (1958) also studied respiration of N. tabacum infected with Potato X virus and observed the same effects as in the 1957 study. Owen has speculated very little on energy relations which might be involved.

Merrett (1962a) studied the effects of 2,4-dinitrophenol and sodium arsenate (which have been found to inhibit energy production or oxidative phosphorylation in the Krebs Cycle) on the multiplication of tomato acuba mosaic virus in leaves of N. tabacum. He postulated that as tomato acuba mosaic virus was unable to multiply when host plant respiration was uncoupled (by 2,4-dinitrophenol) from phosphorylation, it might be that a surplus of high energy phosphate bonds was necessary for continued virus infection.

Merrett (1962b) suggested that nucleotides such as adenosine triphosphate (ATP) are utilized as energy sources for virus multiplication. He postulated that initially this would lead to an

increase in respiration to produce more ATP. Likewise he stated later that utilization of nucleotides by the virus might result in a depletion of nucleotides within the cell and a subsequent decrease in respiration due to the reduction of phosphate acceptors. Here again, no postulation was made as to how this would effect energy requiring reactions such as sulfate reduction.

MATERIALS AND METHODS

Propagation of Plants and the Hydroponic System

Three lots of Nicotiana tabacum L. var. Havana tobacco plants were grown in the greenhouse during the winter and early spring of late 1963 and the first three months of 1964. Each lot was seeded in vermiculite and watered daily with tap water. Vermiculite proved to be a satisfactory medium for starting tobacco plants. Soil and sand were also used for seeding during the first year and found less satisfactory due to soil borne diseases.

The greenhouse was maintained near 21°C and was kept as constant as possible. During the last days of the study (early spring) temperatures rose slightly above 21°C. The day length was maintained at 16 hours of light (for photoperiod) with the use of banks of fluorescent bulbs. Though most days were sunny, there were several cloudy days, and no additional light was used.

Tobacco seedlings for the first 48 hour study were 56 days old when transplanted to nutrient solution in the hydroponic system; seedlings for the second 48 hour study were 66 days old when transferred to nutrient solution; and seedlings for the 72 hour study were 47 days old when transplanted to nutrient solution. However, each lot of seedlings was similar in size to the other two lots and all lots were assumed to be at the same physiological age at the time of transfer.

A photograph of tobacco plants growing in the hydroponic system is shown in Figure 1. The plants were grown in three liter

jars immersed in a water bath in the metal tank shown, and the temperature of the water bath was approximately 21°C. The nutrient solution used was ½ Hoagland No. 1 with micro-nutrient supplement (Hoagland and Arnon 1950). The solution was changed twice during the growth of the plants and previous to inoculation. Two seedlings were grown per jar and each experiment utilized a series of twelve plants or six jars. Usually more plants were transplanted than needed so a series of twelve uniform plants could be selected for each individual study; six plants (three jars) were used for TMV inoculations and six plants were used as healthy controls.

The tobacco plants used for the first 48 hour (after inoculation) study had been growing in nutrient solution a total of 27 days when the leaves were collected for analysis; plants of the second 48 hour study (repeat of the first experiment) had been growing in nutrient solution for 33 days when the leaves were harvested for analysis; and the plants for the 72 hour (after inoculation) study had been in solution a total of 34 days when the leaves were harvested for analysis. Again the physiological ages among individual groups of tobacco plants were similar at the time of harvest.

Inoculation Procedures

To obtain infected N. tabacum L. plants the virus must first gain entrance to the host plant through damaged epidermal cells or leaf hairs. Thus it was necessary to inoculate by mechanically damaging the leaves to aid entrance of virus particles into the

Fig. 1. Nicotiana tabacum var. Havana plants growing in the hydroponic system.

leaves.

The source of TMV was charcoal clarified TMV infected plant sap obtained from Dr. Webster H. Sill of the Botany Department, Kansas State University. The infectivity of the TMV source was checked utilizing the Latin Square technique with N. glutinosa. Infectivity of the source was found to be fairly good. It was found later that the source had lost some of its infectivity, but the reason for this was unknown.

The 9th, 10th, and 11th leaves below the apical portion of each test plant were sprinkled lightly with carborundum abrasive (six plants in all), and rubbed lightly with a cotton swab which had been dipped in the concentrated TMV source. The carborundum abrasive insured rupture of epidermal cells and epidermal hairs providing passage for virus particle into the leaf. The inoculated leaves were lightly sprinkled with distilled water after each rubbing to remove carborundum.

Since this study dealt only with uninoculated leaves at 48 hours and 72 hours after inoculation with TMV, the inoculated leaves were left intact on the plants at the time of harvest. This made it possible to positively check on the success of the inoculation. In the next 3 to 5 days after the removal of the apical portions and leaves from the test plants, new leaves were formed from axillary buds present at various harvested nodes. After 14-15 days the characteristic systemic symptoms of TMV infection could be observed on newly emerged upper leaves, but symptoms were never visible on inoculated leaves. The inoculations in each of the three

studies were successful. It is not known why symptoms took so long to develop, but this may have been due to some initial loss of infectivity of the virus source. Symptoms of TMV on N. tabacum have been observed as early as 5 to 6 days after inoculation.

Methods of Harvesting and Handling the Plant Material Previous to Analysis

In the first year some radioactive uptake studies were conducted which were not successful mainly due to the method of harvesting the plant material. It was found that it was unsatisfactory to collect single leaves from individual plants, because these leaves did not provide enough plant material for satisfactory analysis and averages were difficult to obtain. Therefore an improved method of harvest was devised.

This method entailed the pooling of leaves from corresponding nodes of physiologically similar plants. The method was as follows:

1. Apical portions from the six infected plants were harvested and pooled to make one sample. The same was done with the apical portions of the corresponding control plants. Leaves from the first node below the apex of the six infected plants were harvested and pooled to make one sample, and the same was done for the leaves of the first node of the control plants. This procedure was followed for the next four nodes down to and including the fifth node below the apex.
2. For the two 48 hour studies all samples were brought

into the laboratory for analysis. Each pooled sample of fresh tissue was divided into two parts: one part for quantitative analysis and the other part for extraction of organic sulfur compounds. The part used for sulfur determinations was placed in the oven to dry, at 90-100°C.

3. The 72 hour study was only made to determine sulfur fractions.

Thus this method of sample pooling produced an average for the apex and various nodes collected; it also provided adequate plant material for several analyses.

Quantitative Estimation of Sulfur Fractions

The method of Johnson and Nishita (1952) for the microestimation of sulfur fractions in biological material was selected for use in this study. It required only small samples for analysis and was quite rapid in comparison to other methods for quantitative estimation of sulfur in biological materials. Chemically, the principle of this technique is as follows: Inorganic forms of sulfur are reduced to hydrogen sulfide gas by a reducing agent in a closed, reducing atmosphere of nitrogen gas. The inflow of nitrogen gas through the reducing flask drives the evolved H_2S gas into a gas absorbing solution, and after the reaction is complete the color is developed as methylene blue and is determined spectro-photometrically. The readings are then compared to a previously prepared

standard curve, and the measurement can be determined directly in micrograms (mg) of sulfur.

The apparatus used was identical to that suggested by Johnson and Nishita (1952) in their description of this technique, and is diagrammed in Figure 2. The inflow of nitrogen gas (A) maintained a reducing atmosphere in the reducing flask (B). As nitrogen gas was bubbled through the hot sample-reducing agent mixture (C), it carried evolved H_2S gas up through the water-jacket condensor (D), over through the U connecting tube (E), and into the gas washing column (F) where it was bubbled through the gas wash mixture (sodium dihydrogen phosphate and pyrogallol). Then, after the H_2S gas was bubbled out of the gas wash mixture (driven by N_2 gas) it was forced through the delivery arm (G), and into the absorbing solution (zinc acetate and sodium acetate) in a 100 ml volumetric receiving flask (H). After the reaction was complete the color was developed utilizing p-amino dimethyl-aniline and ferric ammonium sulfate to form methylene blue; the color was then allowed to stabilize. After the color had stabilized it was read on a suitable colorimeter at 670 mu.

For this study a Bauch and Lamb Spectronic 20 colorimeter was utilized, and the color was read in ½ inch I. D., perfectly round cuvettes [manufactured by Biological Research, Inc. of St. Louis, Missouri (No. 33015)]. A standard curve was prepared using various micro-amounts of sulfur in the form of potassium sulfate. The curve was plotted in per cent transmittance versus micrograms of sulfur in 100 ml of methylene blue solution and deionized-distilled

Explanation of Fig. 2.

(A.) Nitrogen input tube.

(B.) Reducing flask.

(C.) Reducing mixture.

(D.) Water condenser.

(E.) U connecting tube.

(F.) Gas washing column

(G.) Gas delivery arm.

(H.) Receiving flask.

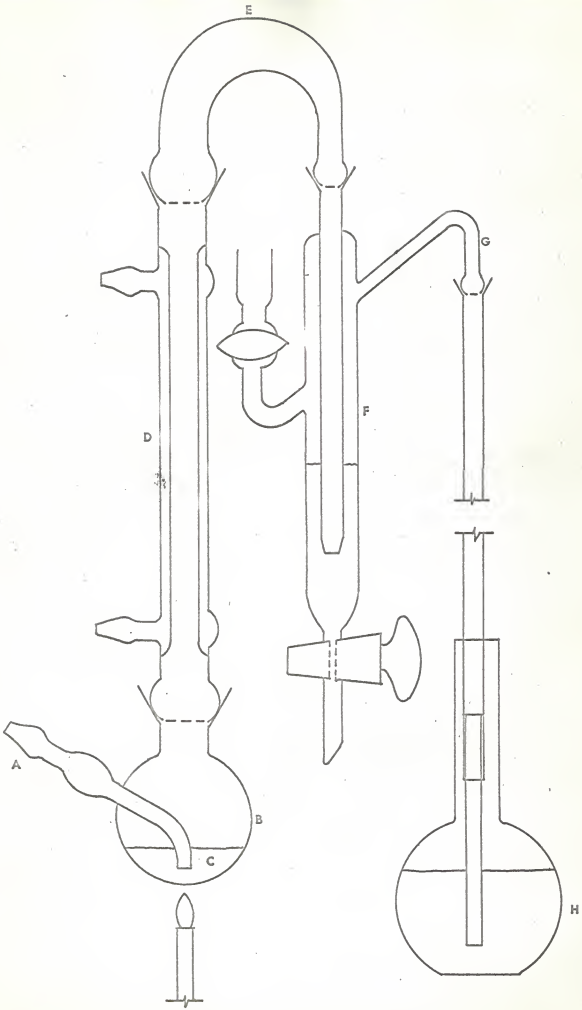

Fig. 2. Sulfur analysis apparatus.

water was used as the blank, thus the curve did not cross the % transmission axis at 100% transmission. The standard curve follows Beer's Law only in a range of from 10 to 35 mg/100 ml. This was thought to be due to the characteristics of this colorimeter.

REDUCING MIXTURE PREPARATION APPARATUS: Johnson and Nishita (1952) have made no specifications concerning the type of apparatus used for the preparation of the reducing mixture. Since this mixture of formic acid, hydridic acid, and red phosphorous is corrosive it was necessary to prepare it in a pyrex glass apparatus. The author designed this apparatus which was constructed by Mr. M. Ohno, glass-blower for the Kansas State University Physics Department. It is diagrammed in Figure 3. (A) is a ground glass stopper with a thermometer well (B) fused into it; (C) is the nitrogen gas input (since the reducing mixture must be prepared in a reducing atmosphere of nitrogen gas); and (D) is the exhaust outlet where generated gas and N_2 gas may escape during the boiling process. The thermometer bulb is always immersed in mercury in the thermometer well (B) to provide better contact between the glass walls of the well and the external solution preparation.

THE FRACTIONS MEASURED: Sulfur must be in an inorganic form, preferably $SO_4^=$, to be analyzed with this technique. Sulfur in reduced forms, such as cysteine sulfur and methionine sulfur, was not attacked by the reducing mixture and could therefore not be converted to hydrogen sulfide gas, hence sulfur in organic forms had to be oxidized before quantitative estimations could be obtained. It was therefore possible to gain the quantitative estimation of inorganic

Explanation of Fig. 3.

(A.) Ground glass stopper

(B.) Thermometer well.

(C.) Nitrogen gas input tube.

(D.) Gas exhaust tube.

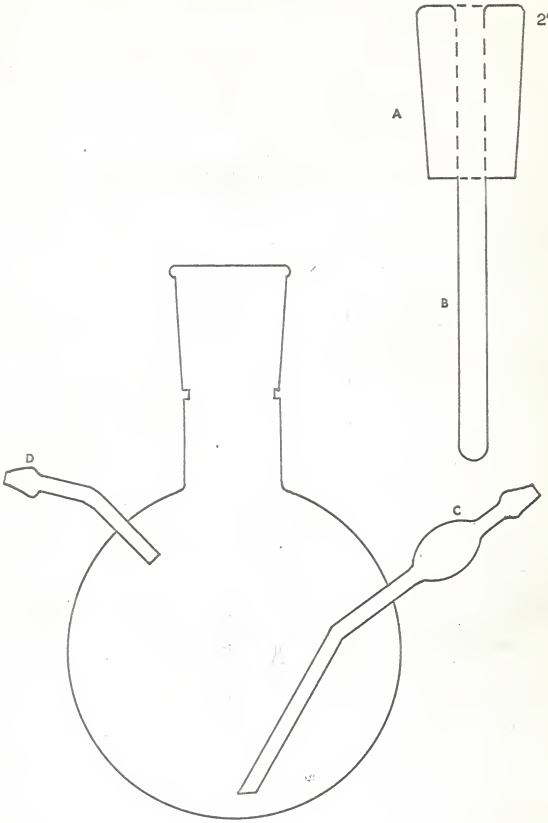

Fig. 3. 500 ml reducing mixture preparation flask.

sulfur directly from a given amount of dry plant material. This could be done without fear of error caused from organic sulfur. Twenty-five mg portions of ground, dry plant material were used for the determination of inorganic sulfur, and three repetitions were run for each sample.

Johnson and Nishita (1952) seemed to indicate that only sulfate sulfur was measured, but Chapman and Pratt (1961) in their report of this technique indicated that forms of inorganic sulfur such as $SO_3^=$ and $S_2O_3^=$ were also attacked and reduced to H_2S gas. This author has reduced quantitate amounts of standard $S_2O_3^=$, but has not had completely successful recovery. This was attributed to the age of the reducing mixture, and there has not been time for further study of this, but it will be assumed that the inorganic fraction of sulfur (including $SO_4^=$, $SO_3^=$, and $S_2O_3^=$) have been measured. Sulfite and thiosulfate sulfur, however, would represent a very small fraction of the inorganic sulfur in comparison to sulfate sulfur.

Total sulfur was obtained in the following manner: A given amount of plant material was first subjected to the nitric acid, perchloric acid, and hydrochloric acid wet ashing technique described by Johnson and Nishita (1952). This converted all forms of sulfur to sulfate sulfur, and volumes were regulated to obtain proper dilutions. The total sulfur was then estimated in the previously described manner; 100 mg portions were digested, and 100 mg of plant tissue was represented in a final volume of 50 ml of deionized-distilled water. One ml aliquots were used for analyses,

and 3 repetitions were run for each sample.

Sulfur 35 Methods

At the time of inoculation 75 micro curies of radioactive sulfur in the form of $S^{35}O_4^=$ were added to the nutrient solution in each jar containing tobacco plants. Thus plants harvested at 48 hours and 72 hours after inoculation had taken up and metabolized $S^{35}O_4^=$ for 48 hours and 72 hours respectively. The original source of radio sulfur used in this study was Oak Ridge Laboratory at Oak Ridge, Tennessee.

Samples were counted for activity using Nuclear-Chicago Geiger-Muller Detector tubes (type D-34, 1.4 mg/cm^2) with Berkley Decimal Scalers (models 100 and 2105). Samples from 48 hour and 72 hour studies were dried, ground in a Wiley-Mill using a 30 mesh screen, and 25 mg portions of each sample were counted to determine total S^{35} activity at each node.

PREPARATION OF EXTRACTS AND HYDROLYSATES FOR 48 HOUR STUDIES: Only the 48 hour studies were investigated for bound and labeled S-amino acids. The method of Burroughs *et al.* (1964) was used for extractions. Immediately after harvest 5 gram or 10 gram samples (fresh wt) of leaves were placed in large test tubes which were immersed in a dry ice-ethanol bath at -20°C or lower. The leaves were frozen and thoroughly ground in a cold mortar. Ten ml of 80% methanol was added, the mortar covered and left over-night in the refrigerator. Duplicate samples from infected and control plants were prepared.

The extracts were filtered using suction, and the residue washed with a small volume of 80% methanol. The volume of methanol extract was reduced under vacuum, and the resulting residue removed by centrifugation. The concentrated solution was then diluted to 1 ml with 0.1% isopropanol. The final concentration was 5 grams fresh wt/ml.

The residue from methanol extraction was transferred to a 50 ml beaker; 15 ml of 6N HCl was added and the material was hydrolyzed in the autoclave for 6 hours. The resulting mixture was filtered, the residue washed, and the filtrate evaporated to dryness. The residue was resuspended in 2.5 ml 10% isopropanol, and any precipitate removed by centrifugation. The dark color of the hydrolyzates appeared to have no effect on subsequent chromatography. Concentrations were adjusted to 2 gm fresh wt/ml.

CHROMATOGRAPHY AND AUTORADIOGRAPHY: The concentrated samples (methanol extracts and hydrolysates) are spotted on Whatman #1 filter paper in 25 ul amounts and chromatographed one dimensionally using two excursions of butanol, acetic acid, and water (25:6:25-V:V:V). The papers were dried thoroughly (4-6 hours) in the hood after each excursion. Dried chromatograms were sprayed with 0.25% ninhydrin in acetone and placed in the hood saturated with moist air at 30°C for 2-3 hours to develop the color reaction. Duplicate, unsprayed chromatograms were autoradiographed on Blue Brand X-Ray film (Kodak). Exposure was 7-10 days with methanol extracts and 3 weeks for hydrolysates. S^{35} labeled spots containing S-amino acids were cut from the chromatograms and the activity was determined.

EXPERIMENTAL RESULTS AND DISCUSSION

Quantitative Determinations of Sulfur Fractions

The data of the quantitative determinations of sulfur fractions in leaves of healthy and TMV infected plants for the first 48 hour study, the second 48 hour study, and the 72 hour study may be found in the Appendix in Tables 1, 2, and 3, respectively. The results for total sulfur and inorganic sulfur fractions at the apex and lower nodes are graphically represented in Figures 4, 5, and 6. It should be noted that sulfur fractions are expressed as micrograms of sulfur per milligram of dry leaf tissue, and organic sulfur is derived by substracting inorganic sulfur from total sulfur. Each sulfur fraction of each node represents a pool of six corresponding leaves from six healthy plants or six infected plants. Total sulfur was also reported as percent of dry weight in Tables 1, 2, and 3. It can be observed that the total sulfur varies from 0.45-1.05% of the dry weight, and is comparable to other species studied by Thomas, Hendricks, and Hill (1950b). Organic sulfur may exceed inorganic sulfur by factor of 1.5 or more. This factor can be less than one, but may be greater than 2. Such variations may be observed in Table 4, and these values compare to values calculated from summary data presented by Thomas (1958).

It was the main purpose of this part of the study to make observations of inorganic sulfur in relation to total sulfur in healthy and noninoculated, TMV infected leaves at corresponding nodes. The fraction of sulfur present as inorganic sulfur was

calculated by dividing inorganic sulfur by total sulfur, and these values for each node of healthy and infected plants may be found in Tables 1, 2, and 3. In order to compare the fraction of sulfur present as inorganic sulfur in corresponding leaves at nodes of healthy and infected plants it was necessary to calculate an Infected to Healthy ratio between the fractions of inorganic sulfur. Such a ratio has been used by Owen (1955a, 1955b, 1956, 1957, 1958) in comparing respiration rates of infected versus healthy TMV infected tobacco leaves. An increase in such a ratio, above one, might indicate an accumulation of inorganic sulfur and a decrease in sulfate reduction in infected leaves compared with healthy corresponding leaves. A decrease in the ratio, below one, might indicate a decrease in inorganic sulfur and an increase in sulfate reduction in infected leaves as compared with healthy leaves. These ratios are reported in summary in Table 8, and appear graphically in Figure 7.

The first 48 hour study represented in Figure 4 suggest a higher concentration of both total sulfur and inorganic sulfur of infected plants in comparison to healthy plants. Note the greater concentration of total sulfur of both healthy and infected plants at the fifth node. Evidently the total sulfur concentration is higher in the lower more well developed leaves of healthy and infected plants. The plants used in this study were harvested at 3 p.m., while the plants used in the second 48 hour study represented by Figure 5 were harvested at 9:00 a.m. These studies were equivalent in all other factors. Note the lower concentrations of

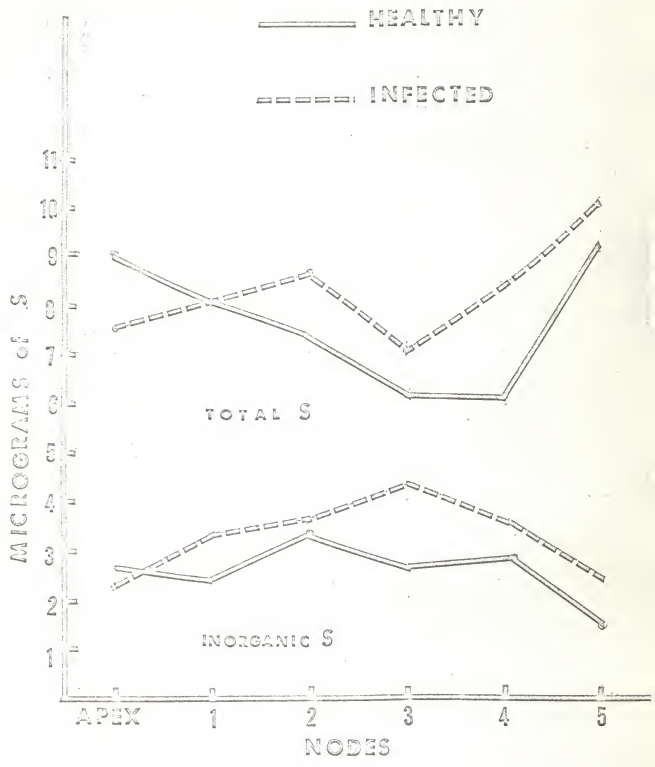

Fig. 4. Total sulfur and inorganic sulfur per milligram dry weight for the first 48 hour study.

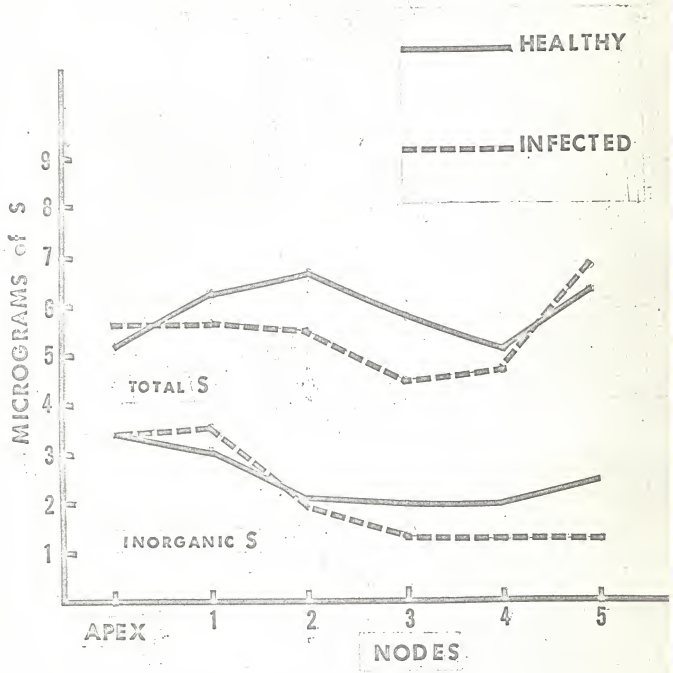

Fig. 5. Total sulfur and inorganic sulfur per milligram dry weight for the second 48 hour study.

both total and inorganic sulfur in the infected series, but the lowest node again shows greater total sulfur in both healthy and infected plants.

The 72 hour study represented by Figure 6 was harvested at 3:00 p.m. This study seems to indicate little change in inorganic sulfur between the healthy and infected, and total sulfur shows variations. Total sulfur was equivalent at the apicies for healthy and infected; lower at the first node for infected, higher at the second and third nodes for infected, and lower again at the fourth and fifth nodes. There was a greater amount of total sulfur for both healthy and infected at the fourth node this time, but it dropped at the fifth node instead of raising.

The results of the I/H ratio of inorganic sulfur can be observed in Figure 7. The I/H ratios per node for the 48 hour studies showed very little agreement between studies even though these studies were nearly equivalent aside from the time of day of harvest. The 72 hour study indicates a great deal of variation from node to node in the I/H ratio.

No conclusions can be drawn from these studies, because it is difficult to know just how the virus particles are affecting metabolism in these leaves. It is assumed that by 48 hours and 72 hours virus particles have moved into every leaf of the plant, and have begun the processes of multiplication, but this is an assumption. Perhaps the results of the quantitative study really suggest variations in sulfur metabolism between normal plants, because it is not known how much virus has accumulated in these uninoculated

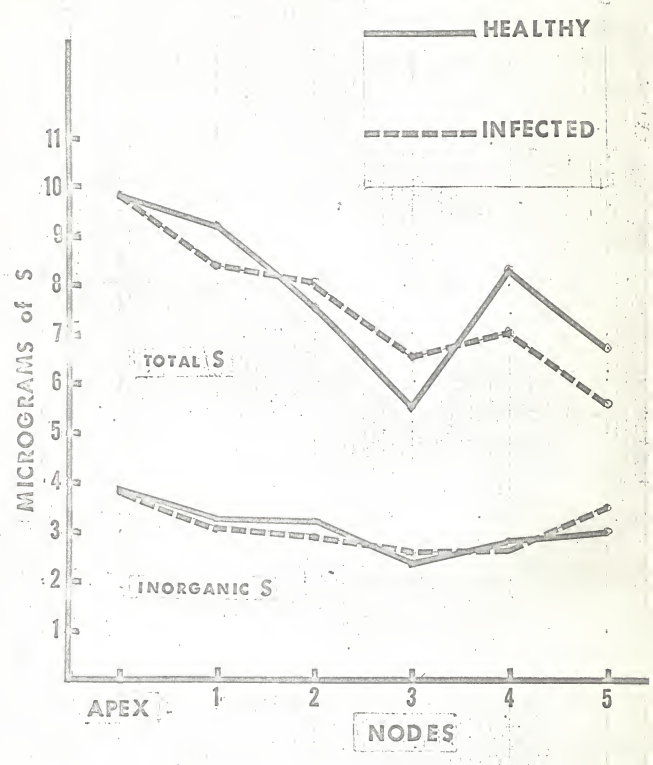

Fig. 6. Total sulfur and inorganic sulfur per milligram dry weight for the 72 hour study.

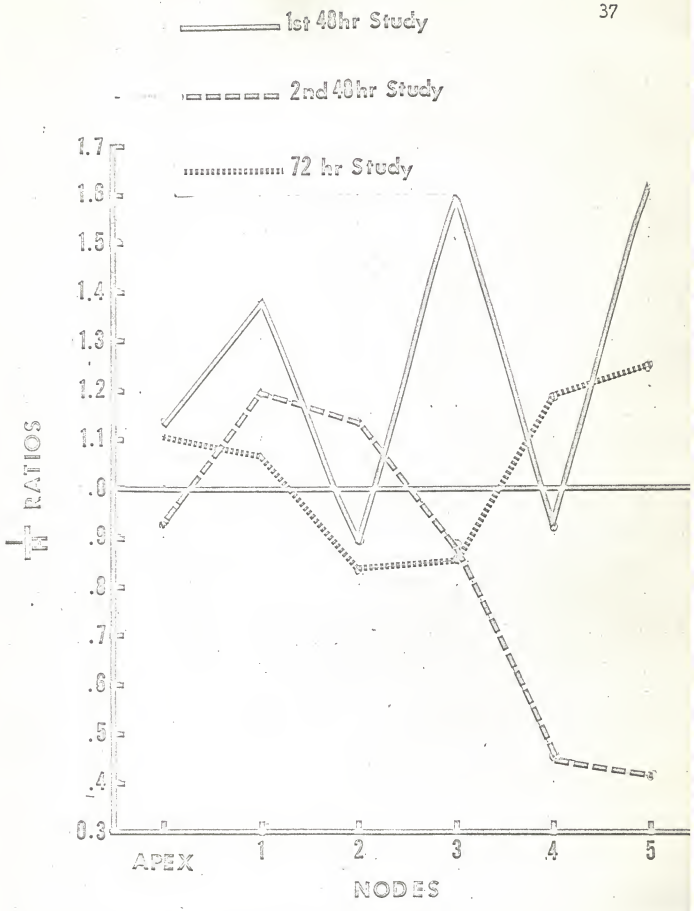

Fig. 7. Infected: Healthy ratios of inorganic sulfur: total sulfur ratios at the apex and the next five lower leaves.

leaves.

The quantitative data agree at least with sulfur fractions measured in other species by other workers such as Nightingale et al. (1932), Eaton (1935, 1941, 1942, and 1951) and Thomas et al. (1950b). Since no other reports of studies dealing with sulfur metabolism accompanying TMV infection except that of Ling and Pound (1962) appear to be available it is difficult to assess the validity of this data; however future investigators will have this work as a comparison. Unfortunately it requires a great deal of time and work to measure sulfur fractions so that one can be certain of results. It may be necessary that further research will require constant environment growth chambers and statistical analysis.

Additional studies should be made at other periods after inoculation starting at 8 hours after inoculation and continuing at 8 hour intervals for as long as 4 to 5 days.

Also studies should be conducted concerning TMV inoculated leaves for which Owen (1955a,b, and 1956) has observed respiration changes. Owen (1956) has found no changes in respiration rates of uninoculated leaves, at an early period after inoculation and this would perhaps indicate no changes in metabolism.

Uptake and Accumulation of Sulfur 35

The results of the uptake studies may be found in the Appendix in Tables 5, 6, and 7 (48 hours, 2nd 48 hours, and 72 hours respectively); and are graphically represented in Figures 8, 9, and 10 respectively. Twenty-five mg samples (dry) were counted to

determine total S^{35} activity, and each sample represented a pool of six corresponding leaves from corresponding nodes of healthy or infected plants.

Figure 8 indicated that S^{35} accumulated per node was greater in the infected than in the healthy plants; and that in both the healthy and infected series S^{35} accumulation was greater in the more actively growing sites such as the apex, thus a gradation of S^{35} accumulation could be observed at each lower node. Figure 9 indicated the reverse situation of Figure 8; in this second 48 hour study the accumulation was greater at each node of the healthy plants in this series as compared with infected plants. There was again a gradation of accumulated sulfur at each descending node indicating that S^{35} was accumulated and metabolized at more actively growing sites in both healthy and infected plants.

The 72 hour study favors the first 48 hour study as Figure 10 indicated; the plants of this study were harvested at 3:00 p.m. as was the first 48 hour study. The greater amounts of S^{35} were found accumulated in the infected nodes as compared to the healthy nodes. Again the accumulation of S^{35} could be observed in greater amounts at the apex and younger leaves in both healthy and infected plants, and the usual gradation was evident.

An I/H ratio of total S^{35} accumulation was computed for all three studies, and the results recorded in Tables 5, 6, and 7 and summarized I/H ratios for all study are found in Table 9. These results of I/H ratios per node are recorded graphically in Figure 11, and show a great deal of variation between each study. The

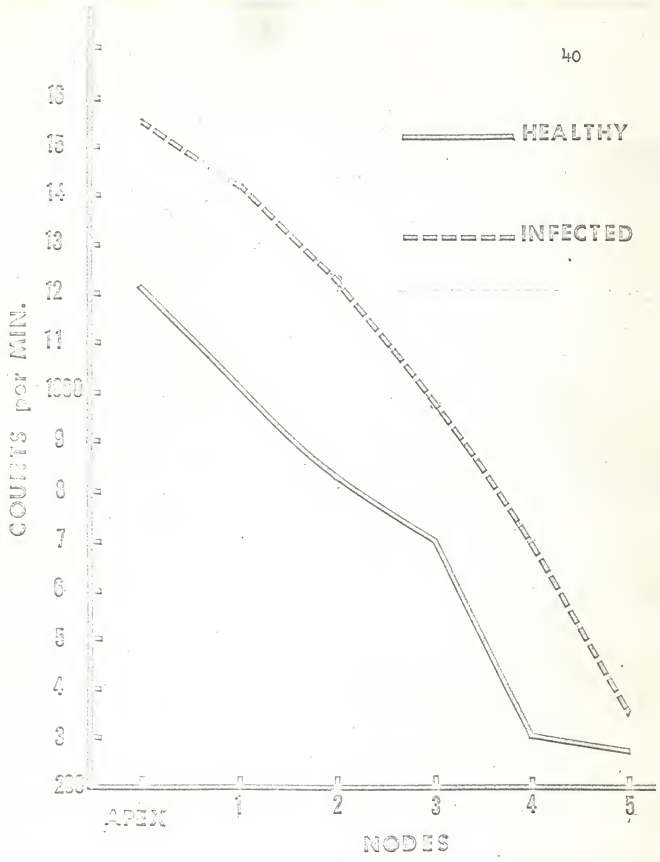

Fig. 8. Accumulation of total sulfur 35 per 25 milligrams dry weight during the first 48 hour study.

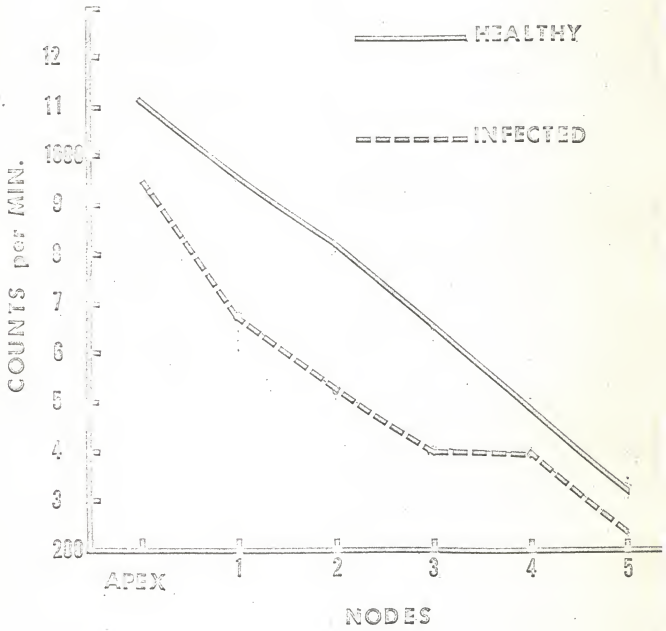

Fig. 9. Accumulation of total sulfur 35 per 25 milligrams dry weight during the second 48 hour study.

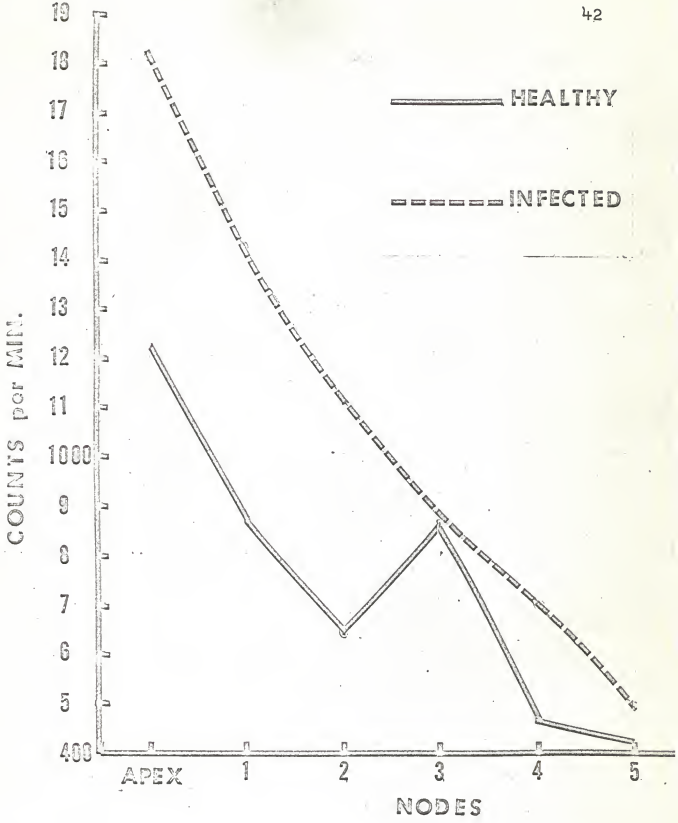

Fig. 10. Accumulation of total sulfur 35 per 25 milligrams dry weight during the 72 hour study.

I/H ratios of S^{35} accumulation were greater than one at all nodes in the first 48 hour study, therefore this indicated an increase in uptake in infected plants as compared to healthy. The second 48 hour study indicated the reverse situation, because all I/H values are less than one. The 72 hour study indicated a peculiar lowering of the I/H ratio toward lower nodes.

Burroughs et al. (Unpublished) have observed an increase in phosphate ($P^{32}O_4^{=}$) ion accumulation in brome mosaic virus infected barley leaves (as compared to healthy) at 48 hours after inoculation and a slight decrease in ($P^{32}O_4^{=}$) phosphate ion uptake at 72 hours. These results agree with this author's first 48 hour study and somewhat with the 72 hour study, but there have been no reports found in the literature concerning $S^{35}O_4^{=}$ accumulation in TMV infected plants. To be sure sulfur 35 did accumulate at more actively growing sites (such as apical portions) than at more mature sites (such as well developed leaves) of both healthy and TMV infected tobacco. Further replications at varying periods after inoculation must be carried out before a true and complete picture of S^{35} accumulation can be drawn. This author has shown that there may be some relationships which are detectable in $S^{35}O_4^{=}$ ion uptake and TMV multiplication.

One other note should be made concerning S^{35} uptake and accumulation in general. As previously stated there is a greater accumulation of S^{35} at more actively growing and metabolizing sites. Harrison et al. (1944) demonstrated this using autoradiography of whole plants. Figures 1, 2, and 3 indicate that the total sulfur

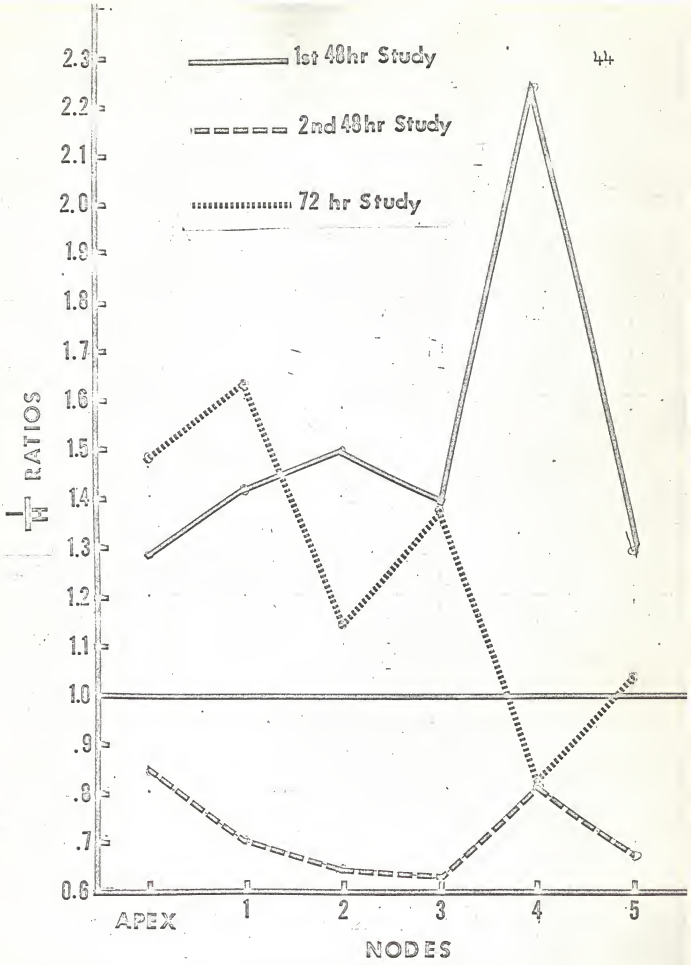

Fig. 11. Infected: Healthy ratios for total sulfur 35 accumulation at the apex and the next five lower leaves.

present may not always be in the greater amounts in the younger parts of the plant. There are more cells per square unit in actively growing areas than in more mature areas, yet the cells in the more mature areas are much larger and may have more sulfur.

Paper Chromatography and Autoradiography

Free Sulfur 35 Labeled Amino Acids.

The purpose of this part of the study was to isolate free sulfur containing amino acids such as cysteine and/or the tripepide glutathione from healthy and TMV infected leaves at 48 hours after inoculation. Much difficulty was encountered in extracting "free" sulfur amino acids, because they were present in tobacco leaves in very minute quantities. Many attempted extractions failed to show S^{35} labeled amino acids (which had been synthesized in leaves of tobacco plants fed with $S^{35}O_4^{=}$) present in the free form, because of problems in volume reduction and mishandling of purification technique.

The technique that was finally used was developed by Rosemary Burroughs in the laboratories where this author conducted his studies. This technique utilized very small volumes of 80% methanol, and volume reduction was done under vacuum. It was an extremely successful and efficient technique for extraction of amino acids which were present in minute quantities. As in other studies conducted, pools of leaves from corresponding nodes of healthy and infected plants were used for analysis.

The results of chromatography and autoradiography for methanol

extracts are reported in the Appendix in Table 10A, and Figures 12 and 13 are drawings of typical autoradiograms from methanol extracts. Figure 13 was a typical autoradiogram of a healthy series from methanol extracts, and the standard sulfur amino acids (developed with ninhydrin) indicated the location of cysteine, cystine, glutathione (reduced) and methionine in relation to one another on a typical chromatogram. Spots indentified by autoradiography corresponded to ninhydrin positive areas from the extracts. Figure 13 was a typical autoradiogram of an infected series from both 48 hour studies, and it could be seen that healthy leaf lots and infected leaf lots were identical to each other.

The apices apparently contained only cysteine in the free form, while the leaves of all other nodes appeared to contain a combination of cysteine and reduced glutathione. The concentration of S^{35} labeled cysteine was greatest at the apical portions and 1st nodes, while it became progressively less toward the lower nodes. $S^{35}O_4^=$ was present at or near the origin in varying amounts, and also tended to grade off from the apical portions toward lower nodes.

The results of paper chromatography and autoradiography are reported in the Appendix in Table 10A. Apparently there was no disappearance of S^{35} labeled sulfur amino acids in the "free form" in infected (Uninoculated) leaves, and apparently no new "free" S^{35} labeled sulfur amino acids appeared in these 48 hour studies. Methionine was absent from all methanol extracts of healthy and infected leaves.

Steward et al. (1951) found S^{35} labeled cysteine in alcohol

Explanation of Fig. 12.

Ninhydrin developed standards are placed next to the autoradiograms for indentification, and arrows indicate identification of unknowns.

Key: (A.) Glutathione (presence in extracts questionable; (B.) Cystine (absent); (C.) Cysteine (could have been a derivative in the extract); (D.) Methionine (absent); (E.) $S^{35}O_4^=$ (it would seem likely that $S^{35}O_4^=$ was present in the extracts).

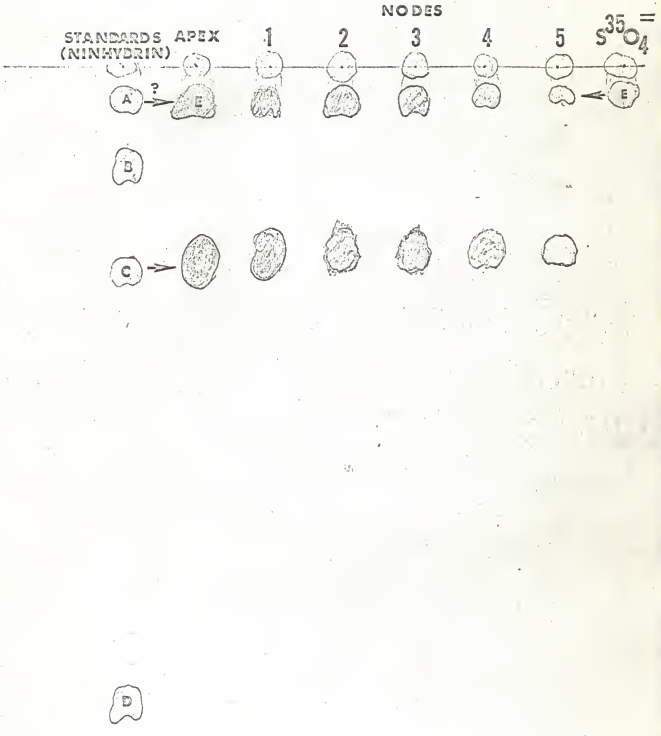

Fig. 12. A diagram of a typical autoradiogram from one dimensional chromatograms for methanol extracts from the first and second 48 hour healthy studies.

Explanation of Fig. 13.

Ninhydrin developed standards are placed next to the autoradiograms for indentification, and arrows indicate identification of unknowns.

Key: (A.) Glutathione (presence in extracts questionable; (B.) Cystine (absent); (C.) Cysteine (could have been a derivative in the extract); (D.) Methionine (absent); (E.) $S^{35}O_4^=$ (it would seem likely that $S^{35}O_4^=$ was present in the extracts).

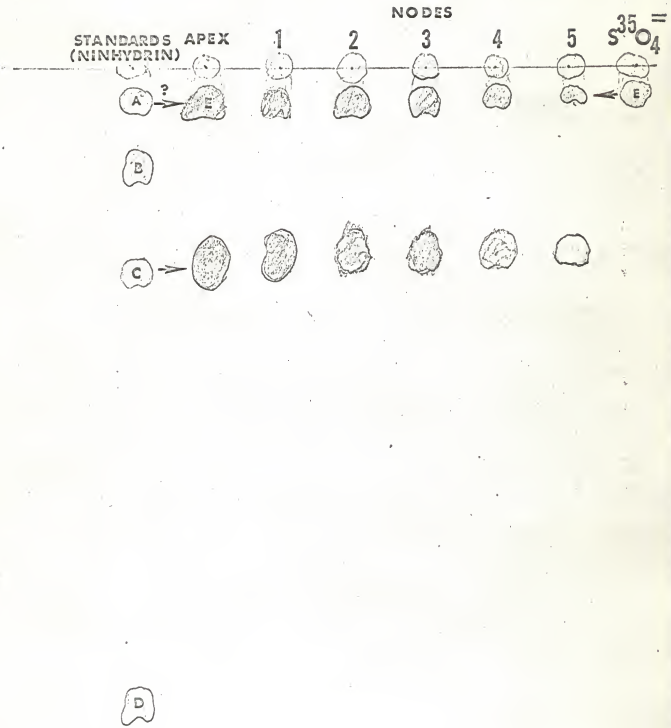

Fig. 13. A diagram of a typical autoradiogram from one dimensional chromatograms for methanol extracts from the 48 hour infected studies.

extracts from alfalfa leaves taken from plants which had been fed $S^{35}O_4^=$, Thomas et al. (1950a) found S^{35} labeled glutathione in methanol extracts of barley leaves from plants which had been fed $S^{35}O_4^=$.

Burroughs et al. (1964) found S^{35} labeled cysteine and/or its derivatives in methanol extracts of $S^{35}O_4^=$ fed barley. Commoner et al. (1953) failed to find cysteine in free form in Nicotiana tabacum var White Burley, but this group had not attempted to use $S^{35}O_4^=$ in their studies, and they were not studying sulfur metabolism. The above workers had all noted the absence of methionine in alcohol extracts, but Kylin (1953) reported methionine in his alcohol extracts from wheat seedling.

Evidently methionine was in extremely small concentration in the free form or entirely absent in the tobacco plants studied here. As Steward et al. (1951) had suggested, perhaps methionine in plant tissue is synthesized directly from cysteine and rapidly incorporated into protein and never present in the soluble nitrogen pool.

No literature has been found by this author which would implicate cysteine or glutathione in virus multiplication, except the report that cysteine is needed by TMV protein in small amounts as peptide chain units (Markam 1959). Perhaps the sulfur amino acids or other sulfur compounds may be affected in some undetectable way by virus multiplication.

Other Studies.

It was decided that the second 48 hour study should be extended beyond the identification of the free, methanol soluble, S^{35}

labeled amino acids. Hydrolysis of residues from the methanol extracts followed by utilization of paper chromatography and autoradiography produced autoradiograms of S^{35} labeled sulfur amino acids from leaf protein. The summary of this chromatographic and autoradiographic study can be found in Table 10B in the Appendix. Figures 14 and 15 are diagramed examples of autoradiograms from healthy series hydrolyzates, and infected series hydrolyzates, respectively, $S^{35}O_4^=$ was present in some hydrolyzates due to incomplete washing of residues during methanol extraction.

It was assumed that the sulfur amino acids indentified in this study were those present due to the results of protein hydrolysis.

It appeared that S^{35} labeled cystine, cysteic acid, and cysteine as well as methionine were present in this second 48 hour study, but it was observed that cysteic acid and cysteine were lacking in the leaves of some lower nodes represented in the infected series. It is known that acid hydrolysis may cause the oxidation of cysteine to cysteic acid or cystine, and some complete destruction of cysteine could also occur. Perhaps hydrolysis had a greater effect on this infected series causing more oxidation of cysteine to cystine, because more cystine is present in the infected series as was shown by counting spots (this will be discussed later). It would seem unlikely that virus biosynthesis at such early hours after inoculation would cause any changes in leaf protein. Oxidation products shown by autoradiography might be due to some breakdown of methionine. Ninhydrin positive areas on paper chromatograms indicated that unknown spots on autoradiograms were amino

Explanation of Fig. 14.

Ninhydrin developed standards are placed next to the autoradiograms for indentification, and arrows indicate identification of unknowns.

Key: (A.) Cystine (present); (B.) Cysteic acid (present); (C.) Cysteine (present); (D.) Methionine sulfoxide (probably absent); (E.) Methionine sulfone (probably absent); (F.) Methionine (present); (G) $S^{35}O_4^=$ (probably present due to incomplete washing of residue from methanol extracts); (H.) unidentified (probably oxidation products).

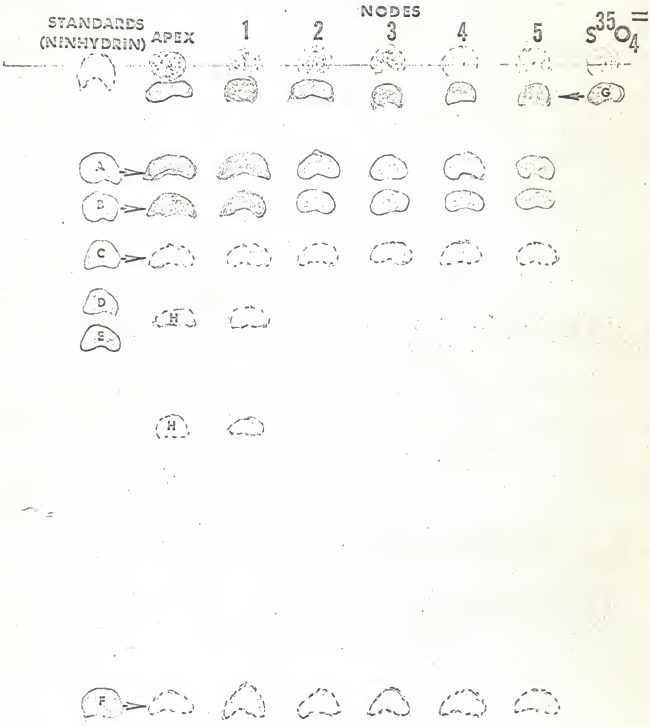

Fig. 14. A diagram of a typical autoradiogram from one dimensional chromatograms for hydrolyzates from the second 48 hour healthy study.

Explanation of Fig. 15.

Ninhydrin developed standards are placed next to the autoradiograms for identification, and arrows indicate identification of unknowns.

Key: (A.) Cystine (present); (B.) Cysteic acid (absent in some nodes); (C.) Cysteine (absent in some nodes); (D.) Methionine sulfoxide (probably absent); (E.) Methionine sulfone (probably absent); (F.) Methionine (present); (G.) $S^{35}O_4^=$ (some traces evident); (H.) unidentified (probably oxidation products).

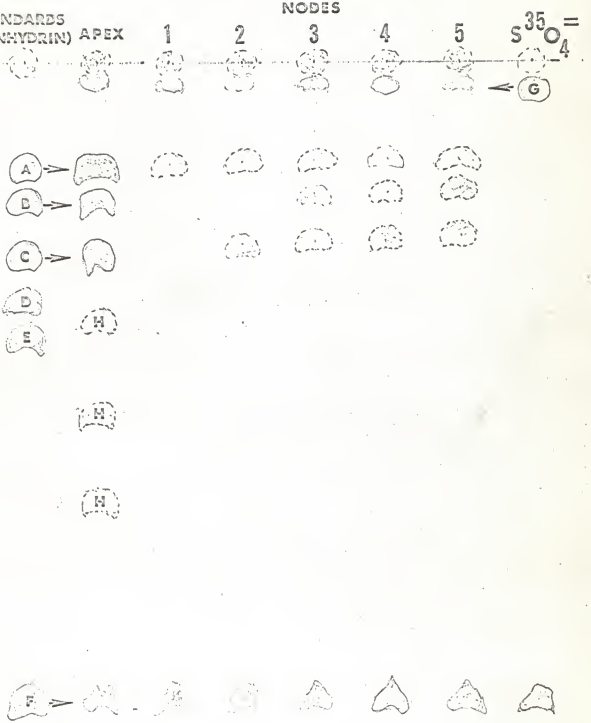

Fig. 15. A diagram of a typical autoradiogram from one dimensional chromatograms for hydrolyzates from the second 48 hour infected study.

acids, and all other sulfur amino acids identified by autoradiography corresponded to ninhydrin positive areas.

Methionine was present in all hydrolysates from healthy and infected leaves. Steward et al. (1951), as well as other workers have observed all of the amino acids containing sulfur, including methionine, in hydrolysates from various plant species.

Comparative quantitative determination of S^{35} labeled amino acids from methanol extracts and hydrolysates of the second 48 hour study.

Visual determination of amino acids, such as cysteine, by use of autoradiograms gave only qualitative results. Quantitative determinations were made by removing spots which had been shown to be radio active by autoradiography, and counting these spots to determine how much labeled sulfur was present. Only the second 48 hour series was used.

The results can be found in Tables 11A and B in the Appendix. Counts per minute for $S^{35}O_4^=$ areas for both methanol extracts and hydrolyzates were reported in Table 11A, because $S^{35}O_4^=$ represented part of the soluble sulfur fraction. The percent S^{35} labeled in cysteine (and/or its derivative) was calculated for infected and healthy methanol extract groups, and reported in Table 11A, and in Figure 16; where the infected series were compared graphically to the healthy series. Except at the apical portion, the healthy series demonstrated a greater percentage of S^{35} incorporation in the free cysteine of all lower leaves. This would indicate that there was a slower rate of sulfate reduction in infected lower leaves at 48 hours after inoculation (as compared with healthy).

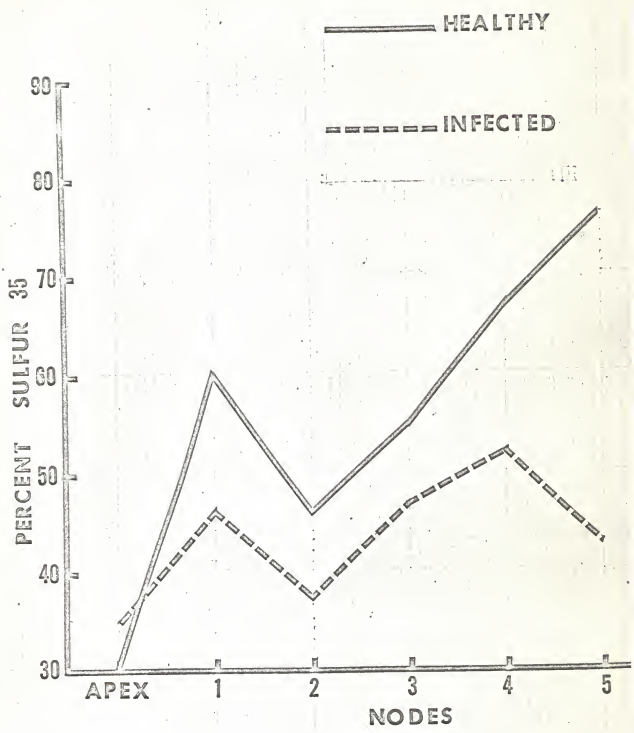

Fig. 16. Percent S^{35} incorporated in cysteine and/or its derivative from methanol extract chromatograms for the second 48 hour study.

Further pursuits of this approach may yield clearer information in respect to the effect of virus biosynthesis on host sulfur metabolism.

Spots of sulfur amino acids from chromatography of hydrolyzates were also removed from chromatograms and counted. The results of this study can be found in Table 11B. The percent S^{35} determined as cystine, cysteic acid, and cysteine was calculated as the total of the three amino acids, and the remaining percentage of S^{35} reported as methionine. The results may be observed graphically in Figure 17. The percentage of methionine sulfur was lower in all leaves than the percent of the combination of cysteine, cysteic acid and cystine for both healthy and infected groups. The percent of S^{35} present as methionine appears to be greater at the apex and 1st and 2nd nodes of the infected as compared with the healthy. Further pursuit of this approach may clarify our knowledge concerning the effect of plant virus biosynthesis and upon the sulfur metabolism of host plants.

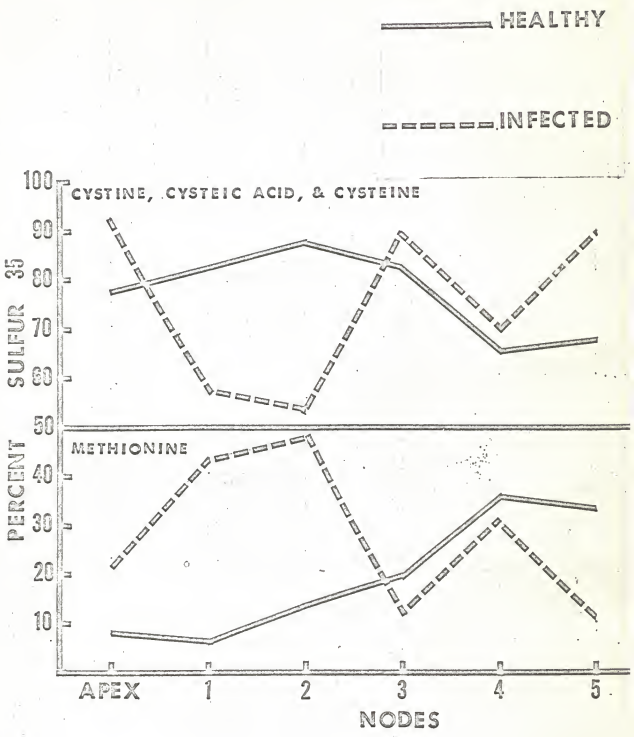

Fig. 17. Percent S^{35} incorporated in cystine, cysteic acid, and cysteine group; and percent S^{35} incorporated in methionine from hydrolyzate chromatograms for the second 48 hour study.

SUMMARY

Certain aspects of sulfur metabolism of plant apices and the first five nodes of Nicotiana tabacum var. Havana were studied with respect to the effect of tobacco mosaic virus infection. These studies were restricted to leaves at 48 hours and 72 hours after infection.

Quantitative determinations were made for total sulfur and inorganic sulfur, while organic sulfur was determined by subtraction. The ratio of inorganic sulfur to total sulfur for each leaf sample was computed. These ratios were compared between healthy and infected nodes by an infected to healthy ratio. Variations in this ratio between the two 48 hour studies occurred, and variations from node to node in the two studies were observed. The I/H ratios in the 72 hour study demonstrated little similarity to corresponding ratios in the 48 hour studies, and these ratios again varied from node to node.

The I/H ratios for total S^{35} accumulation were calculated for apical portions and corresponding nodes. The I/H ratios indicated that during the first 48 hour study, the infected leaves accumulated a greater concentration of S^{35} than the corresponding healthy leaves, but the reverse was true for the second 48 hour experiment. The 72 hour study produced intermediate values. A greater concentration of radioactive sulfur was always observed in the younger leaves, in both healthy and infected plants, and S^{35} showed a decreasing concentration gradient toward the lower, older leaves in both healthy and infected plants.

Methanol extracts were made to isolate and detect S^{35}-labeled compounds in the "free nitrogen pool" of each leaf sample. Autoradiograms indicated the presence of cysteine and/or its derivatives and glutathione in both healthy and infected plants. $S^{35}O_4^=$ was evident at the origins, but labeled cystine and methionine were absent. There appeared to be no loss or gain of new or different S^{35}-labeled compounds in the methanol extracts of the infected leaves.

Autoradiograms of hydrolysates from the second 48 hour study indicated the presence of S^{35}-labeled cystine, cysteic acid, methionine, and traces of unidentified compounds which were probably oxidation products of cysteine and methionine. There appeared to be more labeled cysteic acid in the healthy leaves than in the infected ones.

Areas on the methanol extract chromatograms from the second 48 hr study which corresponded to darkened zones on the autoradiograms were counted for S^{35} activity. The percent S^{35} incorporated in cysteine and/or its derivatives was calculated for both the healthy and infected series. The results suggested a lower rate of S^{35} incorporation in unbound cysteine in the infected lower leaves as compared to healthy lower leaves. The percent S^{35} incorporated into cystine plus cysteic acid plus cysteine was calculated from activity measured from hydrolysate chromatograms. The same was done for methionine, and the results indicated the percent S^{35} incorporated in methionine was less than that incorporated into cystine plus cysteic acid plus cysteine in both the healthy and infected leaves. Some additional increases in the percent S^{35} found

in methionine in the apical portions and the next two lower nodes were observed in the infected leaves with corresponding decreases in the cystine grouping.

ACKNOWLEDGMENTS

I wish to express my appreciation to Dr. James A. Goss, major advisor and plant physiologist, for his untiring guidance and encouragement during the course of my graduate study toward the Master of Science degree. I wish to thank Dr. Webster H. Sill Jr., leader of the research project of which these studies were a part, for his encouragement and for his valuable assistance concerning tobacco mosaic virus.

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APPENDIX

Table 1. Quantitative determinations of sulfur fraction[1] at 48 hours after inoculation.

Leaf Source	Column 1 Inorganic S		Column 2 Organic S[2]		Column 3 Total S		Column 4 Total S as % dry wt		Column 5 Inorganic S/ Total S[3]		Column 6 From Col. 5[4]
	H	I	H	I	H	I	H	I	H	I	I/H Ratio
Apical Portion	2.60	2.39	6.40	5.11	9.00	7.50	0.90%	0.75%	0.29	0.32	1.13
1st Node	2.40	3.30	5.60	4.70	8.00	8.00	0.80%	0.80%	0.30	0.41	1.37
2nd Node	3.10	3.39	4.23	5.14	7.33	8.53	0.73%	0.85%	0.44	0.39	0.89
3rd Node	2.60	4.77	3.50	2.23	6.10	7.00	0.61%	0.70%	0.43	0.68	1.58
4th Node	2.80	3.54	3.20	4.71	6.00	8.25	0.60%	0.83%	0.47	0.43	0.91
5th Node	1.60	3.33	7.53	6.17	9.13	10.50	0.91%	1.05%	0.20	0.32	1.60
Ave. of Cols.	2.52	3.45	5.08	4.67	7.60	8.10	0.76%	0.81%	0.33	0.43	1.28

[1]Fractions are expressed as micrograms of sulfur per milligram dry leaf tissue.

[2]Organic Sulfur obtained: Total Sulfur minus(-) Inorganic Sulfur.

[3]Column 5: derived by placing values from Column 1 in the numerator and corresponding values of Column 3 in the denominator for healthy and infected plants.

[4]Column 6: derived from Column 5 by placing fraction of inorganic sulfur of infected in the numerator and the fraction of inorganic sulfur of healthy in the denominator. This was repeated for each node.

Table 2. Quantitative determination of sulfur fractions at 48 hours[1] after inoculation.

Leaf Source	Column 1 Inorganic S		Column 2 Organic S		Column 3 Total S		Column 4 Total S as % dry wt		Column 5 Inorganic S/ Total S		Column 6 From Col. 5
	H	I	H	I	H	I	H	I	H	I	I/H Ratio
Apical Portion	3.20	3.20	2.01	2.39	5.21	5.59	0.52%	0.56%	0.62	0.57	0.92
1st Node	3.00	3.20	3.21	2.43	6.21	5.63	0.62%	0.56%	0.48	0.57	1.19
2nd Node	2.00	1.90	4.65	3.56	6.65	5.46	0.67%	0.55%	0.30	0.34	1.13
3rd Node	1.90	1.30	3.85	3.16	5.75	4.46	0.58%	0.45%	0.32	0.28	0.88
4th Node	1.90	1.20	3.10	4.39	5.00	5.59	0.50%	0.56%	0.38	0.21	0.55
5th Node	2.20	1.20	4.00	5.47	6.20	6.67	0.62%	0.67%	0.35	0.18	0.51
Average of Each Col.	2.37	2.00	3.47	3.57	5.84	5.57	0.59%	0.57%	0.41	0.36	0.87

[1]Repeat of Experiment One.

Table 3. Quantitative determination of sulfur fractions[1] at 72 hours after inoculation.

Leaf Source	Column 1 Inorganic S		Column 2 Organic S		Column 3 Total S		Column 4 Total S as % dry wt		Column 5 Inorganic S/ Total S		Column 6 From Col. 5
	H	I	H	I	H	I	H	I	H	I	I/H Ratio
Apical Portion	3.80	3.80	6.10	6.10	9.90	9.90	0.99%	0.99%	0.38	0.38	1.00
1st Node	3.20	3.10	6.00	5.30	9.20	8.40	0.92%	0.84%	0.35	0.37	1.06
2nd Node	3.20	2.90	4.30	5.10	7.50	8.00	0.75%	0.80%	0.43	0.36	0.83
3rd Node	2.40	2.50	3.10	4.00	5.50	6.50	0.55%	0.65%	0.44	0.38	0.86
4th Node	2.80	2.70	5.50	4.30	8.30	7.00	0.83%	0.70%	0.33	0.39	1.18
5th Node	3.00	3.50	3.70	2.10	6.70	5.60	0.67%	0.56%	0.45	0.56	1.24
Average of Each Col.	3.07	3.08	4.78	4.48	7.85	7.56	0.79%	0.76%	0.40	0.41	1.03

[1]Sulfur fractions expressed in same units as Tables 1 and 2.

Table 4. Organic Sulfur: Inorganic Sulfur ratio. This ratio indicates the amount of sulfur fixed in the organic form as compared to the inorganic form.

Leaf Source	Organic sulfur/Inorganic sulfur					
	1st 48 hour Study		2nd 48 hour Study		72 hour Study	
	H	I	H	I	H	I
Apical	2.46	2.13	0.63	0.75	1.61	1.61
Node 1	2.33	1.42	1.07	0.75	1.87	1.70
Node 2	1.36	1.52	2.33	1.87	1.34	1.76
Node 3	1.35	0.50	2.03	2.43	1.29	1.60
Node 4	1.14	1.33	1.63	3.66	1.96	1.59
Node 5	4.71	1.85	1.81	4.56	1.23	1.91
Average of 6	2.23	1.45	1.58	2.34	1.55	1.70

Table 5. Uptake and accumulation of S^{35}[1] at 48 hours after inoculation.

Leaf Source	Healthy counts/min Total S^{35}/25 mg dry wt	Infected counts/min Total S^{35}/25 mg dry wt	I/H Ratio[2]
Apical Portion	1213.0	1552.5	1.28
Node 1	1009.0	1418.0	1.41
Node 2	818.0	1222.5	1.49
Node 3	697.0	970.0	1.39
Node 4	300.0	685.5	2.29
Node 5	279.0	362.5	1.29
		Ave. I/H Ratio:	1.52

[1]All samples counted with same detector tube-scolar unit.

[2]I/H Ratio: Infected counts/min: Normal counts/min.

Table 6. Uptake and accumulation of S^{35} at 48 hours[1] after inoculation.

Leaf Source	Healthy counts/min Total S^{35}/25 mg dry wt	Infected counts/min Total S^{35}/25 mg dry wt	I/H Ratio
Apical Portion	1111	936	0.84
Node 1	955	667	0.70
Node 2	815	520	0.64
Node 3	639	395	0.62
Node 4	483	391	0.81
Node 5	321	214	0.67
		Ave. I/H Ratio:	0.71

[1]Second 48 hour study.

Table 7. Uptake and accumulation of S^{35} at 72 hours after inoculation.

Leaf Source	Healthy[1]	Infected[1]	I/H Ratio
Apical Portion	1218	1807	1.48
Node 1	868	1415	1.63
Node 2	639	1117	1.14
Node 3	869	880	1.38
Node 4	459	702	0.81
Node 5	404	475	1.03
		Average I/H Ratio	1.25

[1]Reported as in studies at 48 hours.

Table 8. I/H Ratios of inorganic fractions summarized from Tables 1, 2, and 3.

Leaf Source	I/H Ratio 48 hours	I/H Ratio 48 hours	I/H Ratio 72 hours
Apical Portion	1.13	0.92	1.00
1st Node	1.37	1.19	1.06
2nd Node	0.89	1.13	0.83
3rd Node	1.58	0.88	0.86
4th Node	0.91	0.55	1.18
5th Node	1.60	0.51	1.24
Average I/H Ratio for Apex and all Nodes.	1.28	0.87	1.03

Table 9. I/H Ratios of total S^{35} summarized from Tables 4, 5, and 6.

Leaf Source	I/H Ratio 48 hours	I/H Ratio 48 hours	I/H Ratio 72 hours
Apical Portion	1.28	0.84	1.48
1st Node	1.41	0.70	1.63
2nd Node	1.49	0.64	1.14
3rd Node	1.39	0.62	1.38
4th Node	2.29	0.81	0.81
5th Node	1.29	0.67	1.03
Average I/H Ratio for Apex and all Nodes	1.52	0.71	1.25

Table 10. Sulfur 35-labeled compounds identified by chromatography and autoradiography for studies conducted at 48 hours after inoculation.

A. Methanol Extracts[1] ($SO_4^=$ and Free Amino Acids)

Leaf Source	$S^{35}O_4^=$		Cysteine		Cystine		Methionine		Glutathione(?)	
	H	I	H	I	H	I	H	I	H	I
Apical Portion	+[2]	+	+	+	-[3]	-	-	-	?	?
1st Node	+	+	+	+	-	-	-	-	?	?
2nd Node	+	+	+	+	-	-	-	-	?	?
3rd Node	+	+	+	+	-	-	-	-	?	?
4th Node	+	+	+	+	-	-	-	-	?	?
5th Node	+	+	+	+	-	-	-	-	?	?

[1] From first and second 48 hour studies.

[2] +: indicates presence of compound.

[3] -: indicates absence of compound.

B. Hydrolysates[1] ($SO_4^=$ and Protein-bound Amino Acids)

Leaf Source	$S^{35}O_4^=$[2]		Cystine		Cysteic Acid		Cysteine		Oxidation Products		Methionine	
	H	I	H	I	H	I	H	I	H	I	H	I
Apical Portion	+	+	+	+	+	+	+	+	+	trace	+	+
1st Node	+	-	+	+	+	-	+	-	+	-	+	+
2nd Node	+	-	+	+	+	-	+	trace	-	-	+	+
3rd Node	+	trace	+	+	+	trace	+	+	-	-	+	+
4th Node	+	+	+	+	+	trace	+	trace	-	-	+	+
5th Node	+	+	+	+	+	trace	+	trace	-	-	trace	trace

[1] From second 48 hour study.

[2] $S^{35}O_4^=$ present due to incomplete washing of residue from methanol extract.

Table 11. Determination of activity of S^{35} labeled compounds separated by chromatography from second 48 hour study.

A. Free S^{35} labeled Amino Acids + $S^{35}O_4^=$

Fraction	Leaf Source: Apical Portion H[1]	Apical Portion I[1]	1st Node H	1st Node I	2nd Node H	2nd Node I	3rd Node H	3rd Node I	4th Node H	4th Node I	5th Node H	5th Node I
$S^{35}O_4^=$ from Methanol Extracts[2]	388	255	99	233	103	160	44	89	18	53	10	28
$S^{35}O_4^=$ from Hydrolyzates	80	15	15	0	45	0	33	3	23	13	10	10
Cysteine and/or derivative from Methanol Extracts	185	143	174	200	126	96	93	83	83	72	65	36
Percent S^{35} found as Cysteine and/or derivative	30%	35%	60%	46%	46%	38%	55%	47%	67%	52%	76%	43%

[1] Experimental values reported as counts/min corrected for background.

[2] Methanol Extracts represented 5 gm fresh wt/ml.

[3] Hydrolysates represented 2 gm fresh wt/ml. Counts obtained for $S^{35}O_4^=$ in hydrolysates were multiplied by 2.5.

Table 11. Continued.

B. Protein bound S^{35} labeled Amino Acids

Fraction	Leaf Source: Apical Portion H	Apical Portion I	1st Node H	1st Node I	2nd Node H	2nd Node I	3rd Node H	3rd Node I	4th Node H	4th Node I	5th Node H	5th Node I
Cystine	11	17	17	4	6	5	7	17	4	7	0	3
Cysteic Acid	17	8	17	-	14	-	9	3	6	3	1	4
Cysteine	4	18	4	-	1	3	1	10	1	4	1	1
Unidentified	3	1	3	-	-	-	-	-	-	-	-	-
Unidentified	3	1	3	-	-	-	-	-	-	-	-	-
Unidentified	1	1	3	-	-	-	-	-	-	-	-	-
Methionine	3	3	3	3	3	7	4	4	6	6	1	1
Percent S^{35} found as Cystine, Cysteic Acid and Cysteine	76%	91%	81%	57%	88%	53%	81%	88%	65%	70%	67%	89%
Percent S^{35} found as Methionine	7%	21%	6%	43%	13%	41%	19%	12%	35%	30%	33%	11%

A STUDY OF CERTAIN ASPECTS OF SULFUR METABOLISM OF TOBACCO MOSAIC VIRUS INFECTED *NICOTIANA TABACUM* L.

by

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B. S., Kansas State University, 1962

AN ABSTRACT OF A MASTER'S THESIS

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Nicotiana tabacum var. Havana plants were grown hydroponically under greenhouse conditions in order to study certain aspects of sulfur metabolism in tobacco mosaic virus infected leaves at 48 hours and 72 hours after inoculation. Studies were conducted on the apical portions and the next five lower leaves of both healthy and infected plants utilizing both a quantitative analysis and radioactive sulfur 35 ($S^{35}O_4^=$) studies.

Total sulfur, inorganic sulfur, and organic sulfur (computed by subtraction) were measured at the apex and five lower leaves for both healthy and infected, and an inorganic sulfur: total sulfur ratio was computed for each leaf sample. These ratios were compared at various corresponding apices and leaves by an infected to healthy ratio, because it was thought that a ratio of this sort might reflect sulfate reduction differences in healthy as compared to TMV infected (corresponding) leaves. However a great deal of variation was observed in these ratios at corresponding nodes for all three studies, and no definite conclusions could be drawn concerning sulfate reduction in the infected leaves.

I/H ratios were computed for total S^{35} accumulation in the leaves, and these ratios indicated an increase in S^{35} accumulation in all TMV infected leaves as compared to corresponding healthy leaves in the first 48 hour study, but the second 48 hour study indicated a complete reverse of this situation. I/H ratios of total S^{35} accumulation for the 72 hour study demonstrated little similarity to the 48 hour studies. A greater concentration of radioactive sulfur was always observed in the younger leaves both healthy and

infected plants, and S^{35} showed a decreasing concentration gradient toward the lower, older leaves in both healthy and infected plants.

Methanol extracts were made to isolate and detect S^{35}-labeled compounds in the "free nitrogen pool" of each leaf sample. Autoradiograms indicated the presence of cysteine and/or its derivatives and glutathione in both healthy and infected plants. $S^{35}O_4^=$ was evident at the origins, but labeled cystine and methionine was absent. There appeared to be no loss or gain of new or different S^{35}-labeled compounds in the methanol extracts of the infected leaves.

Autoradiograms of hydrolysates from the second 48 hour study indicated the presence of S^{35}-labeled cystine, cysteic acid, methionine, and traces of unidentified compounds which were probably oxidation products of cysteine and methionine. There appeared to be more labeled cysteic acid in the healthy leaves than in the infected ones.

Areas on the methanol extract chromatograms from the second 48 hr study which corresponded to darkened zones on the autoradiograms were counted for S^{35} activity. The percent S^{35} incorporated in cysteine and/or its derivatives was calculated for both the healthy and infected series. The results suggested a lower rate of S^{35} incorporation in unbound cysteine in the infected lower leaves as compared to healthy lower leaves. The percent S^{35} incorporated into cystine plus cysteic acid plus cysteine was calculated from activity measured from hydrolysate chromatograms. The same was done for methionine, and the results indicated the percent S^{35} incorporated in methionine was less than that incorporated into

cystine plus cysteic acid plus cysteine in both the healthy and infected leaves. Some additional increases in the percent S^{35} found in methionine in the apical portions and the next two lower nodes were observed in the infected leaves with corresponding decreases in the cystine grouping.