DISTRIBUTION OF S^{35} IN LEAVES OF HEALTHY AND VIRUS INFECTED PLANTS

by

PATRICIA WAYNETTE HOOK

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Approved by:

J. M. Grogan Jr. 5th P.

Major Professor

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INTRODUCTION

The following study was undertaken to determine whether sulfur might accumulate in a different pattern in Datura stramonium L. leaves infected with bromegrass mosaic virus than in healthy leaves.

S^{35} was chosen as the isotope since it has been known to accumulate in some diseased or mechanically injured tissue and was well adapted to such studies as autoradiography and chromatography having soft B particles with a fairly long half-life of 87 days.

Datura stramonium plants were grown in nutrient culture to which S^{35} had been added. The plants were then harvested and the amount and distribution of the isotope was studied. Autoradiography of leaves, chromatography and subsequent autoradiography of alcohol extracts, and total counts per minute of dried, ground leaf material were the techniques used. Autoradiography of leaves was also done using tobacco mosaic virus on Nicotiana glutinosa L. another local lesion host, and on Nicotiana tabacum L., a systemic host.

The use of three different hosts, two of which were inoculated with the same virus, allowed a comparison of sulfur distribution as shown by autoradiograms. The information gained from these comparisons when coupled with other work, such as respiration studies or enzyme system studies, will be valuable for understanding plant metabolism during the disease process.

REVIEW OF LITERATURE

Datura stramonium was used as a quantitative bioassay host for bromegrass mosaic virus (BMV) by Chiu and Sill (1959). The typical symptom

expression was local chlorotic rings with light green centers. Systemic symptoms, consisting of local lesions on uninoculated leaves, occurred after the removal of apical meristems. In subsequent papers (Chiu, 1961 and Chiu and Sill, 1963a) the conditions under which satisfactory local lesions could be obtained were reported. The plants were most susceptible during the 4-6 leaf stage. In summer with temperatures up to 35° C. the production of lesions was adversely affected. Under these conditions only faint chlorotic spots developed. Newly unfolded and quite old leaves were nearly immune to the virus. The virus was purified by Chiu and Sill (1963b) and was found to be a polyhedral virus with an average diameter of 32.8 mu.

Tobacco mosaic virus (TMV) according to Burnet and Stanley (1959) is a widespread, extremely infectious and stable virus. It is of economic importance and can parasitize many hosts. TMV was selected for the following study because it caused a systemic infection in N. tabacum and a local lesion infection in N. glutinosa. The sweet potato strain was used as reported by Sill et al. (1960).

The accumulation of S^{35} in virus lesions has been reported by several authors (Yarwood and Jacobson, 1955; Shaw and Samborski, 1956; and Yoshii et al. 1955). In 1955, both Yarwood and Jacobson, at Berkeley and Yoshii et al. in Japan reported an accumulation of S^{35} and P^{32} in the areas of lesions caused by TMV on N. glutinosa. Each also observed accumulation in leaf areas which had been mechanically injured. Yoshii, et al. found an accumulation of both P^{32} and S^{35} in lesions formed by injecting $KClO_3$ or $CuSO_4$ into N. tabacum leaves, although none occurred when leaves were injected with HCl or punctured. Yarwood and Jacobson found no accumulation around TMV lesions on N. glutinosa the

first day after inoculation, but did observe localized accumulation two days after inoculation before the appearance of symptoms. Leaves infected three or more days and showing lesions had marked accumulation in the lesions. Accumulation also occurred with TMV infection of Phaseolus vulgaris. Leaves mechanically injured by pressure and H_2SO_4 also showed accumulation but not as much as the TMV lesions. They reported no accumulation apparent in the systemic infection of TMV on N. tabacum. The presence of the isotopes was detected by the use of both Geiger-Muller counter and autoradiographic techniques. The isotopes were applied, as H_2S and H_3PO_4, to the leaves for eight to 91 hours and the leaves placed between pieces of No-screen X-ray film at 0° C. for varying lengths of time. To determine activity, the leaves were dried and placed under a lead disk with a hole over the region to be counted. TMV lesions on N. glutinosa were found to have 19 times as much S^{35} as did the healthy tissue.

Shaw and Samborski (1956) also reported accumulation of P^{32} and of Ca^{42} in TMV lesions on N. glutinosa. However, systemic infections of false stripe virus on barley and TMV on N. tabacum did not affect P^{32} accumulation. They found that the tracers did not enter necrotic, mechanically wounded, tissue but did accumulate in fresh wounds under conditions where great water loss might occur. The isotopes were injected into the leaves, or excised leaves were placed in solutions containing isotopes. The leaves were dried between filter paper and placed on No-screen X-ray film for several days.

Variations in rate of respiration with virus infection have been reported by several authors. Owen (1955a, b) reported changes in respiration of tobacco leaves with TMV inoculation and of N. glutinosa leaves inoculated with TMV (Owen, 1958). The systemic infection in tobacco resulted in a

respiration change within one hour while the local lesion host (N. glutinosa) showed no change till symptoms appeared at which time respiration rose as much as 30%, depending on the number of lesions. Yamaguchi and Hirai (1959) also reported an increase in respiration in N. glutinosa with the onset of symptoms preceded by decreased respiration just before lesion formation. Since they found a virus increase before the onset of symptoms, they concluded that the respiration increase reflected the formation of local necrotic lesions rather than the formation of virus. A change in respiration has also been observed with other virus infections. Merrett (1960) reported an increase in respiration of tomato stem tissue infected by tomato aucuba mosaic virus.

Such increases in respiration would normally lead one to assume that an increased amount of energy would be available which might be used for the uptake of a mineral nutrient such as sulfur. The ability of plants to accumulate materials against a concentration gradient requires energy. With the energy from increased respiration available one would expect increased accumulation. However, in the presence of an uncoupling agent, such as DNP, salt uptake and accumulation decrease (Beevers, 1961).

In previous work done in this laboratory (Burroughs et al., 1964) it has been shown that the infection of barley plants with BMV brought about changes in the amounts of sulfur in the leaves at various periods after infection. These were related to oxygen uptake. Diseased plants were found to have a higher total sulfur content than healthy plants. Radioactive sulfur decreased before symptom development then increased with the onset of symptoms and remained slightly higher than in infected plants. The rate of oxygen uptake showed a similar curve.

The reduction of sulfate within the plant requires energy as does the accumulation of the sulfur. The sulfate is first converted to sulfhydryl groups which appear in the amino acid cysteine. According to Thomas (1958) sulfate is reduced to sulfide in a reversible reaction. This sulfide may then combine with glycolytic compounds which have undergone amination to form cystine and other amino acids. The reduction of sulfate to sulfide requires 118.5 Kcal. per mole at 25° C. The use of the sulfur containing amino acid in protein synthesis makes this an element of great importance.

Several workers have investigated the free amino acids of plants when studying sulfur metabolism and the effects of disease on sulfur metabolism. Selman et al. (1961) reported increases in total free amino acids up to 180% in tomato plants infected with tomato spotted wilt virus. They did not report any specific increases in sulfur containing amino acids, however. They postulated that the increase was due to a check in growth and might contribute to necrosis. Fife (1956) reported an increase in amino acids in the morning only, in sugar beet plants affected with curly top. Steward et al. (1951) reported on the amino acids in alfalfa using S^{35} tracer techniques. They found cystine detected as cysteic acid and other substances which may have been glutathione which yielded cysteine on hydrolysis. Methionine was not found in the free state. Their work was on healthy plants. No reports of studies of the sulfur metabolism of Datura stramonium were found in the literature.

MATERIALS AND METHODS

Growth of Plants

Datura stramonium, *Nicotiana tabacum*, and *N. glutinosa* were seeded in Terralite in pots in the greenhouse. After germination, the seedlings were given Hoagland's solution (Hoagland and Arnon, 1950) with iron-ethylene-diamine tetracetic acid (Fe-EDTA) once a week until they had at least two leaves. At this time, they were transferred to water culture using quarter-strength Hoagland's solution with iron supplied as Fe-EDTA. The plants were supported by means of cotton pads in one-half inch holes cut in vinyl tiles. One tile, holding two to four plants, was then placed over a three liter battery jar of solution with the roots submerged in the solution. The battery jars were suspended, by means of metal strips, in a large tank of water which served to regulate the temperature of the nutrient solution. Air was supplied to each jar by means of glass tubing attached to a central aerating system. A water trap was placed along the tubing to protect the solution from contaminants in the air piped to the greenhouse. The greenhouse was fumigated weekly and the plants were hand sprayed with Malathion as needed to control insects. The temperature was usually between 20° C. and 30° C. Humidity was maintained above 30% by spraying walks and benches when necessary. The plants were given supplementary light during the winter to prevent flowering of *D. stramonium*, and to maintain the leaves at a stage susceptible to infection by BMV. This 18-hour photoperiod was maintained by fluorescent tubes suspended over the tanks at a distance of about 34 inches. Tubes giving white light as well as those giving red and blue light were used.

After transfer to the nutrient solution the plants were allowed to become established before their use. A preliminary experiment was done without S^{35} to determine the pH at which the D. stramonium plants could best be grown. Groups of three plants each were grown in solutions with pH values of 5.5, 7.5, and 8.5. The original pH of quarter-strength Hoagland's solution made with distilled water was about 5.5. Adjustments were made to 7.5 and 8.5 adding 0.1N KOH. The pH of each jar was checked daily and an appropriate amount of KOH or HCl added to maintain the desired pH. The plants tended to adjust the pH of the solution to about 7.2. They were allowed to grow two weeks at which time the fresh and dry weights of the plants were determined.

In subsequent studies, the solution was renewed weekly. After the first week, N. tabacum and N. glutinosa plants were given one-half strength Hoagland's solution, but D. stramonium plants were kept in one-quarter strength solution. Any plants showing root rot were discarded. To prevent root rot clean battery jars, tiles, and glass tubing were used. All of this equipment was soaked in a solution of chlorine bleach after each usage and was rinsed carefully again just before being put into use.

The plants were allowed to grow in this way until they reached the proper stage for inoculation. N. tabacum and N. glutinosa were inoculated when the leaves had expanded to a desirable size: one and one-half to two inches for N. glutinosa, and three to four inches for N. tabacum. D. Stramonium was inoculated, using the third and fourth true leaves, just before these leaves reached maturity. The rate of growth of all the plants depended on climatic conditions and the time of year. N. glutinosa and N. tabacum required from two to three months from seed to inoculation, and D. stramonium required from five to eight weeks.

Injury and Inoculation

Leaves were mechanically injured in several ways. They were pricked with a dissecting needle, scraped with a knife, or torn with a bottle opener. Some received accidental injury when they became infested with mites or caterpillars or when too much pressure was exerted during inoculation. Some showed unidentified injury which probably was sunburn.

D. stramonium plants were inoculated with bromegrass-mosaic virus (BMV), Chiu's strain (Chiu and Sill, 1959), obtained from infected barley leaves which were stored in the refrigerator over calcium chloride. At the time of use the leaves were ground in a mortar with a pinch of sand and a small amount of distilled water. The extract was filtered through cheese cloth, and the residue reground and refiltered with another small amount of water. The combined extracts were used for inoculation.

Tobacco-mosaic virus (TMV) was used to inoculate N. tabacum and N. glutinosa. This was the sweet potato strain (Sill et al., 1960). An extract was made by grinding live, heavily infected tomato leaves with sand in a mortar. The juice was filtered through cheese cloth and the pulp suspended in a small amount of water, reground and refiltered. These combined filtrates made a potent inoculum which required further dilution when single lesions were desired.

The leaves were inoculated using ready made cotton swabs which were dipped into the extract and rubbed over a leaf, supported on a folded paper towel. Carborundum was added to the virus extracts when inoculating to assure sufficient injury of the cells and entrance of the virus. It was necessary to exert care when inoculating so as not to injure the leaf excessively. N. glutinosa and N. tabacum are especially tender, and

N. tabacum leaves were easily killed by rubbing too hard. After the extract was applied each leaf was rubbed gently once more with a finger to be sure that the entire surface had been covered. Control leaves, of the same age as those inoculated, were rubbed in the same way, using carborundum and distilled water. The leaves were then rinsed with tap water from a wash bottle. Inoculation was done at either 10 o'clock in the morning or 1 o'clock in the afternoon. Since N. glutinosa and D. stramonium are local lesion hosts, all leaves to be used were inoculated. With N. tabacum only one or two leaves were inoculated since the virus was was systemic. Radioactive sulfur was added immediately following inoculation in dosages ranging from 50 to 100 microcuries for each three-liter jar. Sulfur was added as $H_2S^{35}O_4$ in HCl and contained a small amount of P^{32} as a contaminant.

Infected leaves were harvested at the same time of day as inoculated. The time elapsed between inoculation and harvest varied over 24 hour increments. N. glutinosa was harvested as soon as lesions appeared on the leaves, two or three days after inoculation. Leaves were harvested again five days later when greater uptake had occurred and a sharper radioautogram could be obtained. N. tabacum was harvested when mosaic symptoms appeared, about one week after inoculation. D. stramonium was harvested in a series beginning two or three days after inoculation and continuing until lesions were well defined at five to six days after inoculation. In the one case when S^{35} was not added until lesions had appeared, 48 hours uptake was allowed before harvest. In general, these plants took up enough S^{35} in two or three days to give adequate autoradiograms; however, in the winter there is less uptake and the autoradiograms were much fainter than those

made from leaves harvested in the summer. For this reason the duration of exposure to S^{35} was increased during winter with 100 microcuries of S^{35} added per jar.

Autoradiography

Leaves to be used for autoradiography were cut from the plant and laid on paper towels. The midribs were removed and healthy leaves were marked by the removal of an additional large vein. Care was taken to match each diseased leaf with a healthy leaf of the same size and age from a different plant as leaves of different ages were found to take up different amounts of S^{35}. Each leaf was laid top down on a piece of Saran Wrap, and a second piece of Saran was smoothed over the leaf so that the leaf was held flat between the plastic films. The leaves were then taken to the dark room where a small piece of Kodak Blue Brand X-ray film just larger than the leaf was laid against the top surface of the leaf. The edges of the Saran were then wrapped around the film holding the leaf firmly against the film. The pieces of film with leaves were placed in a light tight box and put into the freezer at -10° C. for one week. When several leaves were put into one box, aluminum foil was placed between them to prevent exposure of the neighboring film.

After one week the leaves were removed from the film and the film was developed using Kodak liquid X-ray Developer and Fixer, following the instructions of the manufacturer. After drying, each film was matched with its respective leaf and labeled with a wax pencil. Areas of necrosis and virus lesions were noted along with the length of time of uptake, the condition of the leaf (whether healthy or diseased), and any other pertinent

information. The films were filed in envelopes. The leaves themselves, once frozen and thawed, disintegrated rapidly so observations and comparisons were made as soon as possible. Leaves which did not mold or decompose immediately were kept with the films. Some leaves were photographed before being frozen, but this was undesirable as it increased the time between harvest and freezing and allowed a greater chance for the S^{35} to move in the leaf before the autoradiogram was made.

Photographs were made, using the autoradiograms as negatives by placing them in an enlarger. Shadow prints also were made by placing the leaf itself, made transparent by freezing and thawing, in the enlarger.

Chromatography

For chromatography, whenever possible an extract was made from only half a leaf and the other half was autoradiographed. However, this allowed only very small amounts of tissue to work with, and better results were obtained using whole leaves. Chromatography was done only with D. stramonium, and was done in a series as lesions developed. Leaves were harvested in the greenhouse taking care to match healthy and infected leaves as to age. The midribs were removed and equal amounts of healthy and infected tissue were weighed. D. stramonium leaves lost water very rapidly once harvested and accurate wet weights were difficult to determine. The leaf tissue was ground in a mortar with a pinch of washed sand. When the tissue was thoroughly macerated, a small amount (3-4 ml.) of 80% methanol was added as an extracting medium. The extracts were labeled as healthy or infected, the weights recorded, and the extracts placed in the refrigerator for 24 hours, after which they were filtered and returned to the refrigerator.

When the entire series of extracts had been collected the volume was reduced using either a hairdryer on the "cold" setting or the Bio-dryer and centrifuge. The hairdryer was found to be quite adequate for small amounts. The extracts were taken nearly to dryness and then brought back to the desired volume with 10% isopropanol. Isopropanol was used to prevent spoilage. The final volumes varied according to the original amount of tissue used, but the final concentrations were kept as nearly equal as possible. However, the object of these experiments was to compare healthy and diseased leaves at particular lengths of time following inoculation. Therefore, the concentrations in each healthy-diseased pair were kept equal. The final concentration was usually about one milliliter to one gram of wet weight, but should have been more concentrated.

The extract was spotted on chromatographic grade, Whatman No. 1 filter paper in 25 or 50 lambda aliquots. Each spot was labeled according to time between inoculation and harvest, condition of the leaf, and amount of extract in the spot. Each entire series was put into the chromatographic cabinet at once to obtain uniformity. The papers were run in one dimension only using butanol, acetic acid, and water. One hundred-twenty-five milliliters each of n-butanol and distilled water were shaken in a separatory funnel. Thirty milliliters of glacial acetic acid were added and two layers allowed to separate. The lower water layer was used to help saturate the cabinet or was discarded. The upper layer was used as the mobile phase.

The solvent front moved down the paper in 15 to 18 hours. The papers were then dried in the hood, sprayed with a 0.25% solution of ninhydrin in acetone, and steamed until the ninhydrin positive spots developed.

Eight to ten spots appeared with D. stramonium extracts although not all of these were visible at the lower concentrations. After the spots had developed, the chromatograms were placed on Kodak No-screen X-ray film in X-ray film holders. Several were put into each holder and were separated with aluminum foil. Film was placed on either side of the top chromatogram so that one film could be developed to determine sufficient exposure. Approximately one month was necessary for exposure.

When developing the film the holder was opened carefully and each chromatogram and corresponding film was marked by punching a pattern of holes with a dissecting needle. This proved somewhat difficult and they might have been better marked with small spots of radioactive ink. The films were developed in the same manner as the small leaf autoradiograms except that longer times were necessary. Development took about five minutes, washing ten, and fixing fourteen. The films were then washed in running water for at least two hours. After drying they were matched with their respective chromatograms and labeled.

Total Radioactive Sulfur

For the determination of the total uptake of radioactive sulfur in healthy and diseased tissue, some leaves were harvested at six or eight days after inoculation when lesions were well developed. They were then dried in the oven at 80° C., ground in a Wiley mill, and 25 mg. samples placed in labeled planchets. These were counted, using a thin window detector and scaler and the counts per minute of healthy and diseased tissue on the two days were compared.

RESULTS

Experiments with pH

In these experiments, no significant differences in weight were found as seen in Table 1.

Table 1. Weights of Datura stramonium Plants Grown in Solution at Varying pH Values.

pH	5.5	7.5	8.5
		Wet Weight	
Weight Per Plant	17.31	16.32	15.52
	15.00	16.08	16.63
	18.70	16.95	14.80
Average Weight	17.00	16.47	15.65
		Dry Weight	
Weight Per Plant	3.47	3.27	3.28
	3.30	3.27	3.39
	3.38	3.14	3.35
Average Weight	3.35	3.23	3.37

Because of these experiments it was decided that it would not be necessary to adjust the pH of the solutions in which the plants were grown.

Autoradiography

In the autoradiography study, no accumulation of S^{35} was observed adjacent to any lesions caused by BMV on D. stramonium. There also was no accumulation in spots indicating the areas in which lesions would appear on leaves harvested before the appearance of symptoms. However, in some

cases, the entire autoradiogram of a healthy leaf had more radioactivity than an infected leaf of the same age. This was particularly noticeable in leaves harvested the last week in September and the first week in October, and may have been due to the healthy leaves being younger than the infected leaves (Plate I).

D. stramonium did show accumulation around some mechanical injuries. Pin pricks, sharp cuts, and insect damage did not show accumulation, but, when tissue was damaged in such a way that it collapsed and became necrotic, there was accumulation. In these cases accumulation was noted in the seemingly healthy, green area just around the necrotic tissue (Plate II). Accumulation was also seen in leaves which had been scraped or injured by heat. This was usually less noticeable as shown in Plate III.

N. glutinosa inoculated with TMV showed accumulation in individual lesions only two days after inoculation (Plate IV). Accumulation was also noticed in areas where lesions had coalesced in seven days, but this was between the necrotic areas in the apparently healthy green tissue (Plates V and VI). In the group of leaves from which Plate V was chosen there was also some evidence of accumulation in the areas of individual lesions although it was rather faint.

In both D. stramonium inoculated with BMV and N. glutinosa inoculated with TMV leaves sometimes had the S^{35} localized in the veins more than did the healthy leaves (Plates I and VII).

N. tabacum leaves when inoculated with TMV showed little difference between healthy and symptom bearing leaves. At seven days the healthy leaves did not show more S^{35} than did the infected. At 11 days the infected leaf had a little less sulfur and was somewhat mottled (Plate VIII).

EXPLANATION OF PLATE I

Autoradiogram of halves of healthy (H) and diseased (D) leaves harvested October 3, 1964, six days after inoculation. The healthy leaf was slightly younger. S^{35} shows as white.

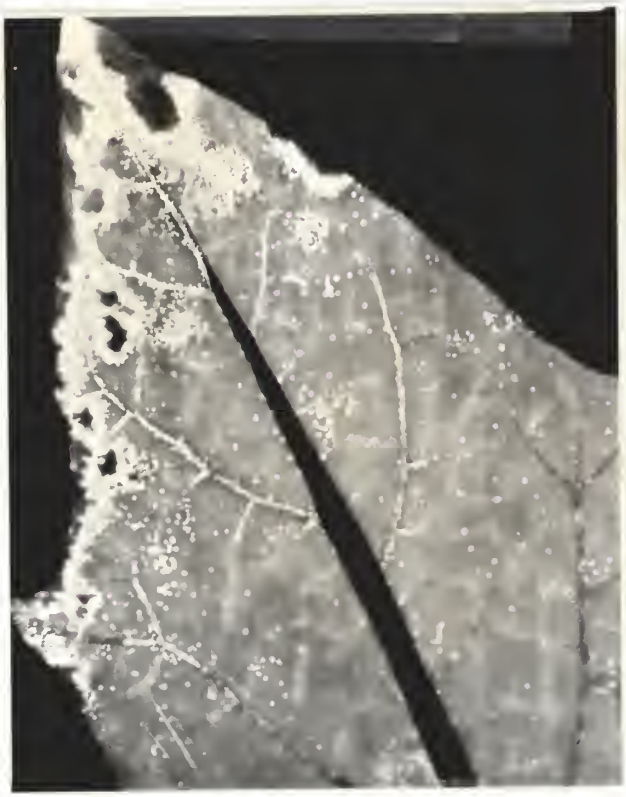
PLATE I

D H

EXPLANATION OF PLATE II

Autoradiogram of leaf harvested in June, 1964, with the edge dead due to unknown causes, possibly sunburn. S^{35} has accumulated around the dead tissue.

PLATE II



EXPLANATION OF PLATE III

fig. 1. Autoradiogram of a scraped leaf harvested in November, 1964, seven days after having been scraped with a paring knife.

fig. 2. Shadowgram of leaf used for autoradiogram; scraped areas show as black.

PLATE III

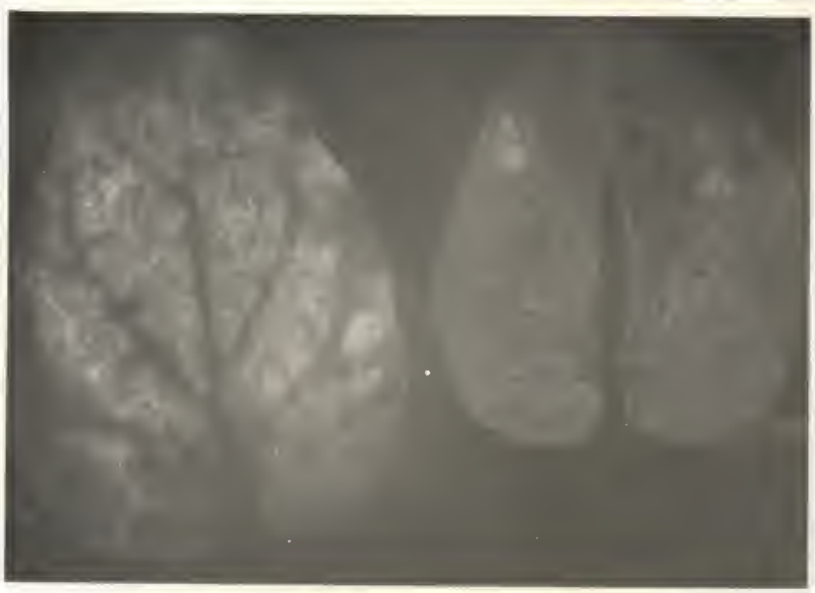
Fig. 1

Fig. 2

EXPLANATION OF PLATE IV

Autoradiogram of *N. glutinosa* leaves harvested in November, 1964, two days after inoculation. S^{35} has accumulated in the lesions. This is particularily clear in the autoradiogram at the left.

PLATE IV



EXPLANATION OF PLATE V

fig. 1. Autoradiogram of N. glutinosa leaf inoculated with TMV harvested in October, 1964, seven days after inoculation. The necrotic areas were quite dry and cracked. S^{35} has accumulated between and around the coalesced lesions.

fig. 2. Shadowgram of the leaf from which the autoradiogram was made - showing lesions.

PLATE V

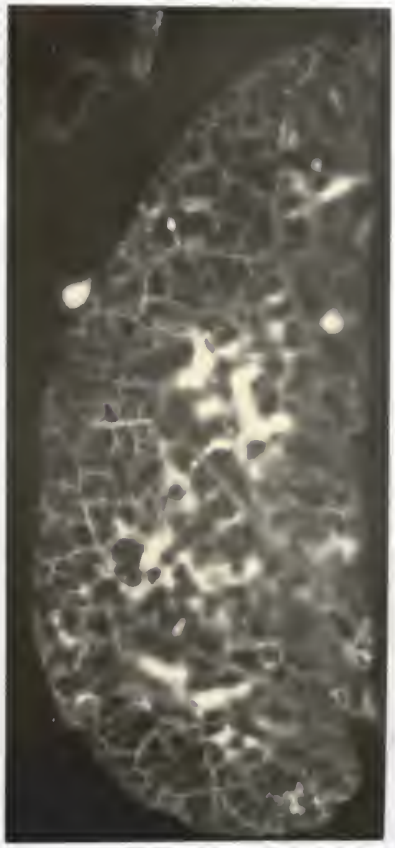


Fig. 1

Fig. 2

EXPLANATION OF PLATE VI

fig. 1. Autoradiogram of N. glutinosa leaves harvested in November, 1964, seven days after inoculation with TMV.

fig. 2. Shadowgram of leaves from which autoradiogram was made.

PLATE VI

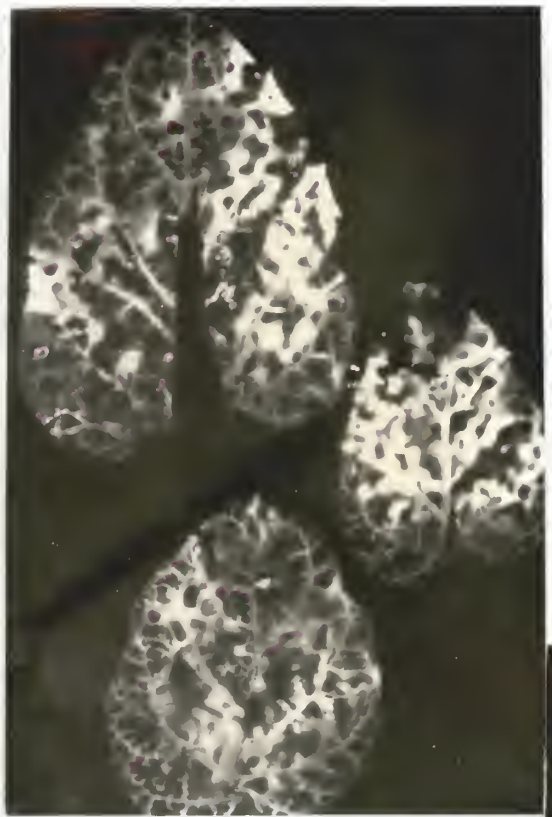


Fig. 1

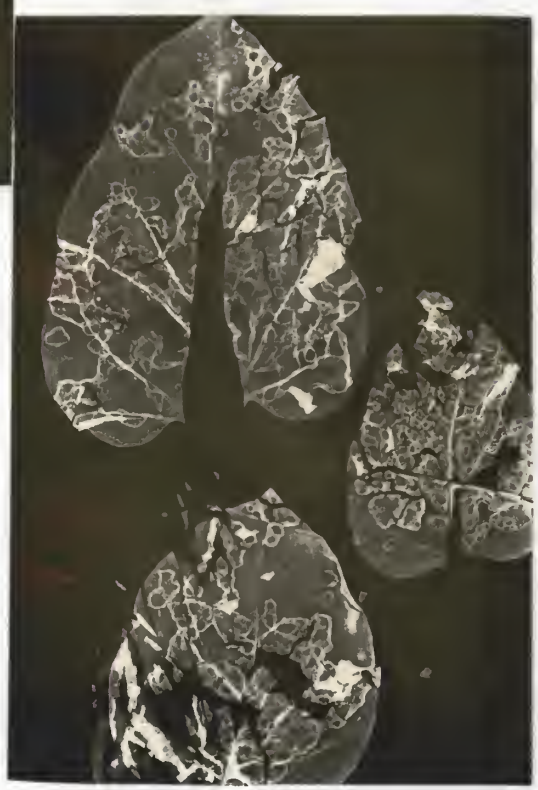


Fig. 2

EXPLANATION OF PLATE VII

N. glutinosa half leaves. The inoculated half leaf on the left has S^{35} localized in the veins.

PLATE VII

EXPLANATION OF PLATE VIII

N. tabacum leaves harvested October 16, 1964, 11 days after inoculation. The diseased leaf on the right, inoculated with TMV, was showing severe mottling. The short, black streaks are leaf hairs.

PLATE VIII

Chromatography

Autoradiograms of chromatograms of alcoholic extracts from leaves harvested in October did not show any consistent evidence of a greater amount of S^{35} in the extracts from either healthy or diseased leaves. These autoradiograms showed two or three dark spots near the origin which may have been cystine, cysteic acid, and glutathione. These spots were slightly ninhydrin positive. Altogether there were eight to ten ninhydrin positive spots on the chromatograms. These were primarily below the S^{35} spots which were always near the origin.

Total Radioactive Sulfur

Leaves harvested in the afternoon on the sixth and eighth days after inoculation were dried and counted. The healthy leaves consistently had higher counts per minute than did the infected leaves. They were matched in pairs as to age (Table 2.).

Table 2. Counts Per Minute of BMV Infected Datura stramonium Tissue on the Sixth and Eighth Days after Inoculation

Replications (leaf pairs)	Healthy	Infected
	c.p.m./25 mg.d.wt.	
	Sixth Day	
1	1957	1921
2	2004	1189
	Eighth Day	
1	2428	1656
2	2757	2226
3	1363	1100

Some of the differences here were quite small, but the counts on healthy leaves were consistently higher.

DISCUSSION

It is possible to conclude from the autoradiograms of leaves that the metabolism of different host-virus systems varied enough to cause differences in the accumulation pattern of an element such as sulfur. TMV in N. glutinosa caused accumulation in lesions and in N. tabacum caused no difference in distribution patterns of S^{35} between healthy and diseased tissue. This has been reported by other workers (Yarwood and Jacobson, 1955; Yoshii et al., 1955; Shaw and Samborski, 1956). D. stramonium with BMV showed no apparent change. Although D. stramonium was an interesting host for these studies, not having been used in quite this way before, it had several disadvantages. The plants gave a local lesion reaction only under certain conditions and were difficult to use during the summer months. The second and third true leaves were the most susceptible, and this

limited the number of leaves per plant which could be used. The very young and very old leaves were not susceptible at all. Supplemental light must be given to keep the plants from flowering. There was also a great difference in amount of uptake by leaves of differing ages. The environmental conditions affecting the formation of local lesions were reported by Chiu and Sill (1963a).

An interesting phenomenon on some autoradiograms with D. stramonium and N. glutinosa was the presence of small sharply defined black spots on autoradiograms of injured leaves (Plates II and VI). They did not correspond to any injury visible to the naked eye but were never seen on autoradiograms of healthy, uninoculated leaves. When leaves which gave the spots were examined under the microscope the area appeared to have a residue of carborundum on the surface, and to be somewhat injured. It would appear that this minor, almost invisible injury is in some way responsible for the appearance of the black spots, although they were not found in connection with all injured leaves. One possible explanation might be the invasion of the tissue by a fungus with the accumulation of S^{35} in the fungal fruiting bodies. No fungus, however, was readily apparent.

Several theories have developed as to why a mineral element such as sulfur accumulates in diseased areas of a plant. Accumulation in the pathogen itself is one possibility, although it does not seem likely that this is the case when local lesion hosts are used. This would not explain the accumulation in mechanical injuries, and in the case of accumulation due to TMV in N. glutinosa only a relatively small amount of virus is being formed. It was suggested by Yarwood and Jacobson (1955) that inorganic

sulfur might accumulate in a diseased area due to a high transpiration rate in that area and accompanying crystallization of the isotope as evaporation occurred. They concluded, however, that this was not the case because accumulation was being achieved even in instances in which the leaf was enclosed and little or no transpiration was occurring. Shaw and Samborski (1956), however, prevented accumulation in mechanical injuries by coating the area with petroleum jelly. They suggested increased translocation as a means of accumulating the element. It should be noted in respect to this that petroleum jelly contains a complex of organic substances which may have had an effect on the plant. The works of Yarwood and Jacobson and Shaw and Samborski differ primarily in that Yarwood and Jacobson achieved accumulation in mechanical injuries under conditions of low transpiration whereas Shaw and Samborski did not, and also in the fact that Yarwood and Jacobson achieved accumulation of S^{35}, which was not used by Shaw and Samborski, when applied in the form of H_2S, which is a respiratory poison.

Yarwood and Jacobson also suggested greater permeability of the diseased area as a cause of increased uptake in instances in which the isotope was taken up by the leaves. We have obtained the same results as they did, however, with root uptake. A higher metabolic rate with increased uptake seems to be the most nearly acceptable theory. This is as Yarwood and Jacobson finally concluded. It is known that there are often variations in respiration during disease development. Owen (1958) stated that in N. glutinosa inoculated with TMV the respiration increased up to 30% at the time lesions appeared, the amount of change depending on the number of lesions. He also stated (1955) that respiration rates may vary according to several factors such as the time after inoculation, the physiological

state of the plants, the environmental conditions during growth, the age of leaves used, and the mode of expression of the results. The fact that respiration increased does not necessarily indicate that more energy was available for uptake.

The presence of uncoupling agents which prevent the formation of useful energy compounds such as ATP may cause increased respiration with the energy lost as heat. It is, therefore, possible to have a very high respiration rate and little uptake. Merrett (1962) has suggested that the increased respiration rate was due to a disorganization of the cell in such a way that more substrate was brought into contact with enzymes rather than simply due to the presence of uncouplers. The disorganization of the cell to allow more substrate in contact with enzymes may also be a factor in lesion formation. Waggoner and Dimond (1956) stated that the degree to which the polyphenyl oxidase system of a plant is active appears to depend on the amount of substrate available in the host plant. Disease may increase the substrate. They suggested that the freeing of phenols from conjugated phenols might accomplish this. The substrate would then be acted on by the polyphenyl oxidases to form quinones which, being toxic, may cause lesions. Although polyphenol oxidases do not occur in all plants, especially not in some legumes (Merrett, 1962) they have been shown to increase during virus infection in some plants (Merrett, 1962; Kammen and Brouwer, 1964; Farkas, et al., 1960; Solymosy, et al., 1959).

It is difficult to determine the relationship between lesion formation due to polyphenol oxidases, if this does occur, and the accumulation of sulfur in these lesions. It would seem that the toxicity brought about would have an uncoupling effect and prevent energy being used for uptake,

and yet S^{35} will accumulate in some lesions. Perhaps these lesions are formed by other than polyphenol oxidase activity, or perhaps cell disorganization causing the increase in oxidase activity is also sufficient to cause a great enough increase in respiration to produce enough useful energy to allow accumulation.

Further work might involve a correlation of respiration, polyphenol oxidase, and mineral uptake data using a single host-virus system. Comparison could then be made with other systems. Mechanical injury of the plant might also be further investigated in relation to polyphenol oxidase activity. Here the cells might be disorganized but would not be subjected to the other aspects of a virus disease such as the use of energy and metabolites in the reproduction of the pathogen. In respect to the work on D. stramonium as compared to N. glutinosa one should determine whether both have the same polyphenol oxidase behavior during infection since N. glutinosa inoculated with TMV shows S^{35} accumulation in virus lesions as shown in this research and by Yoshii et al. (1955), Yarwood and Jacobson (1955) and Shaw and Samborski (1956), but D. stramonium inoculated with BMV showed no accumulation in virus lesions, although there was some on mechanical injuries.

The results of the chromatography were rather unsatisfactory due in part to the low concentrations which made ninhydrin spots difficult to see. Many more replications would be necessary before any definite statements could be made concerning a greater or lesser amount of S^{35} regularly occurring in a particular metabolite on any one day following inoculation.

The lack of S^{35} in diseased D. stramonium leaves as shown by counts per minute would seem to be more in accordance with the presence of an

uncoupling agent than would the accumulation in the local lesions of *N. glutinosa*. Again, one should compare these results with further studies on respiration and polyphenol oxidase activity.

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DISTRIBUTION OF S^{35} IN LEAVES OF HEALTHY AND VIRUS INFECTED PLANTS

by

PATRICIA WAYNETTE HOOK

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The object of the investigation was to determine the pattern of distribution and amount of S^{35} in leaves of Datura stramonium infected with bromegrass mosaic virus (BMV). Similar work done on Nicotiana glutinosa and Nicotiana tabacum with tobacco mosaic virus (TMV) served as a comparison.

Plants were grown in nutrient culture in the greenhouse. S^{35} was added to the solution at the time of inoculation. Leaves were harvested after various lengths of times, and the amount and location of S^{35} was determined by one of three ways; autoradiography of leaves, chromatography and subsequent autoradiography of alcohol extracts, or determination of total S^{35} as counts per minute of ground samples of dried leaves.

No accumulation of S^{35} was found in the areas of lesions caused by BMV on D. stramonium. Accumulation was found, however, around the edges of necrotic tissue caused by mechanical injury such as scraping, tearing, or heating. On N. glutinosa S^{35} was found in newly developed TMV lesions and around the edges of old, necrotic lesions. N. tabacum, not a local lesion host, showed no apparent difference in S^{35} distribution pattern between healthy and symptom bearing leaves.

The chromatography showed no significant differences of amounts of S^{35} or S^{35} containing compounds. This was done with D. stramonium inoculated with BMV only.

The total S^{35} determinations showed that in D. stramonium inoculated with BMV with symptoms well developed the healthy tissue consistently had a slightly higher S^{35} content than did the infected tissue.

It was concluded that the three host-virus systems used vary in their accumulation of sulfur during the progress of the disease. This was attributed to changes in metabolism of the plant as a result of disease.