CHARACTERIZATION OF TOBACCO RINGSPOT VIRUS ISOLATED FROM KANSAS SOYBEAN

by

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I wish to express my deepest appreciation to Professors F. W. Schwenk and J. F. Schafer for guidance and encouragement throughout my period of study. I would also like to thank my good friends Richard Lee and Arvin Ghanekar for assistance rendered during my stay here at Kansas State University.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION

Tobacco ringspot virus (TRSV) was first discovered as the cause of a plant disease in varieties of Nicotiana tabacum L. in Virginia by Frome and Wingard in 1922 (14). TRSV was later found to cause a disease in soybeans by Price in 1934 (26), under experimental conditions. In agriculturally grown soybeans, TRSV induced disease was first observed by Samson (30) in Indiana. Melhus (24) and Johnson (20) found the disease in Iowa and Ohio. The disease of soybeans caused by TRSV was named bud blight by Allington (1) in 1946. Bud blight has been found throughout the soybean producing areas of the midwestern United States. Apparently the disease had existed earlier in soybean fields but investigators failed to recognize it.

Bud blight is a cyclic disease. Athow (3) reported high soybean losses from bud blight from 1943-1947 and from 1955-1957, and low losses during intervening years.

Many host range studies have been conducted on TRSV. Most of these studies have been condensed into one report by De Zeeuw (11) who lists 251 plant species in 54 families as hosts of TRSV. Many common weeds as well as cultivated plants are on the list. Several studies have been conducted concerning the spread of TRSV into and within soybean fields. Johnson (20), while observing damage caused by TRSV, noted that most infected plants were along fences and adjacent highways. Allington (1) also found that the disease advanced inward from the margins of soybean fields and suggested an insect was the vector. Athow (3)

recorded similar results. The advance of TRSV into the soybean fields was greatest when the fields were next to long-established grassy and weedy areas. Crittenden (7) found the largest number of TRSV-infected soybean plants closest to a lane and drainage ditch. Hill (19) using photographs taken of soybean fields from an airplane was able to demonstrate the importance of the prevailing winds in the spread of the disease. When the soybean field was down-wind from a mixed legume-grass-weed field the disease spread into the field 400-600 yards. When up-wind from such fields, the disease spread into the soybeans only 50-100 yards. An aerial vector was proposed. He also noted that mixed fields of legumes (particularly red clover and alfalfa), grasses, and annual and perennial weeds, favored the spread of the disease into the soybean fields.

Several vectors, insects and nematodes, have been implicated in the transmission of TRSV between plants. Fulton (15) discovered the American dagger nematode, Xiphinema americanum Cobb, to be a vector of TRSV to cucumber. Bergeson (5) discovered the nematodes ability to transmit TRSV to soybeans.

Dunleavy (13) was the first to demonstrate insect transmission of TRSV. He reported very low transmission of TRSV by the grasshopper Melanoplus differentialis Thos., between soybeans. The tobacco flea beetle Epitrix hirtipennis Melsheimer, was implicated as a vector of TRSV by Schuster (35), who reported transmission from eggplant to eggplant, cucumber, and black-eyed peas (cowpeas). In 1964, Bergeson (5) reported limited transmission of TRSV to soybeans by thrips, Thrips spp. and Frankliniella spp. Thomas (37) reported transmission of TRSV from soybeans to several other plant species by the red spider

mites Tetranychus telarius L. and T. desertorum Banks.

The evidence presented by these authors indicates a possible answer to the source and method of spread of TRSV into soybeans. The weeds of adjacent fields have been shown to be hosts of TRSV and several possible vectors have been found. The cyclic nature of the disease may be caused by the reduction in the population of the vector due to severe conditions or simply to its preference of other plants for food during wetter years.

Seed transmission of TRSV was investigated in this study and is the subject of Part II of this report. In Parts I and III, isolates of TRSV from Kansas were examined for similarities in host range and physical properties.

GENERAL METHODS AND MATERIALS

Nine isolates of TRSV were collected from soybeans in south east Kansas during July and August 1971. They were confirmed as TRSV by serology, using anti-serum provided by S. H. Smith, Pennsylvania State University. The isolates were designated Cherokee Co. #1, MV#8, MV#6, MV#2, MV#9, Field#2, MV#1, Field #3, and Field #4. Four strains from tobacco in North Carolina, NC-38, NC-39, NC-87, and NC-72, were provided by G. V. Gooding Jr., North Carolina State University.

All isolates and strains were maintained on Vigna sinensis Endl. (cowpea var. Early Ramshorn) in the greenhouse. All transfers were made during the period of greatest expansion of the primary leaves. Inoculations were accomplished using carborundum as an abrasive, 0.02 M $KHPO_4^-$ (pH7) as the buffer, and the pestle as the applicator.

PART I HOST RANGE STUDIES

A study was undertaken to deduce any differences in the host ranges of the nine Kansas isolates, and four strains of TRSV from North Carolina.

Procedure: Nineteen plant types were tested for susceptibility to the Kansas isolates and North Carolina strains. The plant types used are listed in Table 1.

In most of the tests, the primary leaves were inoculated, but older leaves were inoculated on Lycopersicon esculentum Mill. (tomato), all Nicotiana spp., Physalis floridiana Rydbg., Glomphrena globosa L. (globe amaranth), and Lens culinaris (lentils).

Inoculated plants were kept for varying lengths of time due to their different growth rates. All plants were tested for local as well as systemic infection by re-inoculating to cowpea.

For plants not susceptible to a particular TRSV isolate or strain after the first test, the test was repeated again in the greenhouse and then again, if still negative, in the growth chambers. If all isolates and strains tested negative, no further tests were made.

The only factor considered in this test was whether or not the particular plant type was susceptible to the various TRSV isolates and strains. Variation in symptoms between isolates and strains was not considered as a reliable indication of difference, due to the variable environmental conditions in the greenhouse. For example, under winter conditions, cowpeas developed large dark brown local lesions. Under summer conditions, however, the lesions were surpressed and

Table 1. Reaction of plant types to inoculation of Kansas isolates and North Carolina strains of TRSV.

		Local infection	Systemic infection
1	Lycopersicon esculentum (tomato)	-	-
2	Nicotiana tabacum (tobacco var. Turkish)	+	-
3	Nicotiana tabacum (tobacco var. Xanthi)	+	-
4	Nicotiana rustica (tobacco)	+	+
5	Physalis floridiana	+	+
6	Citrullus vulgaris (watermelon)	-	-
7	Glomphena globosum (globe amaranth)	+	+
8	Cucurbita maxima (squash var. Acorn)	+	+
9	Cucurbita maxima (squash var. Butternut)	+	-
10	Pisum sativum (pea var. Early Alaska)	+	+
11	Pisum sativum (pea var. Laxton's Progress)	+	+
12	Lens culinaris (lentils)	+	+
13	Cucumis sativus (cucumber var. National Pickling)	+	+
14	Phaseolus vulgaris (bean var. Lima)	+	+
15	Phaseolus vulgaris (bean var. Black Valentine)	+	+
16	Phaseolus vulgaris (bean var. Pinto)	+	+
17	Phaseolus vulgaris (bean var. Michigan Navy)	+	+
18	Phaseolus vulgaris (bean var. Red Kidney)	+	+
19	Phaseolus vulgaris (bean var. Great Northern)	+	+

appeared as cresents or yellow necrotic areas. More variation occurred between similar plants inoculated with the same virus than among similar plants inoculated with various isolates.

Results: When a particular plant type was inoculated with each of the Kansas isolates and North Carolina strains of TRSV, there was no variation in susceptibility. If the plant type was not susceptible, it was not susceptible to any isolate or strain of TRSV used. Tomato is an example of such a plant type. The same pattern held true for those plant types locally susceptible as well as those systemically susceptible.

PART II SEED TRANSMISSION

The condition known as mottling in soybeans has been known for many years. It was first described by Woodworth (40) in 1922, as "the formation of irregular patterns or streaking of black and brown pigments on yellow or green seed coats." It was concluded that mottling was affected by the environment. Owen (25) came to similar conclusions. He found that rich loam soil and thin planting favored mottling while sandy soil and thick planting did not. Some of the plants producing severe mottling, were suspected of having virus infections, but this could not be confirmed.

Recent studies have shown an association between soybean mosaic, mottling, and seed transmission. Ross (29) found that mottled Lee soybean seeds produced 18.2% soybean mosaic virus (SMV) infected plants while normal appearing seeds from the same plants produced 8.2% infected plants. Kennedy (21) studied the genetics of mottling and found that mottling is controlled by a single gene. Resistance to mottling was dominant. However, plants resistant to SMV-induced mottling were still susceptible to SMV infection. Quiniones (27) using three varieties of soybean, confirmed the earlier report of Ross.

Gordon (16) demonstrated that severe bud blight of soybeans was caused by infection of both TRSV and SMV. Many investigators have shown a relationship between TRSV infection and seed transmission of TRSV. Desjardins (10) using Lincoln variety soybeans, reported seed transmission of 78 and 54% respectively in two tests. Athow (3) examined naturally infected Harosoy variety

soybeans, and found 10% seed transmission of TRSV. This investigation revealed that the time of infection was important to seed transmission. The earlier the infection, the greater the chance of transmission through the seeds. Crowley (8) using several varieties of soybeans and a Fulton isolate of TRSV, found that seed transmission of TRSV was less when the plants were inoculated after flowering had begun. Athow (2) studied the seed transmittance of TRSV-infected Harosoy variety soybeans grown from infected seed. In his two trials, he found transmission to be 93 and 88%. Randles (28) using an Australian TRSV isolate and Lincoln variety soybeans found seed transmission in 13 of 15 trials. This agrees with the earlier work of Desjardins. Unlike SMV, no association has been reported between TRSV infection and mottling.

Other species of plants have been found to transmit TRSV through their seeds. These include petunia (Petunia violacea Lindl.) Henderson (18), tobacco (Nicotiana tabacum L.) Valleau (39), lettuce (Lactuca sativa L.) Grogan (17), and dandelion (Taraxacum officinale Weber) Tuite (38).

Procedure: For each of six Kansas TRSV isolates, 10 seeds of soybean varieties Calland, Dare, Wayne, Kent, Cutler, and Columbus were planted on 1/19/72 and again on 2/1/72. On 2/17/72, 10 plants of each of the 2-week and 4-week-old soybean plants were inoculated with an isolate of TRSV; this was repeated for each of the six isolates. The infected soybean plants were maintained in the greenhouse. On 5/14/72, all the soybean pods were harvested and placed in paper bags and allowed to dry.

The dried soybean seeds were removed from the pods, counted, and then graded as dark (mottled) or light (non-mottled).

During the period 10/25/72 to 2/15/73, these soybean seeds from inoculated plants were planted in a staggered fashion. Twenty seeds were planted for each isolate-variety combination. The proportion of dark to light seeds planted, was proportionate of the total seed yield.

Upon reaching the 2nd or 3rd trifoliate leaf stage, the soybean leaves were assayed for infection on cowpea. During this time, abnormal looking soybean plants and their corresponding cowpea assay pots, were labeled. These tests were carried out in the greenhouse.

Results: The age of the soybean plants at the time of inoculation affected both yield and mottling of the seeds (Table 2-A). The soybean plants inoculated at four weeks yielded substantially fewer seeds than did the controls. In the case of the 2-week-old plants, the loss was more severe. Several of these isolate-variety combinations produced no seed at all. This confirmed earlier reports by Athow (2).

Seed-coat mottling, as found in these experiments, is associated with TRSV infection in soybeans. The age of the plant at the time of inoculation can affect the expression of the mottling symptom. Many of the soybean plants inoculated at 4 weeks produced mottled seed, while none of the control plants did. Also, none of the 2-week-old-TRSV-infected soybean plants produced mottled seed.

Seed coat mottling was associated with transmission of TRSV through soybean seeds in all but two instances. Seed produced by the 4-week as well as the

Table 2-A. Correlation of variety, virus isolate, and time of inoculation on yield, seed coat mottling, and seed transmission of TRSV in soybeans.

Isolate	Variety	# Seeds	%Light	%Dark	Transmission Light	Transmission Dark	Reduction of Yield
Cherokee	Dare	0					100%
Co. #1	Wayne	0					100
2-weeks	Columbus	0					100
	Cutler	0					100
	Calland	0					100
	Kent	0					100
Cherokee	Dare	19	100	0	0	-	80
Co. #1	Wayne	64	38	62	0	0	39
4-weeks	Columbus	24	0	100	-	3	87
	Cutler	51	8	92	0	2	66
	Calland	73	68	32	0	0	51
	Kent	30	10	90	0	1	80
MV#8	Dare	0					100
2-weeks	Wayne	0					100
	Columbus	0					100
	Cutler	0					100
	Calland	0					100
	Kent	0					100
MV#8	Dare	16	100	0	0	-	83
4-weeks	Wayne	32	0	100	-	0	70
	Columbus	8	0	100	-	0	96
	Cutler	43	35	65	0	0	71
	Calland	14	0	100	-	0	91
	Kent	7	100	0	0	-	95
MV#6	Dare	0					100
2 weeks	Wayne	0					100
	Columbus	0					100
	Cutler	0					100
	Calland	0					100
	Kent	0					100

Table 2-A (con't.)

Isolate	Variety	# Seeds	%Light	%Dark	Transmission Light	Transmission Dark	Reduction of Yield
MV#6	Dare	17	100	0	0	-	72
4 weeks	Wayne	21	24	76	0	0	80
	Columbus	6	0	100	-	0	97
	Cutler	44	48	52	0	0	70
	Calland	56	80	20	0	0	58
	Kent	0					100
MV#9	Dare	0					100
2 weeks	Wayne	0					100
	Columbus	0					100
	Cutler	0					100
	Calland	0					100
	Kent	0					100
MV#9	Dare	0					100
4 weeks	Wayne	56	100	0	0	-	47
	Columbus	2	50	50	0	0	99
	Cutler	0					100
	Calland	4	0	100	-	0	97
	Kent	32	100	0	0	-	79
Field #2	Dare	0					100
2 weeks	Wayne	17	100	0	0	-	84
	Columbus	23	100	0	0	-	87
	Cutler	0					100
	Calland	23	100	0	0	-	83
	Kent	26	100	0	0	-	83
Field #2	Dare	15	100	0	0	-	86
4 weeks	Wayne	14	0	100	-	0	87
	Columbus	5	0	100	-	0	97
	Cutler	62	81	19	0	0	58
	Calland	54	46	54	0	0	59
	Kent	28	93	7	0	0	81

Table 2-A (con't.)

Isolate	Variety	# Seeds	%Light	%Dark	Transmission Light	Transmission Dark	Reduction of Yield
Field #3	Dare	0					100
2 weeks	Wayne	40	100	0	0	-	62
	Columbus	45	100	0	0	-	75
	Cutler	44	100	0	0	-	70
	Calland	42	100	0	0	-	68
	Kent	48	100	0	0	-	68
Field #3	Dare	75	100	0	0	-	21
4 weeks	Wayne	77	92	8	0	0	27
	Columbus	90	85	15	1	0	50
	Cutler	87	82	18	0	0	41
	Calland	70	100	0	0	-	47
	Kent	70	76	24	0	0	53
Field #4	Dare	0					100
2 weeks	Wayne	0					100
	Columbus	38	100	0	0	-	79
	Cutler	0					100
	Calland	29	100	0	1	-	78
	Kent	24	100	0	0	-	84
Field #4	Dare	1	100	0	0	-	99
4 weeks	Wayne	52	42	58	0	0	50
	Columbus	10	0	100	-	0	94
	Cutler	52	0	100	-	1	65
	Calland	29	51	49	0	0	78
	Kent	29	83	17	0	0	81

2-week TRSV infected soybean plants, transmitted the virus (Table 2-B).

Discussions and Conclusions: This report both confirms and conflicts with some of the earlier literature on the subject. TRSV was transmitted by the soybean seeds but not at the high rate reported by some authors. This would indicate that with the isolates tested and under these conditions, seed transmission plays the role of maintaining a small source of inoculum. It probably has little to do with the cyclic epidemics reported, because the pattern of spread into the fields indicated the presence of an aerial vector bringing the disease in from the surrounding weeds.

TRSV can induce the soybean plants to produce mottled seed in plants inoculated at four weeks of age, but not in plants inoculated at two weeks of age. The reason for this is not known.

Table 2-B. Correlation of TRSV-isolate-soybean-variety with seed transmission of TRSV and seed grade.

Isolate-Variety	% Transmittance	Seed Grade
Cherokee Co. #1-Columbus	15	Mottled
Cherokee Co. #1-Cutler	10	Mottled
Cherokee Co. #1-Kent	5	Mottled
Field #3-Columbus	5	Non-mottled
Field #4-Cutler	5	Mottled
Field #4-Calland (2 weeks)	5	Non-mottled

PART III PHYSICAL PROPERTIES OF TRSV ISOLATES

Purification: A standard purification procedure was followed. Phaseolus vulgaris var. Red Kidney bean, supplied by Chester B. Brown Co., Morril, Nebraska, U.S.A., 69358, was used as the host plant from which the virus was purified. All plants were grown in sterilized sandy loam soil in 4-inch plastic pots, 6-9 plants per pot. The seeds were maintained in the greenhouse until emergence. At this time, they were placed on plastic trays and moved into a growth chamber. The plants were watered once a day by flooding the plastic tray. Growth chamber temperature was maintained at 30 C (day) and 25 C (night).

All inoculations were made onto the rapidly expanding primary leaves as described before, at five to seven days after planting. The plants were allowed to grow for 12 days, then harvested and purified immediately.

Purification was achieved in the following manner.

(1) Add 100 ml 0.02M $KHPO_4^-$(pH7) buffer; add 1 g ascorbic acid; adjust pH back to 7.0 with concentrated K_2HPO_4 solution; tissue (fresh) 100 g; blend 60 seconds in Waring blendor.

(2) Add 100 ml each of chloroform and butanol; blend 60 sec.

(3) Centrifuge 10,000 rpm (10,000 g) 20 minutes in 250 ml bottles in the JA-14 rotor; save aqueous phase.

(4) Centrifuge 35,000 rpm (96,000 g) 4.3 hours in the Al 35 rotor; resuspend the pellet in buffer.

(5) Centrifuge 10,000 rpm (8000 g) for 15 minutes in the JA-20 rotor; discard pellet.

(6) Centrifuge 60,000 rpm (256,000 g) for 75 minutes in the 60 Ti rotor; resuspend the pellet in buffer containing .01 M sodium ethylenediaminetetraacetate (EDTA).

(7) Centrifuge 10,000 rpm (8,000 g) for 10 minutes in the JA-20 rotor; discard pellet.

(8) Centrifuge 60,000 rpm (256,000 g) for 75 minutes in the 60 Ti rotor; resuspend the pellet in buffer.

(9) Centrifuge 10,000 rpm (8,000 g) for 5 minutes in the JA-20 rotor; discard pellet.

All 10,000 rpm centrifugations were performed in a Beckman model J-21 centrifuge and all 35,000 or 60,000 rpm centrifugations were performed in a Beckman model L2-65 B centrifuge. All rotor numbers refer to Beckman rotors. The final low-speed centrifugation (step 9) was performed using 15 ml Corex tubes.

A problem was encountered with purification during the first attempts. The purification procedure established by Stace-Smith (36), modifications of which most investigators of TRSV since have followed, failed to work. This procedure differed from the one described in that .02 M sodium diethyldithiocarbamate (DIECA) and .02 M 2-mercaptoethanol (2-mert) were present in step 1 as a chelating agent and an anti-oxidant, respectively. A density gradient separation of the purified virus preparation using this method revealed the presence of an unexpected peak, figure 1-A and B. This was true for the two hosts tried, Phaseolus vulgaris (bean var. Black Valentine) and P. vulgaris (bean var. Red Kidney) using several of the isolates. C. L. Niblett suggested that this might be degraded virus. This possibility was tested as follows. Two hundredths ml of RNase was added to .3 mg of the purified virus sample. The mixture was incubated at 30 C for 30 minutes in a water bath and then stored at 4 C overnight. Density gradient separation of this material showed that the peaks were removed, as seen in figure 2-A and B. This

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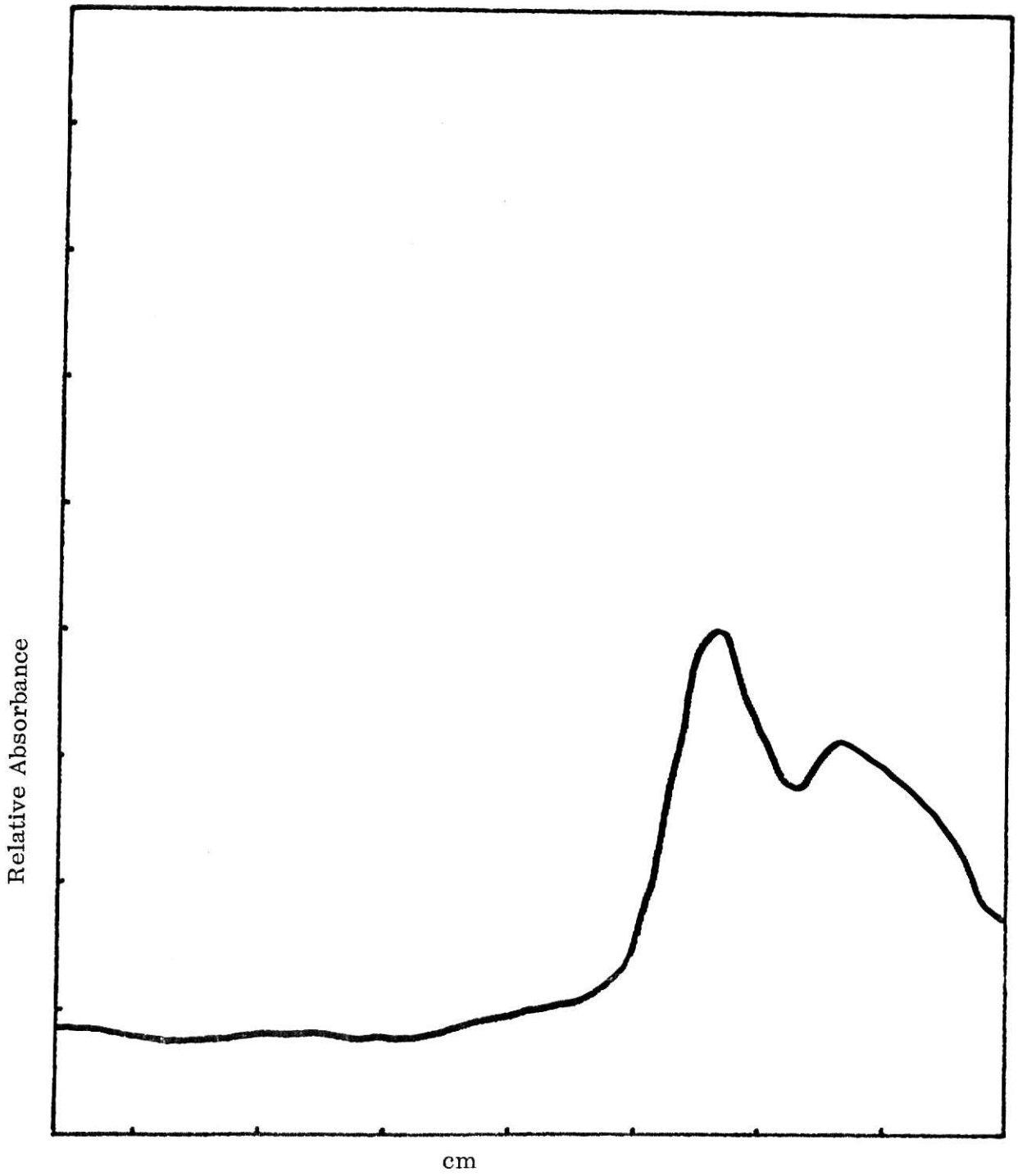

Figure 1-A. Six hour density gradient centrifugation of TRSV isolate MV#6 purified by the 2-mert and DIECA method. The host used was red kidney bean.

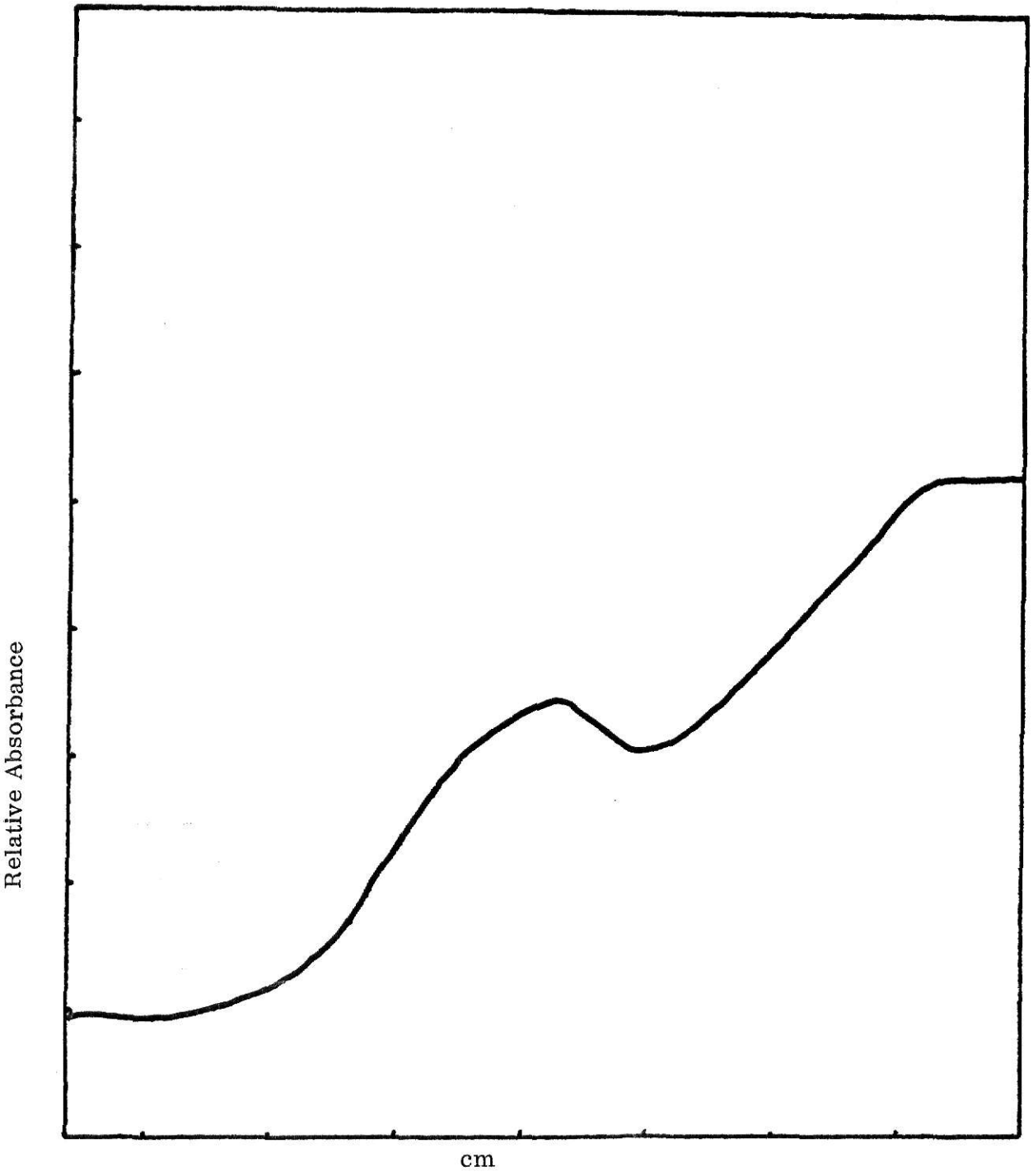

Figure 1-B. Six hour density gradient centrifugation of TRSV isolate Cherokee Co. #1 purified by the 2-mert and DIECA method. The host used was black valentine bean.

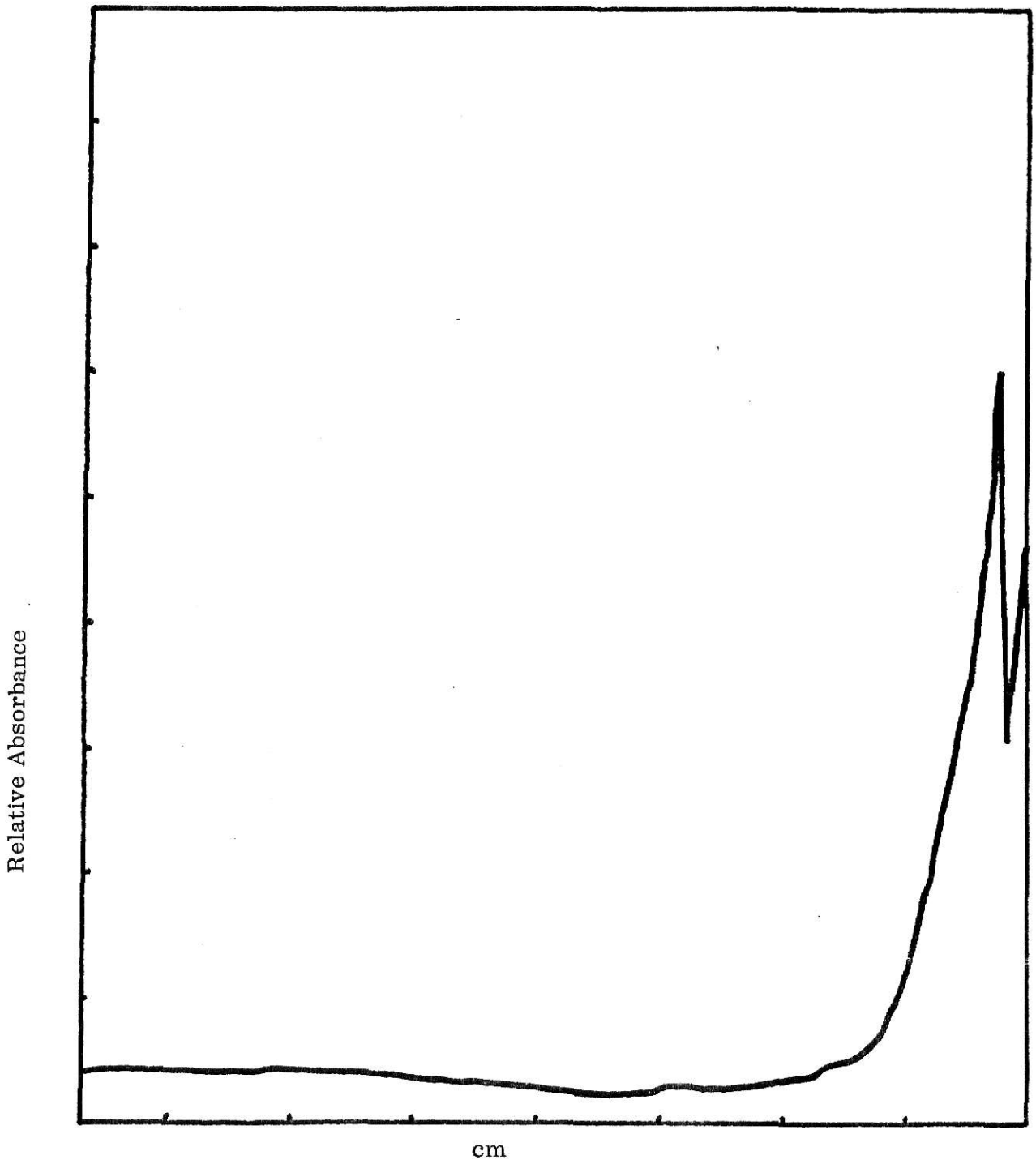

Figure 2-A. Six hour density gradient centrifugation of TRSV isolate MV#6 purified by the 2-mert and DIECA method, after incubation with RNase.

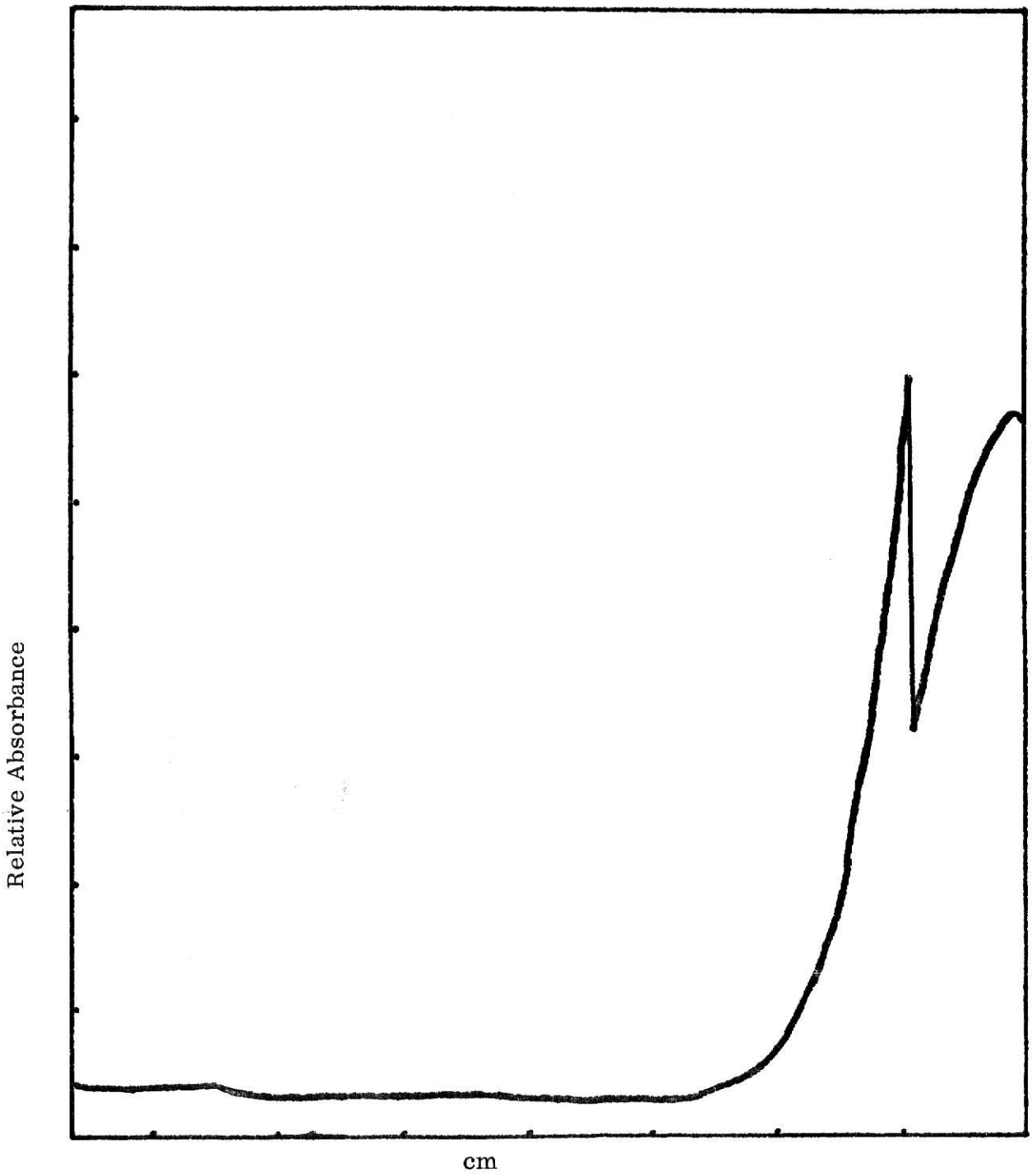

Figure 2-B. Six hour density gradient centrifugation of TRSV isolate Cherokee Co. #1 purified by the 2-mert and DIECA method, after incubation with RNase.

proved that the TRSV was partially denatured when purified by the 2-mert and DIECA method.

The following experiment was run to attempt to determine why the 2-mert and DIECA purification method failed to work. Isolate MV#6 purified by the ascorbic acid method was used. One third mg of purified virus in .04 ml buffer was added to solutions of 2-mert and DIECA in the following manner.

Tube #1 - 0.04 ml MV#6 sample + 2 ml 0.01 in DIECA

Tube #2 - 0.04 ml MV#6 sample +2 ml 0.01 M 2-mert sol.

Tube #3 - 0.04 ml MV#6 sample +1 ml 0.01 M DIECA sol. +1 ml 0.01 M 2-mert sol.

Tube #4 - 0.04 ml MV#6 sample + 0.02 M phosphate buffer.

All solutions were incubated at room temperature for 30 minutes and then stored at 4 C overnight. One ml aliquoits of each were then layered onto sucrose gradients and centrifuged at 82,000 g for 3 hours.

The results, as seen in figure 3-A, B, C, and D, indicate that the 2-mert and DIECA have no harmful effects upon the purified virus. The large absorbance pattern seen at the top of the gradient of tubes #1, 2 and 3 represents 2-mert and DIECA, not degraded virus.

The protection mechanism afforded by the 2-mert and DIECA, as the anti-oxidant and chelating agents, failed to work. Preliminary experiments with substitutes indicated that ascorbic acid would work and this was used in all succeeding purifications, in the manner described in purification.

Using the results of six isolate purifications, purity and yield averages

were obtained. The average 260/280 reading was 1.84, placing it in the acceptable range for TRSV as reported by Ladipo (22). Based on an extinction coefficient of 10 (32), the average yield was 1 mg of virus for every 13.3 g of bean tissue.

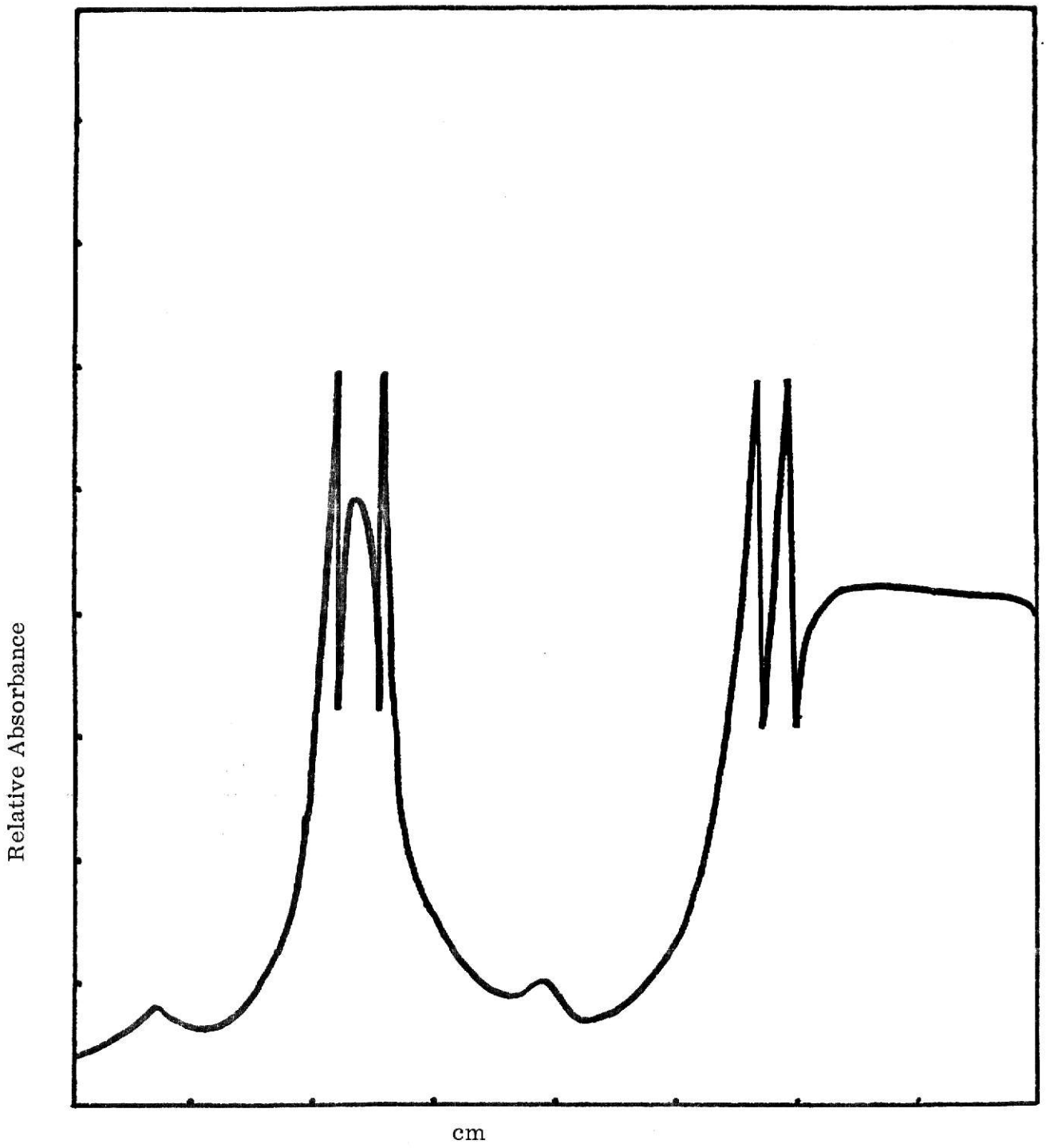

Figure 3-A. Three hour density gradient centrifugation of TRSV isolate MV#6 purified by the ascorbic acid method, after incubation in DIECA.

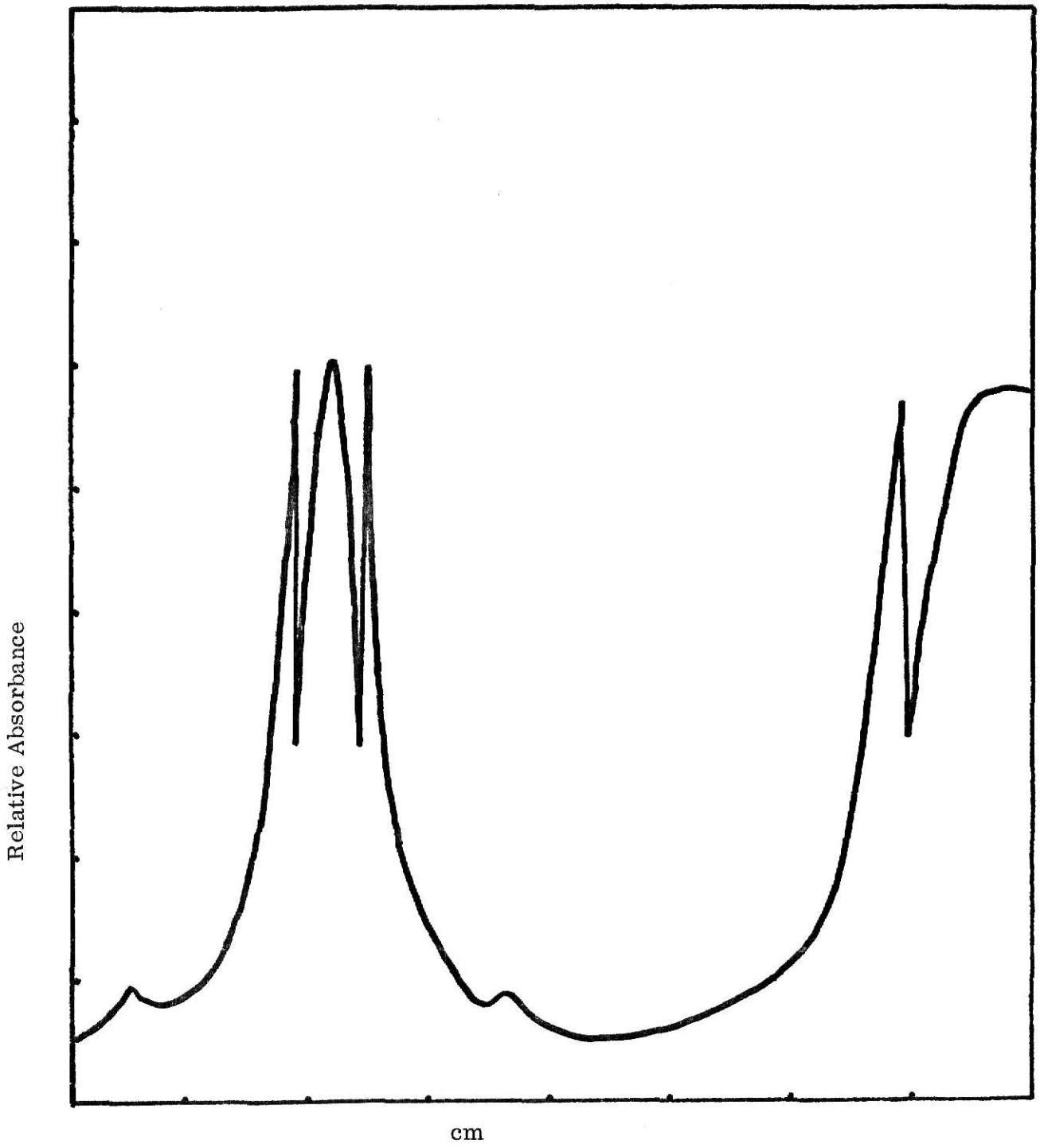

Figure 3-B. Three hour density gradient centrifugation of TRSV isolate MV#6 purified by the ascorbic acid method, after incubation in 2-mert.

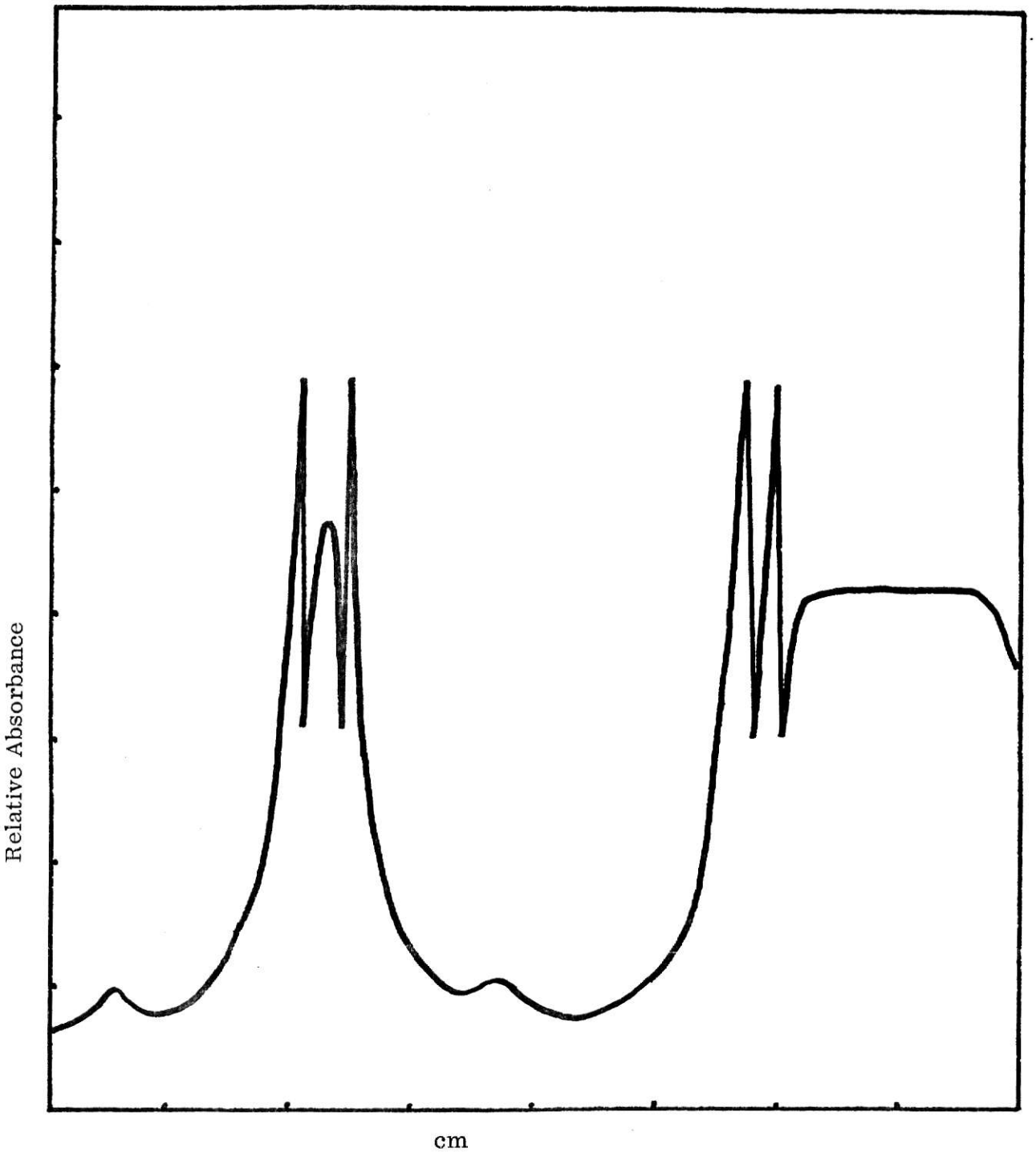

Figure 3-C. Three hour density gradient centrifugation of TRSV isolate MV#6 purified by the ascorbic acid method, after incubation in DIECA and 2-mert.

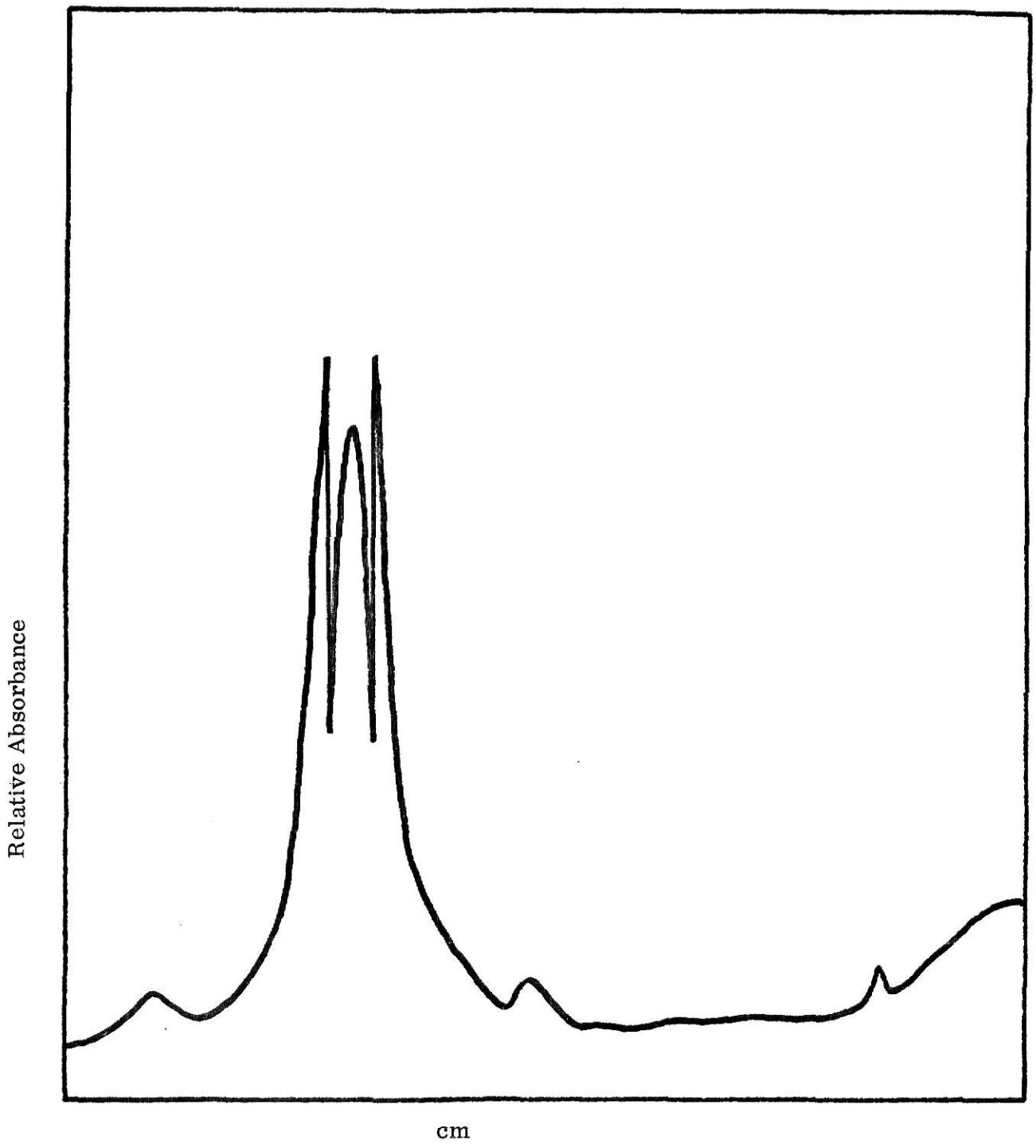

Figure 3-D. Three hour density gradient centrifugation of TRSV isolate MV#6 purified by the ascorbic acid method, after incubation in phosphate buffer.

DENSITY GRADIENT CENTRIFUGATION AND SATELLITE VIRUS

Density gradient centrifugation was undertaken to elucidate any differences in the sedimentation patterns of the nine Kansas isolates of TRSV. All the isolates have similar sedimentation patterns composed of three components, designated top (T), middle (M), and bottom (B). The s unit for each component was calculated.

Log-linear sucrose gradients were used as developed by Brakke (6) (Table 3). Each sucrose solution was layered under the preceeding solution by the use of long thin funnels. The gradients were allowed to stand for 48 hours at 4 C. A Beckman 27 swinging bucket rotor was used.

Immediately prior to the run, 1.5 ml of the gradient solution was removed from the top of the gradient and replaced with 1.5 ml buffer containing 1.5 to 3 absorbance units of sample. The tubes were centrifuged for 3 hours at 25,000 rpm (82,000 g). At the end of the centrifugation all tubes were scanned using an Isco Model D Density Gradient Fractionator and Isco U. V. scanner set at 254 mm, attached to a Model 490 Power Supply (Instrumentation Specialities Co., Inc., Lincoln, Nebraska, U.S.A.) and a Colman 165 Recorder.

All nine Kansas TRSV isolates have similar sedimentation patterns, all of which demonstrate three components. This is in agreement with the patterns reported by Stace-Smith (36) and Schneider (33). The sedimentation patterns for three representative isolates are illustrated in figure 4-A, B and C. The three isolates used are Field #3, Field #4, and MV#9. All illustrate the three peak arrangement.

The s unit for each peak was determined. Cowpea mosaic virus (CPMV) and

Table 3. Formula for making Log-linear sucrose density gradients as developed by Brakke.

Tube and Sol. No.	Grams Sucrose	Ml Buffer	Amount Layered on Gradient
1	0	18	3
2	3.0	23.2	3.5
3	9.2	44.5	5.5
4	11.5	43.1	7
5	13.5	41.9	7.8
6	19.0	51.1	8.4
7	8.35	20	3
			38.2 ml

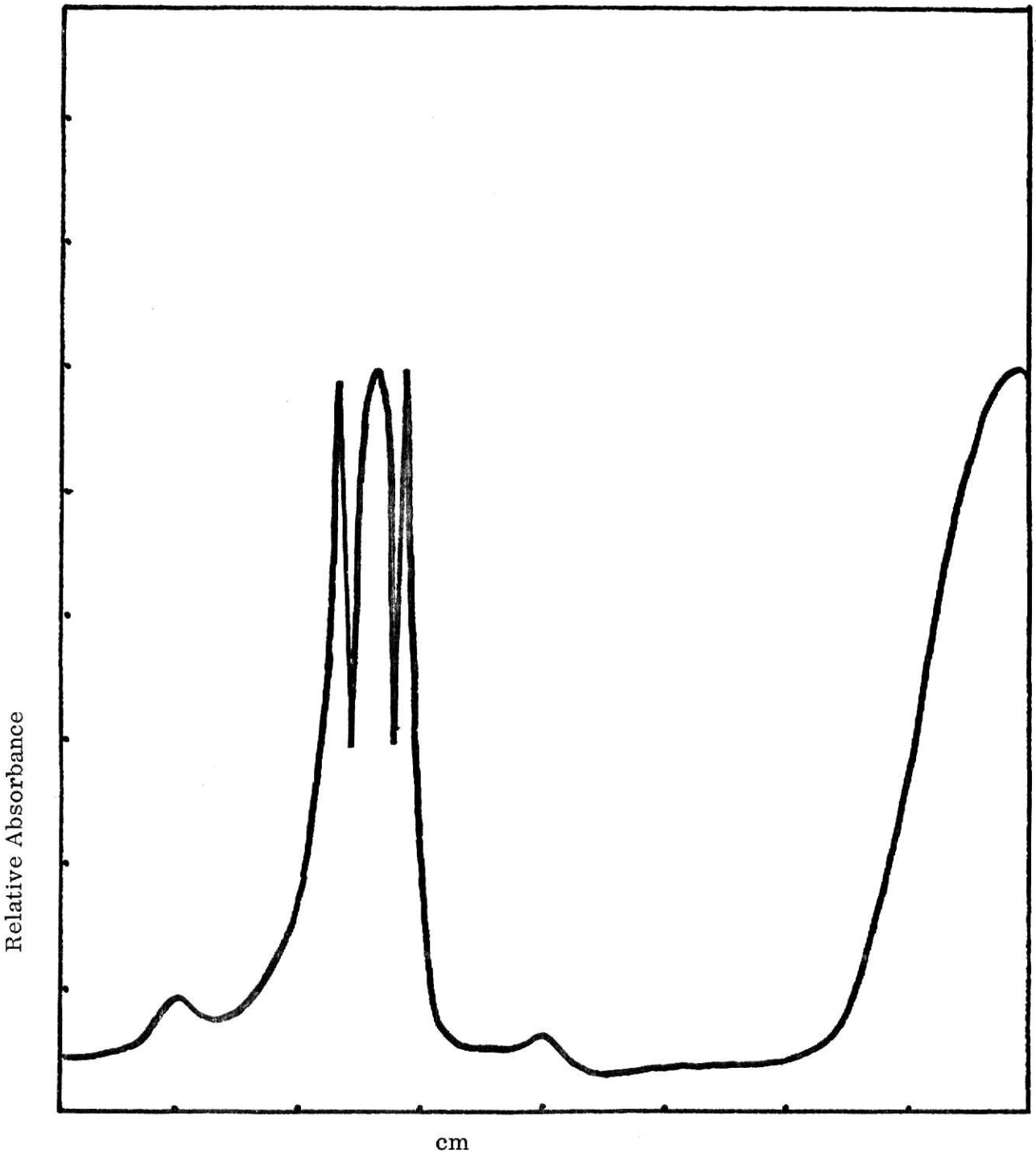

Figure 4-A. Sedimentation pattern for Kansas TRSV isolate Field #3. Centrifugation time, 3 hours.

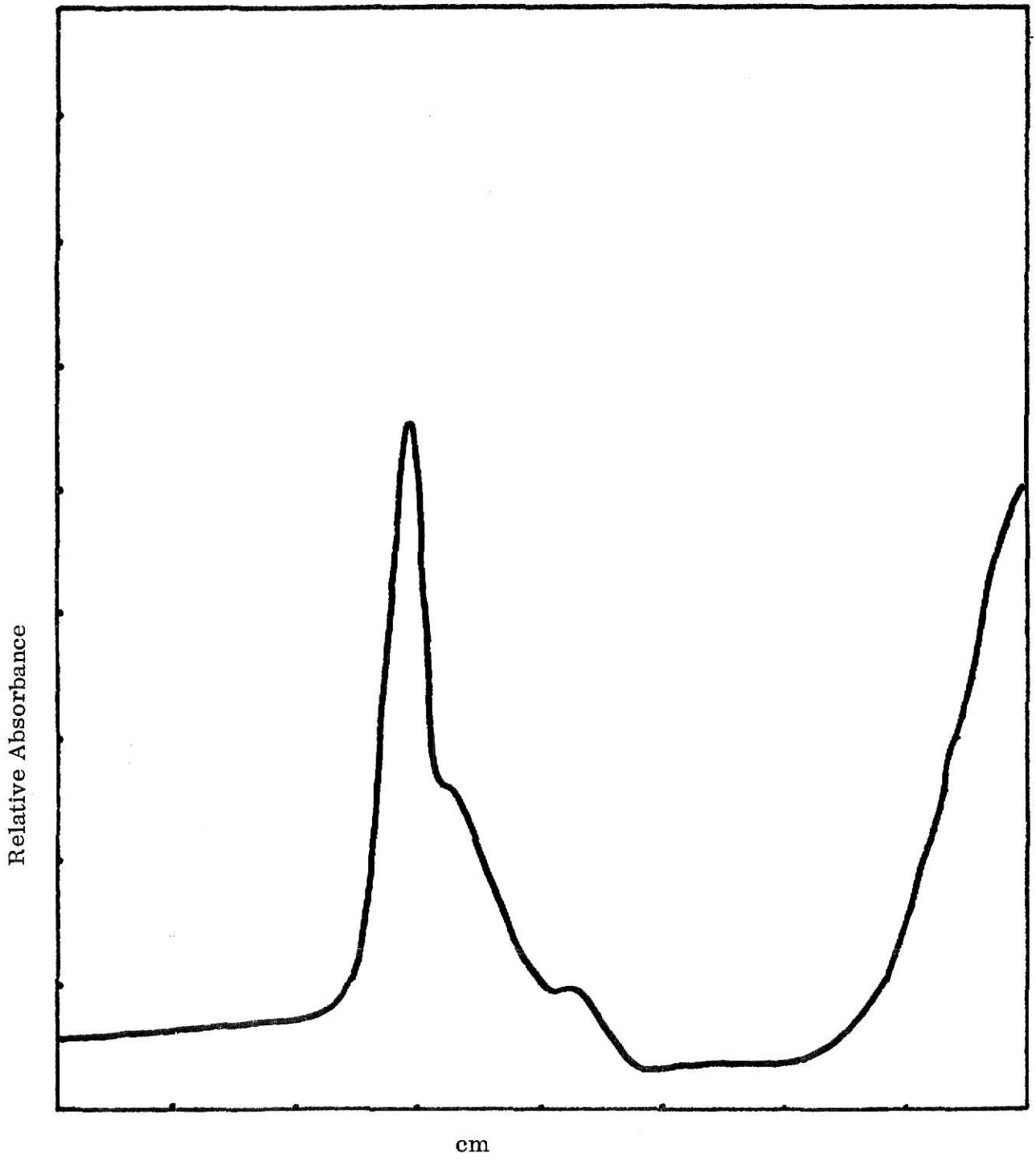

Figure 4-B. Sedimentation pattern for Kansas TRSV isolate Field #4. Centrifugation time, 3 hours.

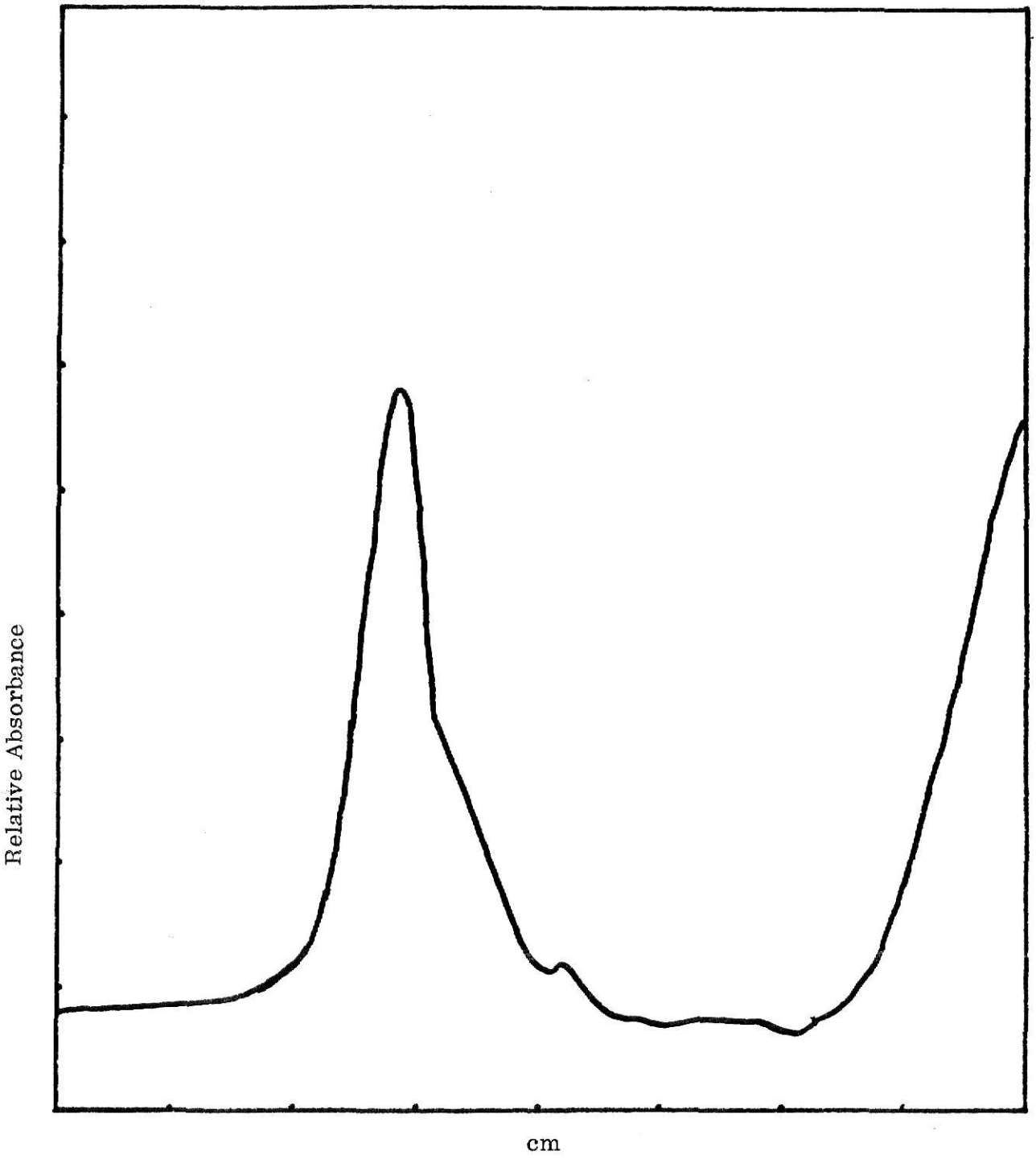

Figure 4-C. Sedimentation pattern for Kansas TRSV isolate MV#9. Centrifugation time, 3 hours.

brome mosaic virus (BMV), with known s units were centrifuged on the standard gradient. Using the number of cm the viruses advanced from the starting point on the chart paper, and plotting these figures against the known s units on graph paper, a standard curve was obtained.

The s unit for each component of the Kansas isolates was calculated using the standard curve. The results are 57.75 s for the top component, 96 s for the middle component, and 128.8 s for the bottom component. These values agree closely with the 53, 94, and 128 s values of Stace-Smith (36) and the 53, 91, and 126 s values of Schneider (33).

Some variations appear in the sedimentation patterns from the Kansas isolates. The top component appears as either one or two low peaks. The middle and bottom components are present in all samples. There are, however, two more unidentified peaks which appear at random in the purified isolates.

The first of these unidentified components appears below the bottom component and is designated sub-bottom. A good example of this peak is in figure 4-A. The sub-bottom component has a calculated s value of 173. This component is erratic in its appearance in the purified samples. It appears in isolate Field #3, during the s value determinations but failed to show up in later purifications. Also, it is not limited to isolate Field #3, but appears sporadically in other isolate purifications. Due to the erratic nature of this peak, it is suggested that it is an artifact of purification. Its s unit value suggests that it may be an aggregation of the top (53 s) and bottom (126 s) components. Ladipo (22) reports finding a 4th component as well as the normal three. This agrees with the data presented.

The second of the extra peaks which appear in the isolates in a sporatic manner can best be observed in figure 4-B. It appears as a shoulder on the 3rd component peak between the 2nd and 3rd components. This peak is suspected to be satellite virus.

The satellite virus of TRSV (S-TRSV) was originally discovered by Schneider (31) in a laboratory isolate. A second isolate was discovered by Schneider (32) in an isolate of TRSV from tobacco in North Carolina. S-TRSV is serologically identical to the isolate of TRSV with which it is multiplying.

Schneider (31) reported the s value for intact S-TRSV as 122, and the s value for S-TRSV RNA as 7.3. Also reported was a peculiar pattern of growth for S-TRSV. When grown in mixed populations with TRSV, the S-TRSV became the dominant particle present in systemically infected Black Valentine bean tissue 7 days after inoculation and represented 90% of the virus yield. Using this information, several isolates from Kansas, Field #3, Field #2 and Field #4, were examined for evidence of S-TRSV.

As was reported earlier, several isolates of TRSV exhibit a shoulder or hidden peak on the 3rd component. Using the standard curve, the s value for that peak was found to be 123.5.

The growth pattern of all three isolates was examined. All isolates were maintained in Red Kidney bean in the growth chamber. For isolates Field #2 and Field #3, one half the unifoliate leaves were harvested on day 8, after inoculation, and the other half on day 10. On day 12, the trifoliate leaves were harvested. Leaves of plants inoculated with isolate Field #4 were harvested in a similar

sequence of days, 5, 7, and 10.

Isolates Field #2 and Field #3 both exhibited sedimentation patterns consisting of three components. The pattern from day 12, purified virus from systemically infected leaves, showed no dominant peak between the middle and bottom components as Schneider had found. The sedimentation patterns for the 5, 7, and 10 day samples of isolate Field #4 exhibited only the bottom component. Why the middle and top peaks failed to show up is not known. This evidence, however, indicated the lack of a satellite virus in the samples.

An examination was made of the RNA content of the 5, 7 and 10 day samples of isolate Field #4. This was accomplished using a modified phenol-detergent method devised by Schneider (33). Sodium laural sulfate was used in place of sodium dodecyl sulfate.

To calculate the s values of the RNA, RNA with a known s value (P. Ahana, Kansas State University) was run along with the TRSV samples in a linear-log sucrose gradient (6). The IEC 41,000 swinging bucket rotor and tubes were used. All samples were run for 6 hours at 14 C. Figure 5-D shows the standard RNA with 18 and 28 s components. A standard curve was calculated. Figure 5-A, B and C show the sedimentation patterns of the TRSV RNA from the 5, 7 and 10 day samples.

Diener (12) reported that whole TRSV contained 2 RNA particles, 24 s (noninfectious) and 32 s (infectious). The 24 s RNA particle represented 80% of the sample. As noted earlier, S-TRSV has a 7.3 s RNA particle.

Calculations made from the 5, 7 and 10 day samples reveal the presence of

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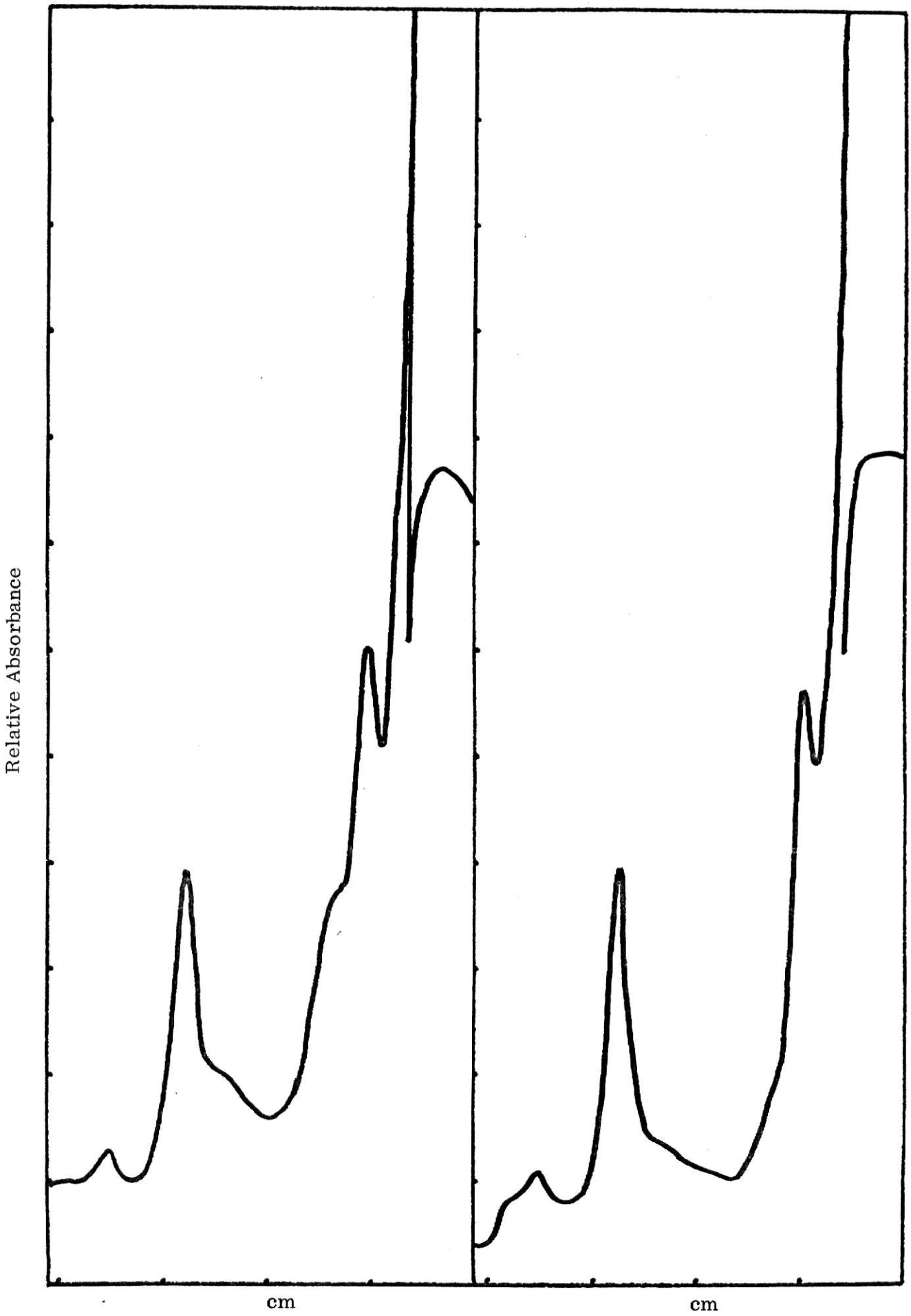

Figure 5-A and B. Six hour centrifugation patterns for isolate Field #4 RNA preparation.

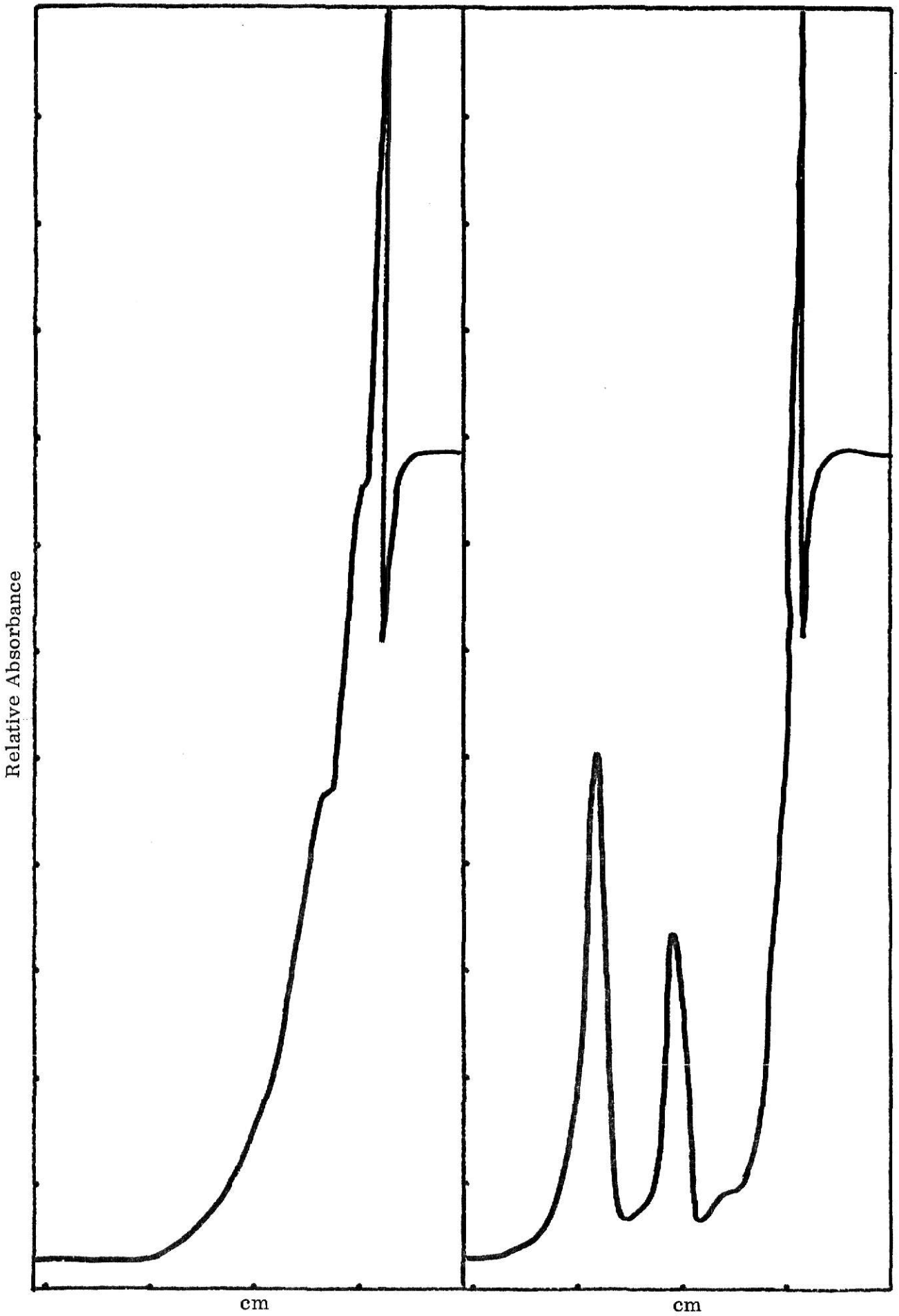

Figure 5-C and D. Six hour centrifugation patterns for isolate Field #4 and known

three RNA particles of about the same s units in each sample. The values are slightly but consistantly high, suggesting an error in the procedure rather than the conclusion. The s units calculated are 9.5, 26.5, and 33.5. The 26.5 s RNA particle was definitely the dominant particle as would be expected in TRSV.

In figure 5-C, the 10 day sample, the 26.5 and 33.5 s RNA particles are absent. This agrees with the previously described growth pattern for S-TRSV whereby the satellite became the dominant particle in the systemically infected tissue. Based on this evidence, it is assumed that S-TRSV RNA was contained in the samples.

The evidence presented indicates the presence of S-TRSV, of the type discovered by Schneider, in the isolates of Kansas TRSV. This conclusion is based principally upon the sedimentation coefficients of whole S-TRSV and S-TRSV RNA.

ELECTROPHORESIS

The procedure followed was that of Davis (9) with 2.8% polyacrylamide sample gels polymerized with 10% ammonium persulfate as described by Lee (23). Glass tubes, 6 x 63 mm, were used. All samples were run at 300 volts (constant) for 45 minutes. Aniline blue black (1 g/200 ml 7% acetic acid) was used as the protein stain. Gels soaked overnight were destained by exposing the stained gels to constant current (30 mV) for approximately 3-4 hours. Three percent acetic acid was used to fix the virus in the gel prior to scanning with the Gilford spectrophotometer. Two hundredths molar K_2HPO_4-KH_2PO_4 (pH7) buffer was used to maintain the integrity of the virus prior to grinding and inoculation onto cowpeas. An 18 hour interval was allowed between the grinding and inoculation to facilitate the release of the virus from the gel. Glass beads aided the grinding.

The protein stain and spectrophotometric scan revealed four bands in the gel. All nine isolates produced similar patterns, figure 6-A, B, C and D. Gels were ground up in an attempt to recover the virus. The first attempt was made without the aid of glass and the 18 hour interval, and was unsuccessful. The second attempt was more successful and revealed the presence of the virus in the slowest band (Table 4).

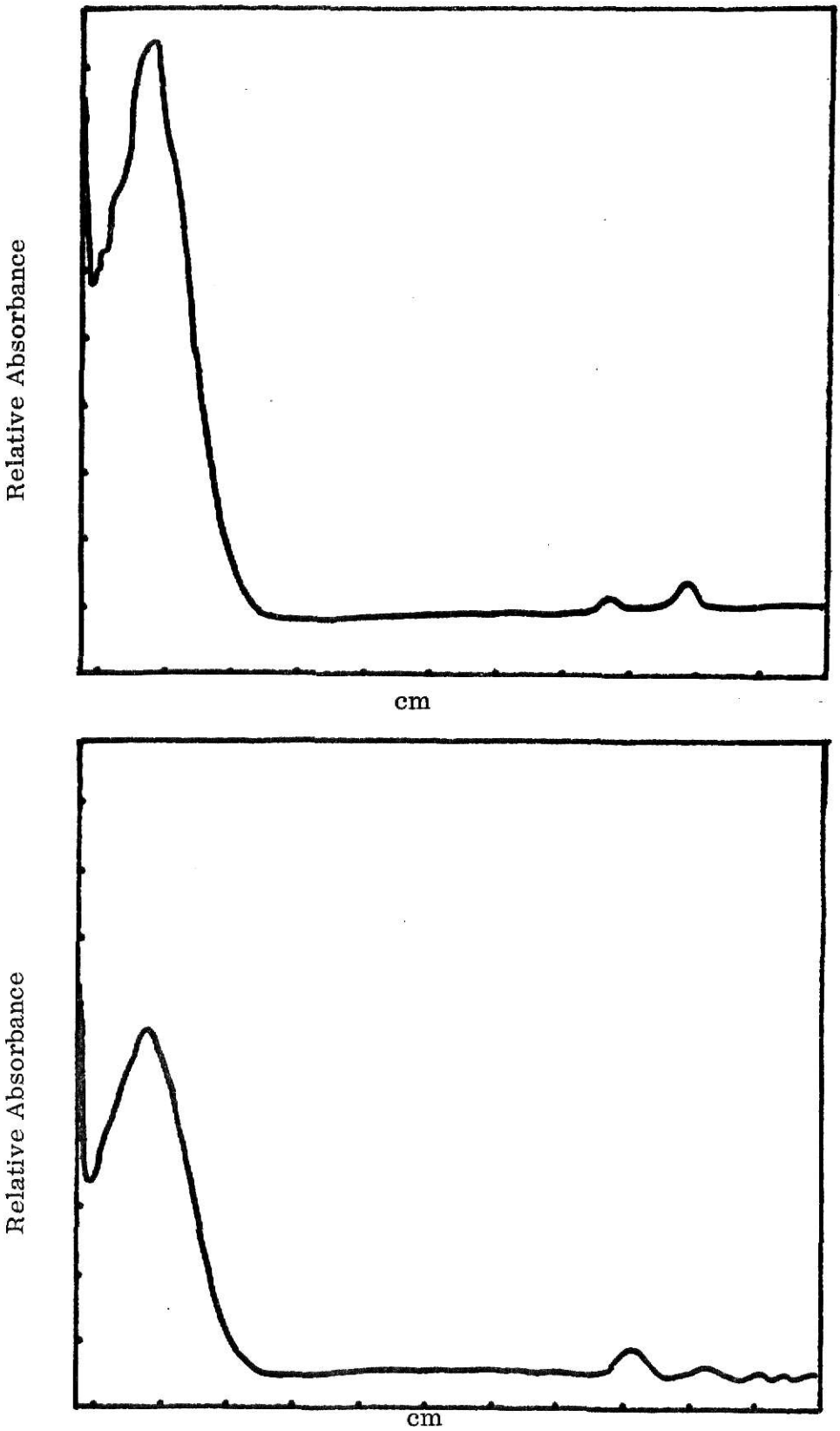

Figure 6-A and B. Electrophoretic gel scans of TRSV isolates Cherokee Co. #1 and MV#6.

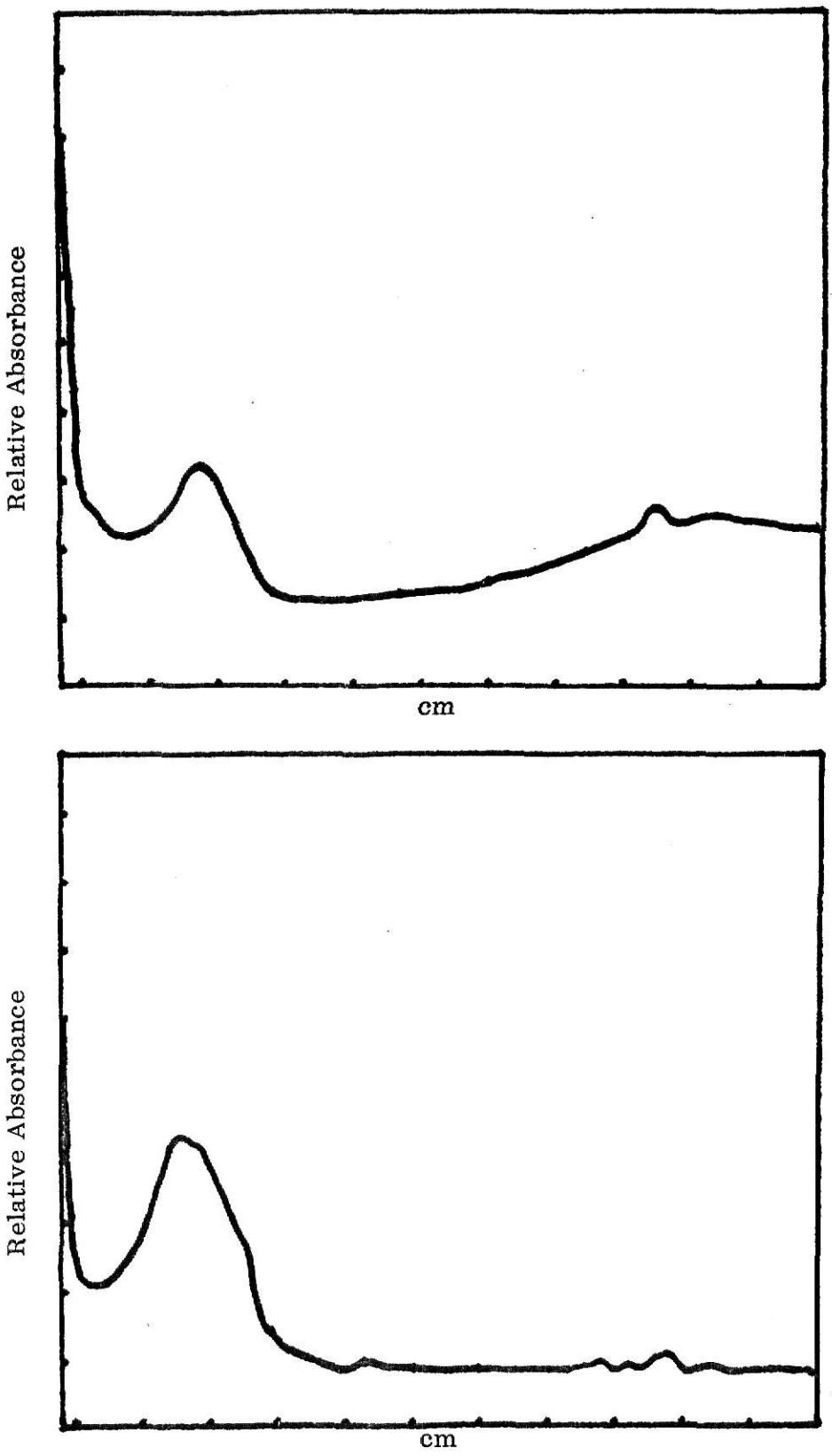

Figure 6-C and D. Electrophoretic gel scans of TRSV isolates Field #3 and Field #2.

Table 4. Correlation between bands and TRSV content of gels.

Isolate		1st Attempt		2nd Attempt	
		Top	Bottom	Top	Bottom
1	Cherokee Co. #1				
2	MV#8			+	
3	MV#6			+	
4	MV#2				
5	MV#9	+		+	
6	Field #2				
7	MV#1	+		+	
8	Field #3			+	
9	Field #4				
10	NC-38				
11	NC-39			+	
12	NC-87			+	

SEROLOGY

Anti-serum was produced in rabbits against two of the Kansas isolates, Cherokee Co. #1 and Field #3. Field #3, based upon the seed transmission experiment, appeared to be less destructive than the other isolates, and was chosen for this reason. Cherokee Co. #1 was chosen as a representative of the other isolates. Agar double diffusion tests indicated a difference between Cherokee Co. #1 and Field #3.

Cherokee Co. #1 and Field #3 isolates were increased in Red Kidney bean in the growth chamber and purified as described before. Both isolates were further purified by collecting the virus fractions from sucrose density gradient centrifugations. The virus was pelleted out of the sucrose by one 60,000 rpm centrifugation in the 60 Ti rotor, and resuspended in .02 M phosphate buffer.

Injections into rabbits were made as described in Table 5. Intraveinal injections were made in the ear after raising the vein with xylene. Intramuscular injections were made in the upper hind leg, using equal quantities of virus preparation and Freunds incomplete adjuvant.

The rabbits were bled twice. Serum was collected from the clotted blood. Its effectiveness was established. The titer for both anti-serum types was 1:256.

An Ouchterlony agar double-diffusion test was prepared as described by Ball (4). Sodium azide, 0.01 M was substituted for merthiolate.

Using the test, a difference between Cherokee Co. #1 and Field #3 was detected. The precipitation zone between Cherokee Co. #1 and the anti-serum, ex-

tended beyond, as a spur, the precipitation zone between Field #3 and the antiserum. It is thus concluded that while Cherokee Co. #1 and Field #3 are both TRSV, they are distinct strains.

Table 5. Schedule and method of injection.

Isolate	Date	Amt. Inject.	Where Inject.
Cherokee Co. #1	6/20/73	1 mg	vein
	6/28/73	1 mg	muscle
	7/ 5/73	1 mg	muscle
	7/13/73	1/2 mg	muscle
	7/18/73	1/2 mg	muscle
Field #3	6/20/73	1 mg	vein
	6/28/73	1 mg	muscle
	7/ 5/73	1 mg	muscle
	7/13/73	1/2 mg	muscle
	7/18/73	1/2 mg	muscle

DISCUSSION AND CONCLUSION

The purpose of this study was to examine Kansas isolates of TRSV in the areas of host range, seed transmission through soybeans, and physical properties.

The host range study indicated that all nine isolates had similar host range patterns with the hosts used. The common Kansas soybean varieties, Dare, Wayne, Columbus, Cutler, Calland, and Kent, were all susceptible to all the TRSV isolates. The lack of symptom variation between isolates suggested that all isolates were similar.

Seed transmission of TRSV through soybeans was very low. Because of this, seed transmission can maintain a small source of inoculum in the field. It would not be expected, however, to play a primary role in the periodic epidemics of bud blight. As reported by other authors, the pattern of spread of the disease into the field indicated the source of inoculum to be outside the field. An aerial vector appeared to be involved.

Mottling of soybean seeds can be caused by TRSV. Mottled seeds failed to develop on soybeans inoculated at the age of two weeks while plants inoculated at four weeks developed mottled seeds. The mechanism by which the younger plants failed to produce mottled seed was not determined.

Mottling developed among the different isolates as well as among the different varieties. For example, mottling was associated with Cherokee Co. #1 isolate inoculated 4-week old soybeans of varieties Wayne, Columbus, Cutler, etc. Also, mottling was associated with 4-week soybean variety Wayne inoculated with isolates Cherokee Co. #1, MV#8, MV#6, and others.

Soybean variety Dare failed to produce any mottled seed.

The physical properties revealed few differences. Similar patterns were noted in all sucrose density gradient centrifugations as well as electrophoretic patterns of whole virus. Serology revealed a difference between the two isolates used. No other differences were noted between these two isolates except in the seed transmission experiment. In this study, Field #3 is less pathogenic than the other isolates used, and reduces yield less. The significance of this is not known.

The satellite virus discovered in Kansas isolate Field #4 had similar properties of the satellite virus discovered by Schneider. It was concluded therefore, to be a satellite of TRSV.

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CHARACTERIZATION OF TOBACCO RINGSPOT VIRUS ISOLATED FROM KANSAS SOYBEAN

by

JONATHAN RONALD MUNDT

B. S., Kansas State University, 1970

AN ABSTRACT OF A MASTER'S THESIS

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requirements for the degree

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Manhattan, Kansas

1973

The purpose of this study was to examine nine Kansas isolates of tobacco ringspot virus (TRSV) in the areas of host range, seed transmission through soybeans, and physical properties.

The host range study indicated that all nine isolates had similar host range patterns with each host used. The common Kansas soybean varieties, Dare, Wayne, Columbus, Cutler, Calland and Kent, were all susceptible to all the TRSV isolates. On this basis, all nine isolates appear to be similar.

Seed transmission of TRSV through soybeans was very low. Because of this, seed transmission can maintain a small source of inoculum in the field. It would not be expected, however, to play a primary role in periodic epidemics of bud blight.

Mottling of soybean seeds can be caused by TRSV. Mottled seeds failed to develop on soybeans inoculated at the age of two weeks while plants inoculated at four weeks developed mottled seeds.

Few differences in physical properties were found among the nine isolates. Similar spectrophotometric scanning patterns were noted in all sucrose density gradient centrifugations as well as electrophoretic patterns of whole virus. Serology revealed a difference between the two isolates tested in this manner.

A satellite virus discovered in one Kansas isolate had properties similar to those of the satellite virus discovered by Schneider. It was concluded to be a satellite of TRSV.