

Exploratory Studies on the Use of Paper Chromatography and Electrophoresis for Detection of the Yellow Virus in Sugar Beet¹

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Virus yellows is generally considered to be the most serious virus disease of sugar beet (*Beta vulgaris* L.) in western Europe where it has been an important problem for many years (18)³. Definite evidence of its occurrence in the United States was reported by Coons (4, 5) in 1952. His results show that the disease was widespread at that time, and he stated that it probably had existed in this country as early as 1940—possibly much earlier. Published reports of damage appraisal studies conducted in 1952 and 1953 (2, 6, 15) and unpublished results of more recent studies (3, 10) have shown conclusively that the disease causes substantial losses in root yields and sucrose percentages in parts of Colorado in certain years, and that it is especially serious in parts of California.

In virus disease studies, particularly in breeding for resistance, a positive means of identifying virus-infected plants is highly desirable. Symptoms in sugar beet more or less resembling those of virus yellows may result from various nutritional and other environmental conditions. Furthermore, individual plants or inbred strains may be essentially symptomless carriers. In Europe, such factors have led to extensive use of a serological test for detection of the virus in sugar beet plants (4). A positive reaction from such a test is generally accepted, by Europeans familiar with the method, as proof of virus yellows infection. A negative reaction is considered as inconclusive since a relatively high concentration of the virus in the plant is required to give a positive reaction.

A recent review of the present status of procedures for determination of the presence of viruses in plant juice extracts has been made by Pirie (17). Two general techniques have been developed—serological determination and assay by physico-chemical methods. Serological determination, although highly specific, is estimated to be in error 30 percent of the time. Physico-chemical techniques have been developed for relatively few viruses—notably tobacco mosaic, potato virus X, and a few others. All physico-chemical techniques so far suggested depend on determination of the protein or nucleic acid that remains in a system after impurities have been re-

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³ Numbers in parentheses pertain to list of references.

moved by some fractionation procedure. None of these techniques appears adapted to plant viruses which occur in low concentration, and it is apparent that either entirely new approaches or improvements in old ones are needed. Two new methods involving paper chromatography and electrophoresis show promise. These techniques have been suggested by Gray (13), by McAnelly et al. (14), and Fife (7).

The studies reported in this paper were undertaken in order to investigate the possibility of utilizing paper chromatography or electrophoresis for detection of the sugar beet yellows virus. The work has been exploratory in nature and has not as yet led to the development of a proven testing procedure. However, it is felt that the results obtained thus far may be of interest to others working in this field.

Methods

Sugar Beet Strains and Field Methods

In 1954 a number of sugar beet strains were grown in 1-row, 45-foot plots in a field near Fort Collins, Colorado, chiefly for virus yellows resistance comparisons. Rows were spaced 40 inches apart in order to promote symptom development and to facilitate yellows reaction readings. Stands were thinned to approximately 12-inch spacing in the row. During the 2-day period, June 21-22, all plants in a 20-foot section of row in each plot were exposed to inoculation by means of green peach aphids (*Myzus persicae* Sulz.) which had been reared on yellows-infected sugar beet plants, a comparable 20-foot section in each plot serving as a control sub-plot. The yellows virus or yellows virus complex involved was representative of that naturally occurring in commercial fields in northern Colorado. Techniques employed in rearing the aphids and in applying them to the plants in the field were similar to those described for a yellows experiment conducted at Fort Collins in 1953 (6). The entire experimental area was sprayed frequently with Parathion, beginning on June 25 and ending September 3. Aphid control by this means was relatively satisfactory, yellows being confined almost entirely to the inoculated sub-plots where susceptible strains approached 100 percent obvious infection. Mosaic was not a factor.

Late in September, three inbred strains, representing a wide range in apparent reaction to virus yellows, were chosen for special study. McFarlane 3502 (now NB1) was classed as resistant; US 201-B (53126-0), intermediate; and 461020, highly susceptible. As used in this paper the terms resistant, intermediate, and susceptible pertain only to degrees of yellowing caused by the virus and do not necessarily indicate comparable degrees of resistance to other virus effects.

On September 28, three composite leaf samples, representing old, medium, and young leaves, respectively, were taken from apparently healthy plants of each of the three inbreds in the control sub-plots—a total of nine samples, each based on 10 plants. The term, "old," as used here, refers to outer leaves, not senile; youngest leaves, approximately fully expanded, were classed as "medium" and leaves classed as "young" were only about one-fourth as large as the medium age group. A comparable set of nine

composite samples was composed of leaves from plants in the inoculated sub-plots presumed to be infected with virus yellows. In the intermediate and susceptible strains, plants definitely infected were chosen. In McFarlane 3502, in which both percentage and intensity of symptom expression were very low, the presence or absence of symptoms was disregarded. The 18 leaf samples were placed in separate plastic bags, closed tightly, quickly frozen, and subsequently stored at approximately -17° C.

Yellows comparison plots, similar to those described above, were set up in 1955 with only minor deviations in timing and technique, but with essentially a new set of sugar beet strains. Late in October, seven strains were chosen for chromatographic use. This set consisted of one commercial variety (Acc. 1359, GW 359) and six inbreds, two of which were among those used for such studies in 1954. Two of the seven strains were classed as susceptible (461020 and 54323); two as intermediate, Acc. 1359 and US 201-B (Acc. 2013); and three as resistant (Accessions 1264, 1344, and 2005). On October 31, 1955, leaf samples were taken from each strain in about the same way as in the preceding year, with two exceptions: (1) the age class was medium; and (2) presence or absence of yellows symptoms was disregarded entirely. In regard to the latter, it should be stated that, as in 1954, susceptible strains such as 461020 showed a high percentage of obvious yellows infection in the plants of the inoculated sub-plots and only a very small percentage of such plants in the non-inoculated sub-plots, indicating relatively satisfactory virus transmission and aphid control.

Laboratory Methods

Paper Electrophoresis

A Wieland type of electrophoretic instrument, modified by Grassmann and Hannig (11), was used to produce zone electrophorograms. The pH of the cacodylic buffer⁴, which served as the electrophoretic electrolyte, was adjusted to 6.93, just above the isoelectric point of the beet protein, so that the protein fraction moved anodically. The ionic strength was 0.1.

The frozen beet leaf samples were thawed and the juice dialyzed with distilled water under constant stirring for four hours to eliminate the sugars and free amino acids. Then the samples were lyophilized to concentrate them 12 to one. A 5-microliter aliquot of the juice, dialyzed and concentrated 12 to 1, was pipetted near one end of a buffer-dampened filter paper strip (Whatman No 1) one and one-fourth inches wide. The instrument was run six hours at 300 volts and five milliamperes, after which the paper

⁴ Cacodylic Buffer:

4.68 g. of sodium chloride
4.28 g. of sodium cacodylate
0.46 g. of cacodylic acid

Dissolved in enough water to make one liter.

⁵ Dye bath

2.5 g. of amido black 10 B
450.0 ml. of acetone-free absolute methanol
50.0 ml. of glacial acetic acid

strips were taken out and put immediately (that is, without drying) into a bath of amido black 10 B⁵ (Farbenfabriken, Bayer Leverkusen, Germany) for 10 minutes, where they were dyed a dark color. Three 10-minute baths of 5-percent phenol were used to destain the strips of paper, followed by fixation in 10-percent concentrated glacial acetic acid in absolute methanol. The strips then were dried, leaving the protein zones with a dark blue color.

The relative amounts of proteins in the leaf juices were measured by passing the dried electrophorograms through a densitometer (Welch and Company Densichron) at 3 mm. intervals followed by plotting the optical density data on graph paper. Since the amount of amido black 10 B adsorbed is directly proportional to the amount of protein on the paper (12), the optical density curves were used to compare healthy and diseased samples.

Paper Chromatography

Ascending one-dimensional paper chromatography was used, as described by Williams and Kirby (20). Small sheets (20 x 20 cm.) of Whatman No. 1 filter paper were used. The solvent was 40-percent ethyl alcohol. Twenty microliters of 12:1 concentrated juice were placed on the paper, dried, and chromatographed in a closed container at constant temperature. After the solvent had ascended the paper, the paper was removed and put immediately, that is without drying, into a dye bath of amido black 10 B, fixed and destained as described for the electrophorograms.

These one-dimensional chromatograms of the leaf proteins were used both years to determine the maximum optical density of the principal protein and the R_f (the distance the protein travels up the paper divided by the distance the solvent travels up the paper) of the principal protein.

The sugar attached to the leaf proteins was determined by cutting out, of the one-dimensional chromatograms, the principal protein spot of the healthy and yellows-infected material. These pieces of filter paper, containing the principal protein spot, were placed in separate test tubes and hydrolyzed with 1 ml. of 1 N H_2SO_4 for 2 hours at 100° C. A 10-microliter portion was chromatographed in a solvent containing a mixture of 4 parts of butanol, 1 part of glacial acetic acid, and 2 parts of water. When the solvent front approached the top of the paper, the paper was removed and dried until all odor of the solvent had dissipated. The paper then was sprayed with Partridge's aniline hydrogen phthalate reagent (16) and, after subsequent drying, was developed at 120° C. for 15 minutes.

Two-dimensional paper chromatography was carried out according to the techniques described by Franklin *et al* (8, 9). A 0.1 M. sucrose solution and 0.1 M. sodium potassium tartrate solution were used as the two solvents. One drop of Tween 81 was added to 1 ml. of the concentrated beet juice before placing 20 microliters on Whatman No. 1 paper. The juice spot was allowed to dry on the paper before the latter was placed in the first solvent. After removal from the second solvent, sodium potassium tartrate, the papers were dyed immediately in amido black 10 B as previously described.

Results and Discussion

Electrophorograms

Figure 1 shows typical zone electrophorograms from two healthy and two yellows-infected sugar beet sub-plots in 1954. Medium size leaves were used in each case. This was done because, by experiment in 1954 with young, medium, and old leaves, it was found that the difference between healthy and diseased samples was greatest in medium leaves. The principal movement in the electrophorograms was toward the anode and shows a definite increase in total proteins in the yellows-infected samples.

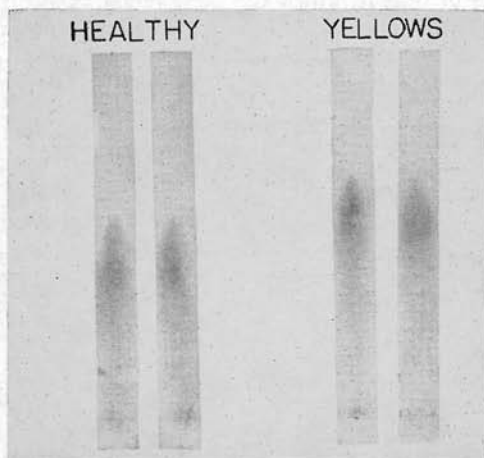


Figure 1.—Typical electrophorograms from leaf juice of healthy and yellows-infected sugar beet plants, 1954.

The electrophorograms were read on the densitometer and graphed. In Figure 2 typical optical density curves are shown. It can be seen from these curves that the main protein component of the healthy plants is present in a decreased amount in the yellows-infected sugar beets. This decrease in amount of the normal protein with increase in a different protein fraction, in the yellows samples, is in agreement with Wildman *et al* (19) and Gray (13).

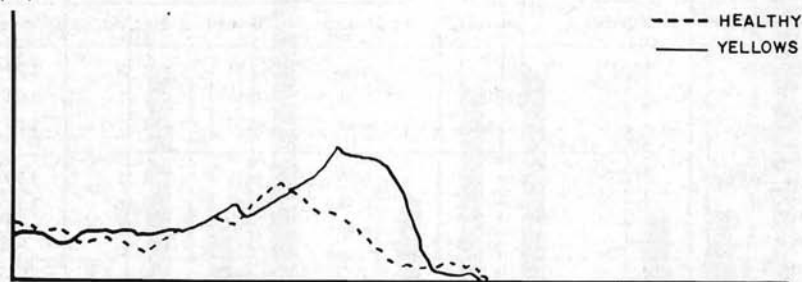


Figure 2.—Typical optical density curves from electrophorograms showing the protein fractions of healthy and yellows-infected sugar beet leaf juice. Curves were obtained by plotting optical density readings from paper electrophorograms against distance moved, cathode at left, anode at right.

This new protein Wildman found to be infective. The new fraction in our electrophorograms has not been proved to be the virus. Such proof might be difficult to obtain because of the nature of its transmission.

One-dimensional Chromatograms

Figure 3 shows typical contrasts between healthy and yellows-infected sugar beet plants for the principal protein fractions in 1-dimensional chromatograms of medium leaves in 1954 and 1955. The developing solvent used was 40-percent ethyl alcohol. This concentration of alcohol was selected following a series of tests in which the percentages of alcohol used were 20, 30, 40, 50, 60, and 70 respectively. The main protein fraction did not move

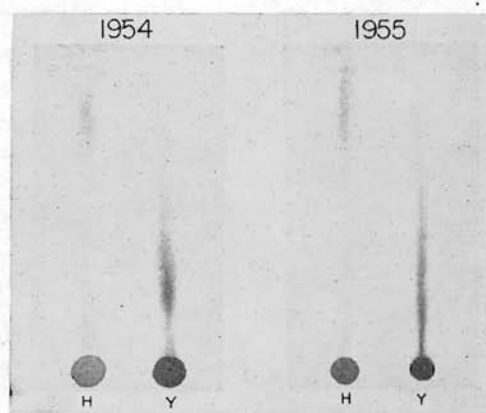


Figure 3.—Typical one-dimensional chromatograms of leaf juice from healthy and yellows-infected sugar beet plants, 1954-1955.

Table 1.—Effects of virus yellows in old, medium, and young leaves, on the maximum optical density of R_f of the principal protein in sugar beet plants, 1954.

Variety		: Age of : Leaves	: R_f		: Optical Density	
Classification	: Number		: Healthy	: Diseased	: Healthy	: Diseased
Resistant	3502	Old	0.42 ¹	0.36	1.58	1.76
		Medium	0.34	0.10	1.87	2.00
		Young	0.44	0.50	1.91	1.97
Intermediate	53126-0	Old	0.15	0.15	1.31	1.55
		Medium	0.48	0.26	1.30	1.71
		Young	0.49	0.36	1.55	1.89
Susceptible	461020	Old	0.33	0.12	1.62	1.77
		Medium	0.57	0.13	1.36	1.91
		Young	0.49	0.57	1.30	1.19

¹ Each value is average of duplicate determinations.

at 70-percent in either healthy or virus-infected samples. There was slight movement at 20-percent. Best movement for comparative tests appeared to be 40-percent. Gray (13) observed a similar phenomenon.

The greatest differences in the R_f between healthy and yellows samples in 1954, appeared in the medium leaves. In 1955 only medium leaves were used. The data for both years are shown in Tables 1 and 2, and bargraphs of the data in Figures 4 and 5. These results show that the R_f for medium leaves was lower in the yellows samples for all strains in both years.

Table 2.—Effects of virus yellows on the R_f and maximum optical density of the principal protein in sugar beet leaves, 1955.

Variety		R_f		Optical Density	
Classification	Number	Healthy	Diseased	Healthy	Diseased
Resistant	Acc. 1344	0.34 ¹	0.18	1.68	1.89
	Acc. 2005	0.50	0.32	1.74	2.18
	Acc. 1264	0.66	0.20	1.10	1.65
Intermediate	Acc. 1359	0.54	0.47	1.53	1.56
	Acc. 2013	0.62	0.54	1.29	1.39
Susceptible	461020	0.54	0.42	1.50	1.80
	54323	0.54	0.39	1.47	1.70

¹All samples are from medium leaves. Each value shown is the average of duplicate determinations.

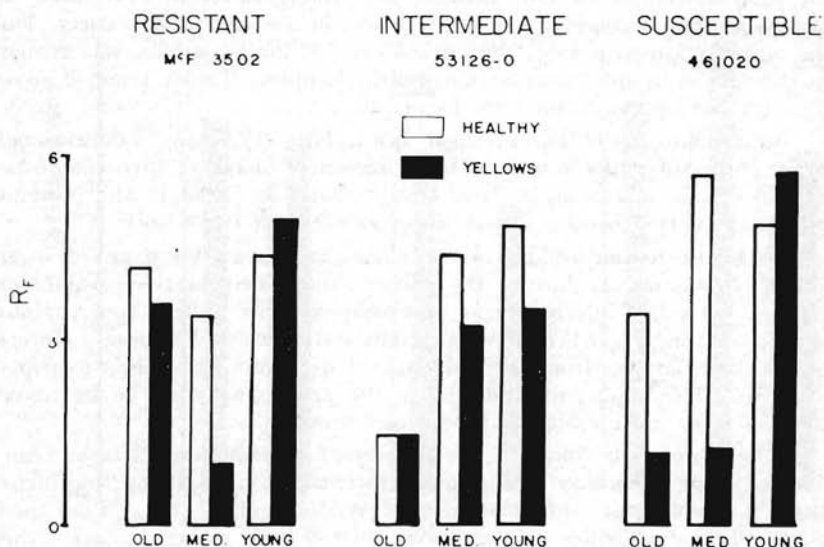


Figure 4.—Histograms comparing the R_f of the principal protein in old, medium, and young leaves of healthy and virus-infected sugar beets, 1954. Each value shown is the average of 8 chemical determinations.

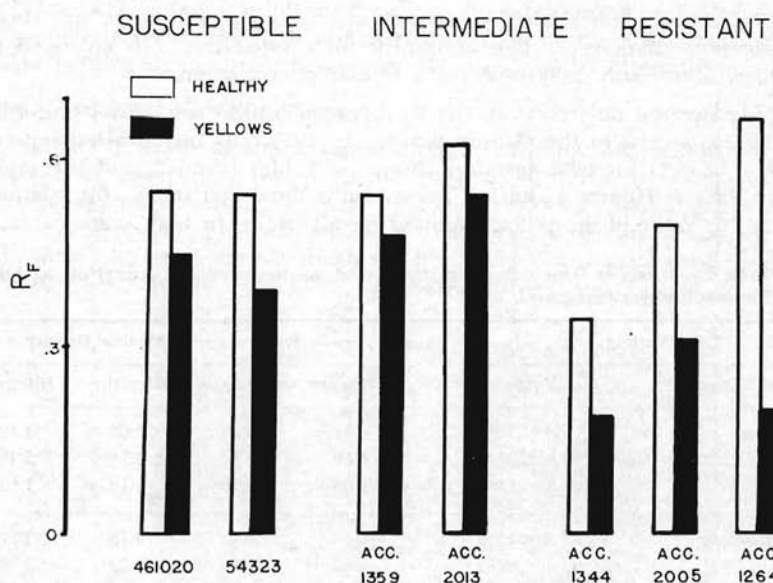


Figure 5.—Effects of virus yellows on the R_f of the principal protein in medium-age sugar beet leaves, 1955. Each value is the average of duplicate determinations.

The maximum optical density of the principal protein was greater in the yellows samples for old, medium, and young leaves in 1954, than in healthy samples, except for the young leaves in the susceptible variety. For the medium leaves in 1955, the optical density, for all strains, was greater in the yellows samples than in the healthy samples. Tables 1 and 2 show the data and Figures 6 and 7 the bargraphs.

In studying curly top in sugar beets, Fife (7), using 1-dimensional paper chromatography, found striking differences in the relative concentration of certain amino acids. The change that was found in the proteins of yellows-infected beets is not in disagreement with Fife's work.

In Figure 8 comparable chromatograms are shown for a mixed sugar sample containing D-glucose, D(-)-ribose, and 2-deoxy-D-ribose, and for healthy and yellow-infected sugar beet samples. The protein spot cut out of 1-dimensional sugar beet chromatograms and hydrolyzed showed a glucose spot in both samples from the hydrolysis of the paper (compared to paper controls). The sugar hydrolyzing from the protein appeared to be ribose and was more concentrated in the yellows samples.

The increase in ribose in the hydrolyzed protein samples from virus-infected sugar beets may be due to an increase in virus ribonucleoprotein. If so, it would agree with the results of Wildman *et al* (19). They used electrophoretic scanning diagrams and showed that tobacco mosaic virus increases at the expense of normal protein. Presumably this virus follows the known pattern of several other plant viruses (1) of multiplying in functional tissue where cell division is at a minimum (medium leaves).

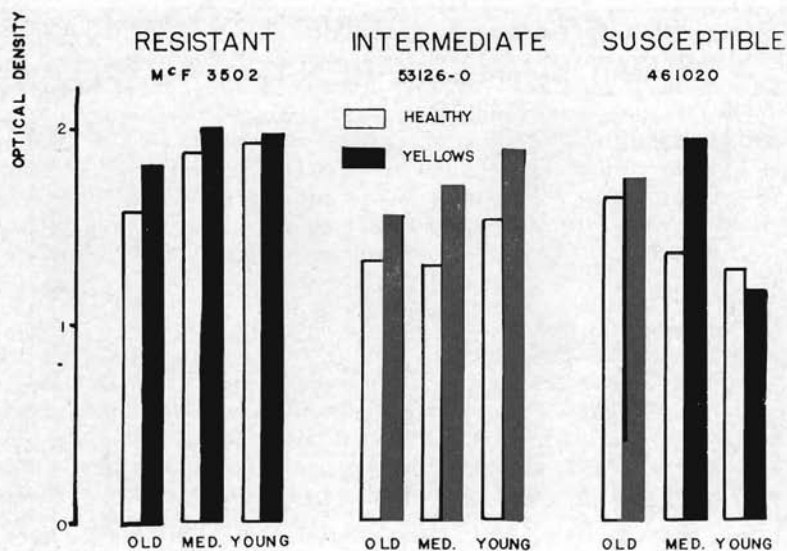


Figure 6.—Histograms comparing the maximum optical density of the principal protein in old, medium, and young leaves of healthy and virus-infected sugar beets, 1954. Each value shown is the average of 8 chemical determinations.

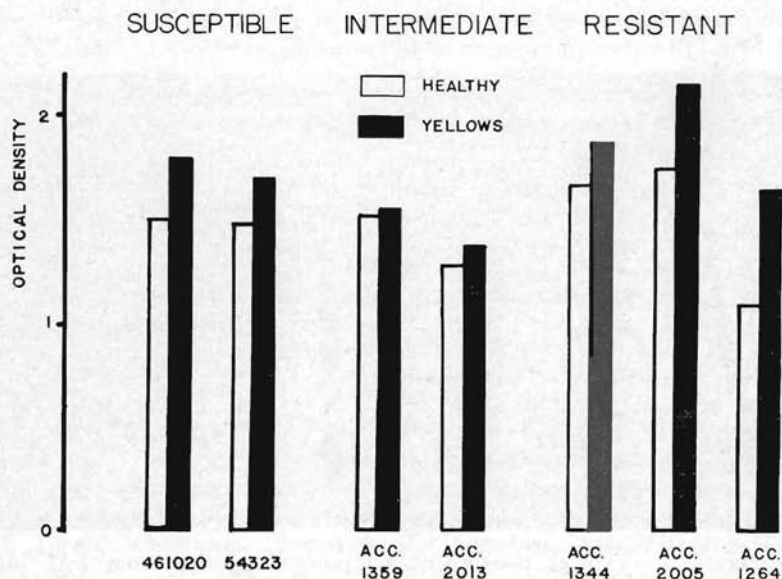


Figure 7.—Effects of virus yellows on the maximum optical density of the principal protein in medium-age sugar beet leaves, 1955. Each value is the average of duplicate determinations.

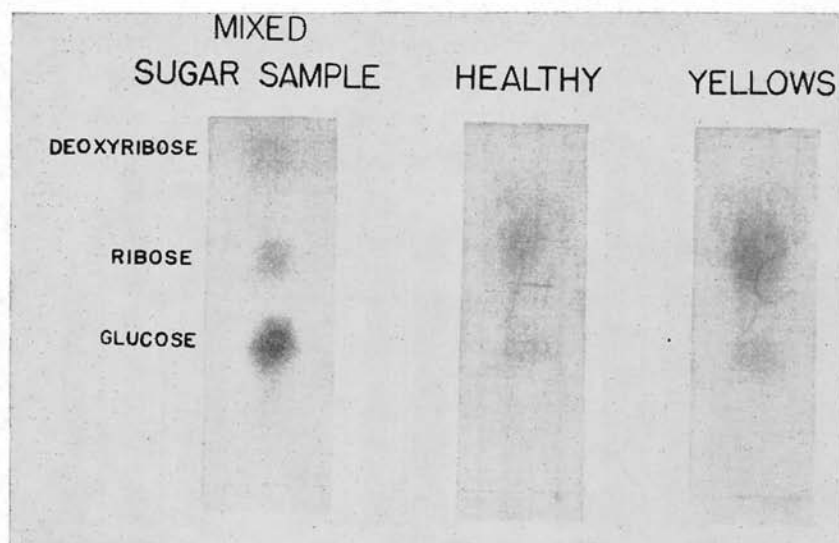


Figure 8.—Typical one-dimensional chromatograms, showing the sugar attached to the principal protein of leaf juice from healthy and yellows-infected sugar beet plants. (Glucose spot is from hydrolyzed filter paper.) The position of pure glucose, ribose, and deoxyribose is also shown for a mixed Sample.

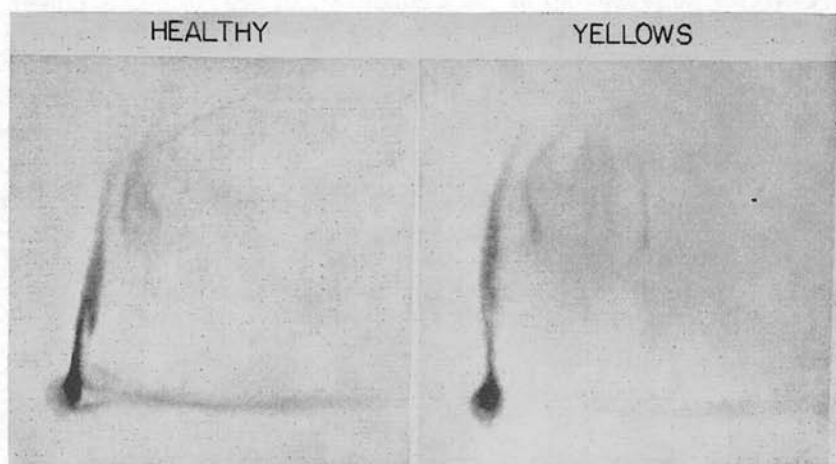


Figure 9.—Typical two-dimensional chromatograms from leaf juice of healthy and yellows-infected sugar beet plants.

Two-dimensional Chromatograms

Two-dimensional chromatograms of healthy and yellows-infected sugar beet samples are shown in Figure 9. It is noted that the number of protein fractions from the healthy sample is less than from the yellows-infected sample. In 1954, 2-dimensional chromatograms were run for all medium-leaf samples. The number of protein fractions for the healthy plants averaged 4.20, and 6.42 for the yellows-infected plants. Here again, whether or not one or more of these new fractions in the yellows infected samples is actually virus protein has not been proved.

Summary

Leaf juice samples from healthy and virus yellows infected plants of eight sugar beet strains which differed widely in apparent reaction to the disease were compared by chromatographic and electrophoretic techniques. The studies were exploratory in nature, and the results were not subjected to statistical analysis. However, certain definite trends were observed. Those trends were most pronounced for medium-age leaves and may be summarized, for that age class, as follows:

1. In zone electrophorograms, the main protein fraction in the diseased samples moved anodically at a substantially faster rate than in the healthy controls.
2. In 1-dimensional paper chromatograms, prepared with 40-percent ethyl alcohol as the solvent, the diseased samples, as contrasted with the controls, were consistently (a) lower in R_f of the main protein fraction; (b) higher in maximum optical density of that fraction; and (c) higher in amount of D (-)-ribose in hydrolyzed protein.
3. In 2-dimensional paper chromatograms, the diseased samples averaged 6.42 protein fractions and the healthy samples, 4.20.

On the basis of these results, chromatographic or electrophoretic techniques appear promising as means of detecting the presence of the yellows virus in sugar beet plants. However, before definite conclusions in this regard can be made, population studies are needed. Such studies should include both infected and healthy plants, representing a relatively wide range of sugar beet genotypes, with the chemical determinations performed on an individual-plant basis.

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