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Some Properties of Cocoa Swollen-Shoot Virus

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SUMMARY

Partially purified preparations of the Kofi Pare isolate of cocoa swollen-shoot virus (CSSV) were usually inactivated after 10 min. at 50°, but not after 10 min. at 45°. Infectivity of freshly made preparations was greatly lessened by diluting 1/10, and lost at 1/100. The infectivity of preparations increased after storage for 24 hr; after 96 hr infectivity was retained at 0–4° but soon lost at 25°. The virus survived freezing *in vitro* and, with some loss of infectivity, freezing in leaves and storage in leaves dried over aluminium oxide. Infective material was precipitated from dilute extracts by half saturation with ammonium sulphate at 25°; it appeared to be equally stable over the range pH 6 to pH 8. Preparations of the symptomatologically distinct Kofi Pare, Mampong, Dawa, Nsaba and Bosomuoso isolates all contained similar rod-shaped particles of size about 121 × 28 mμ.

INTRODUCTION

The availability of infective preparations of cocoa swollen-shoot virus (Brunt & Kenten, 1963) has made it possible to examine the properties of the virus *in vitro*. We report here some of the properties of partially purified preparations of the Kofi Pare isolate, and the electron microscopy of this and four other symptomatologically distinct isolates.

METHODS

The cocoa swollen-shoot virus (CSSV) isolates from Kofi Pare, Bosomuoso, Mampong and Nsaba in Ghana were derived from cultures described previously (Brunt & Kenten, 1963); an isolate from Dawa (Eastern Region, Ghana) was also used. The Kofi Pare isolate, which is indistinguishable from Posnette's (1947) swollen-shoot virus strain A or virulent New Juaben strain, was used throughout, and the others for electron microscopy only.

The viruses were extracted from the leaves of infected cocoa seedlings and partially purified by methods similar to those described previously (Brunt & Kenten, 1963). Twenty-five g. infected first-flush cocoa leaves were ground in a Waring Blendor for 1 min. in 500 ml. of a solution at pH 8·0–8·2 containing 0·05 M-disodium hydrogen phosphate, 0·05 M-thioglycollic (mercaptoacetic) acid, 0·005 M-sodium diethyldithiocarbamate and 1 or 2% (w/v) hide powder (Hopkin & Williams

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Ltd.). This hide powder was first thoroughly dispersed in the solution by running the Waring Blendor at full speed for 3 min. before adding the leaves. The suspension was squeezed by hand through cotton cloth and clarified by centrifugation at 8000 *g* for 5–10 min. The virus was then sedimented by centrifuging the supernatant fluid at 75,000 *g* for 2 hr. The pellets were usually dispersed in a solution at pH 8·0 containing 0·01 M-disodium hydrogen phosphate and 0·01 M-thioglycollic acid, using about 1/35 of the volume of the original extract.

Because the original difficulties in transmitting CSSV by sap inoculation (Brunt & Kenten, 1960, 1962*b*) might have been caused by instability of the virus, at first all extracts were made as quickly as possible, with chilled apparatus and solutions. However, later extractions were made at room temperature (about 25°), after it was found that such extracts were usually more infective than those made below 8°.

All infectivity tests were made by inoculating whole cocoa beans; to increase the number of infections a little 'Celite' 545 (Johns Manville Ltd.) was added to all inocula.

Electron microscopy was done at Rothamsted Experimental Station, with samples of virus preparations made and tested for infectivity in Ghana and sent to the United Kingdom by air in chilled vacuum flasks. On arrival they were clarified by centrifugation at 8000 *g* for 10 min. and the virus again sedimented from the supernatant fluid by centrifuging at 75,000 *g* for 2 hr. The greenish pellets were dispersed in 1–2 ml. of 0·1 M-ammonium acetate and used for electron microscopy after brief centrifugation at 8000 *g* to remove insoluble material. Preliminary experiments showed that satisfactory shadowcast mounts could not be made from these preparations because they still contained mucilage even after two cycles of differential centrifugation. All electron microscopy was therefore done with mounts made by a negative staining technique (Brenner & Horne, 1959). Most microscopy was done at × 20,000 magnification; some pictures were taken at × 80,000. Particle lengths were measured either from the original photographic plates by using a scale graduated in 0·5 mm. steps and a hand lens, or were estimated by measuring the image projected by a calibrated photographic enlarger.

RESULTS

Properties of the virus in vitro

The properties of the virus were determined by using preparations made from clarified cocoa leaf extracts by one cycle of high-speed centrifugation. The sedimented virus was dispersed in phosphate + thioglycollate solution of 1/35th the original volume; any further dilutions were also made in the same phosphate + thioglycollate solution. Since the leaves had originally been macerated in 20 volumes of the extracting solution, each 1 ml. of these partially purified preparations was derived from 1·75 g. fresh leaf.

Dilution end-point. When tested fresh, the infectivity of preparations was greatly diminished by diluting 1/10 and abolished by diluting 1/100. After storage for 24 hr, however, infectivity increased (see below) and infections were obtained at 1/100 (Table 1).

Stability in vitro. Preparations of virus were kept in loosely stoppered test tubes at 0–4° or 25° and tested for infectivity after 24, 48 and 96 hr. After 24 hr the

infectivity of both samples increased; on storage up to 96 hr there was little further change in the infectivity of samples stored at 0–4° but those kept at about 25° lost much of their infectivity.

Thermal inactivation. One-ml. samples of virus preparation were heated for 10 min. to various temperatures. Infectivity, seemingly unaffected by heating up to 45°, was lost at 50° or above, except for a single infection which was obtained from one sample heated to 55° (Table 2).

Table 1. *Stability of cocoa swollen-shoot virus in vitro at room temperature (about 25°) and 0–4°*

	Duration of storage (hr)						
	0	24		48		96	
		Temperature					
Dilution of preparations*		0–4°	25°	0–4°	25°	0–4°	25°
		Infectivity (infections/beans tested)†					
1/1	34/60†	42/60	45/59	35/59	29/57	35/59	5/59
1/10	7/59	22/60	21/60	17/59	15/56	6/60	1/59
1/100	0/57	2/59	2/60	0/56	3/54	2/59	0/59

* Dilutions were made after storage with a solution (pH 8) containing 0·01 M-disodium hydrogen phosphate and 0·01 M-thioglycollic acid.

† The denominator is the total number of whole cocoa beans inoculated in two experiments and the numerator the number of plants which were infected. Beans failing to germinate were omitted from the denominator.

Table 2. *Thermal inactivation of partially purified preparations of cocoa swollen-shoot virus*

		Preparations heated for 10 min.			
Dilution of preparations*	Unheated	45°	50°	55°	60°
		Infectivity (infections/beans tested)			
1/1	19/60	23/59	0/60	1/58	0/60
1/10	6/60	5/60	0/60	0/60	0/60

* Dilutions made with a solution (pH 8) containing 0·01 M-thioglycollic acid and 0·01 M-disodium hydrogen phosphate after heating 1 ml. virus samples.

Effect of freezing and desiccation. Preparations of virus from leaves which had been frozen for 24 hr or dried *in vacuo* over aluminium oxide before they were extracted were infective, but slightly less so than those made from fresh leaves (Table 3). Freezing had little effect upon the infectivity of clarified sap or the partially purified preparations.

Precipitation with ammonium sulphate. In preliminary tests (Brunt & Kenten, 1962*a*) the sparse precipitate formed when ammonium sulphate was added to clarified cocoa leaf extracts, prepared by grinding leaves in extracting fluids containing no added protein, was not infective. However, when extracts made with fluid containing added protein (2%, w/v, hide powder) were half-saturated with ammonium sulphate at 25° the bulky precipitate produced was infective. After

dialysing the resuspended precipitate against phosphate + thioglycollate solution (pH 7·5–8·0), and removing insoluble material by low-speed centrifugation, highly infective preparations were obtained by sedimenting the virus from the supernatant fluid.

Effect of pH value. Preparations were dispersed in phosphate + thioglycollate solution adjusted to pH 6, 7 and 8, and samples tested for infectivity immediately and after 24 and 48 hr. All were similarly infective (Table 4).

Table 3. *Effects of drying or of freezing infected leaves on virus*

In each experiment thirty whole cocoa beans were inoculated with each of six samples. Infected leaves were frozen for 24 hr. Shredded leaves were dried *in vacuo* for 24 hr over aluminium oxide.

	Leaves processed		
	Immediately	After freezing	After desiccation
	Infectivity (infections/beans tested)		
Expt. no.			
1	32/164	20/174	32/172
2	66/180	48/179	16/136
3	63/171	57/180	—
Total	161/515	125/533	48/308
Mean (%)	33·3	23·5	15·6

Table 4. *Effect of pH value on stability of partially purified virus*

	Duration of treatment (hr)		
Suspending solution at pH	0	24	48
	Infectivity (infections/beans tested)		
6	17/40	16/40	11/30
7	22/38	24/40	13/30
8	14/39	23/40	15/30

Electron microscopy

Presence of particles. All virus preparations made from plants infected with the CSSV isolates tested contained rod-shaped particles; such particles were never found in preparations made in the same way from healthy plants. No formal correlation between particle numbers and infectivity was attempted, but more such particles were found in highly infective preparations than in less infective ones.

Morphology of the particles. The particles were straight or slightly curved, with rounded ends (Plate 1). Preparations of the isolates from Kofi Pare, Mampong and Nsaba contained enough particles to permit making histograms showing the length distribution (Fig. 1); particles of the Dawa and Bosomuoso isolates, although too few to construct histograms, gave indications of a similar length-distribution pattern. Because the distribution is skew, no valid estimate of the most common length can be made by taking the mean of all particles measured. We have overcome this difficulty by selecting a narrow range of sizes symmetrically placed about the most common length shown in the histogram, and using only particles falling within the range to estimate the most common length (Brandes & Paul, 1957).

Table 5 shows the mean value obtained for the three isolates by this method. The lengths of particles in preparations made from cocoa plants infected with the Kofi Pare, Mampong and Nsaba CSSV isolates are all similar.

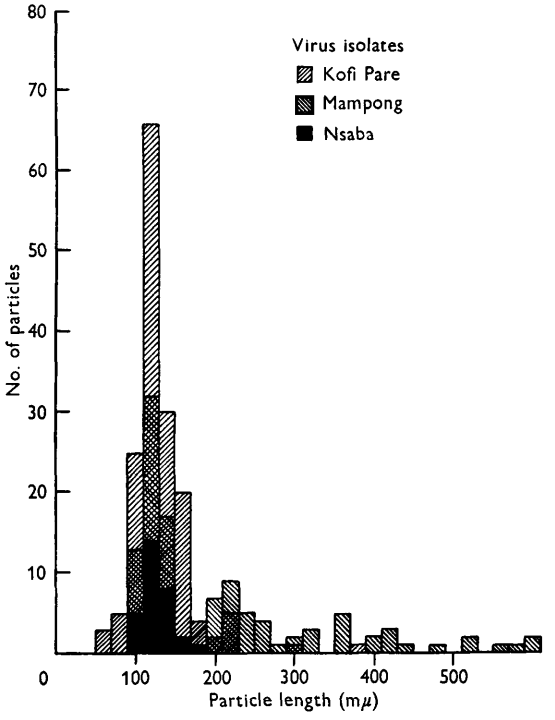

Fig. 1

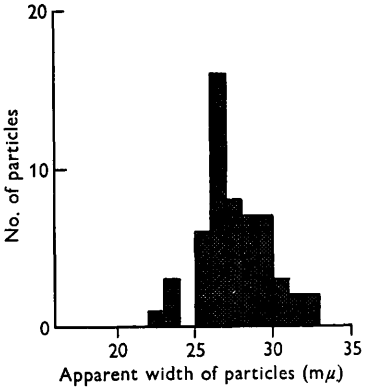

Fig. 2

Fig. 1. Length distribution of particles found in virus preparations from plants infected with three isolates of cocoa swollen-shoot virus.

Fig. 2. Widths of particles seen in electron micrographs of cocoa swollen-shoot virus preparations.

Table 5. *Dimensions of virus-like particles extracted from cocoa leaves infected with three isolates of cocoa swollen-shoot virus*

Virus isolate	Number of particles measured	Particles in 91–150 mμ group (%)	Mean length of 91–150 mμ particles	Width (mμ)*
Kofi pare	102	60·8	119·6	26·7 ± 0·4
Mampong	162	82·1	122·2	29·1 ± 1·8
Nsaba	30	90·0	122·7	28·9 ± 4·0
All isolates together	294	75·9	121·0	27·9

* Particle widths estimated from micro-densitometer traces across the images.

Estimates of particle width were taken from micro-densitometer traces made across the images on the original plates. The values obtained (Fig. 2) almost certainly underestimate the true particle width, because of penetration of the phospho-

tungstate (PTA) into the outer parts of the particles; this occurs even with extremely stable viruses such as tobacco mosaic, particles of which appear to be only 12 mμ wide instead of 15 mμ when measured from shadowed mounts. The particles appear to be tubular, with their centres filled with PTA (Plate). The rounded ends are an unusual feature and we know of only one other rod-shaped virus particle (lucerne mosaic) with rounded ends (Gibbs, Nixon & Woods, 1963). Whether the rounded ends of the CSSV particles represent an essential part of their structure, or merely show that the ends of the particles are degraded, is uncertain; we incline to the latter view because a few particles in some of our better extracts have had one or both ends cut off square. No other structures could be seen in the particles, even in micrographs of good quality made at $\times 80{,}000$.

Although it is not known that the particles observed in the electron microscope are those of CSSV, their size, shape, general appearance, and the fact that their presence appears to be correlated with infectivity, all suggest that they are the virus.

DISCUSSION

Cocoa swollen-shoot virus (CSSV), cocoa mottle leaf virus and strains A and B of the virus which occurs in Trinidad (Baker & Dale, 1947) are the only viruses known with certainty to be transmitted by mealybugs. It is therefore of particular interest that at least one isolate of CSSV can be studied and characterized *in vitro*. Because we have determined some of the properties of CSSV in partially purified preparations, no direct comparison with those of most other viruses is possible. However, our results clearly show that CSSV is moderately stable once it has been separated or protected from some constituents of cocoa leaves. Initial difficulties in transmitting the virus by mechanical inoculation (Brunt & Kenten, 1960, 1962*b*) therefore probably reflected the release of inhibitors of infection when cocoa leaves were macerated, rather than any intrinsic instability of the virus or an unusually small virus concentration in the leaf. Indeed preparations, which on a leaf weight basis were less than twofold concentrated, were not only infective but were so when diluted 1/10 and occasionally 1/100. No attempt was made to identify these inhibitors but, since 'vegetable tannins' occur abundantly in cocoa leaves (Holden, 1957) and inactivate other viruses (Cadman, 1959; Thresh, 1956), Brunt & Kenten (1963) suggested that these are the leaf constituents which inactivate CSSV when cocoa leaves are macerated. The protective effect of protein was explained by postulating that the 'vegetable tannins' combine with this rather than with the virus. However, fixation of tanning substances by proteins is related to the charge carried by the protein, fixation by vegetable tannins increasing with net positive charge. By contrast, fixation of aldehydes by proteins increases with net negative charge, so that at or about pH 8 it might be expected that proteins would fix aldehydes more readily than vegetable tannin. For this reason leaf aldehydes may also be important inhibitors of infection by CSSV in alkaline extracts of cocoa leaves.

Although our attempts to prepare antisera to the Kofi Pare CSSV isolate failed, now that the virus is known to be relatively stable *in vitro*, it will be worth trying to prepare antisera by using more concentrated preparations as antigens. In making such preparations the need to ultracentrifuge large volumes of clarified extracts could

be avoided by making a preliminary concentration by precipitating the virus from dilute extracts with ammonium sulphate.

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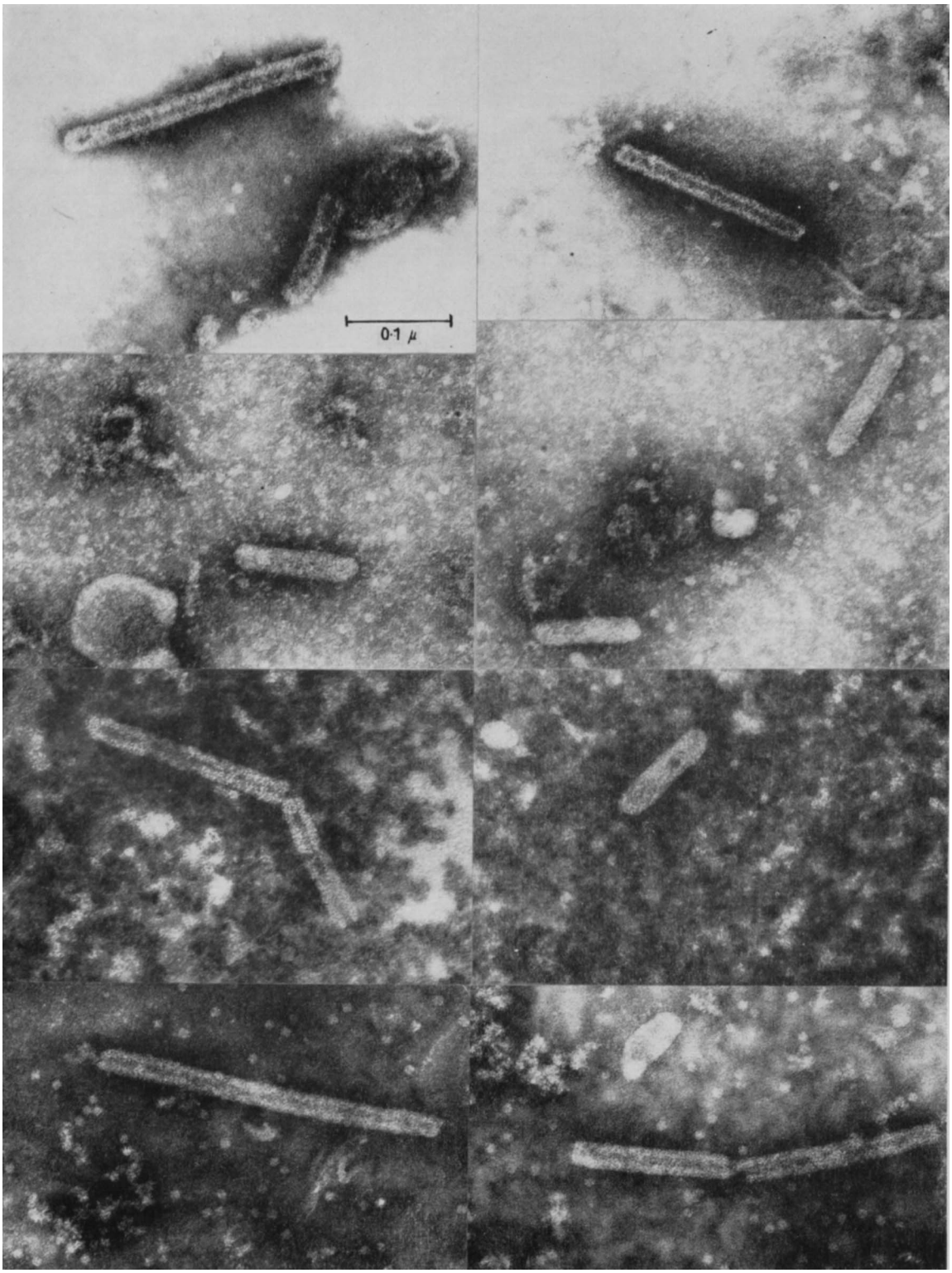
EXPLANATION OF PLATE

Electron micrograph of particles found in preparations from plants infected with the Kofi Pare isolate of cocoa swollen-shoot virus. Negatively stained with phosphotungstate.



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