Purification of Cocoa Necrosis Virus from Cocoa Leaves

D. Adomako, G. K. Owusu, and K. K. Oduro

First and second authors: Research Biochemist, and Research Virologist, respectively, Cocoa Research Institute, P. O. Box 8, New Tafo, Ghana. Third author: Lecturer in Biochemistry, Department of Biochemistry, University of Ghana, Legon, Ghana.

Published with the permission of the Director of The Cocoa Research Institute.

We are grateful to Mr. A. J. K. Gbewonwo of the Electron Microscope Laboratory, University of Ghana Medical School, Korle-Bu for help with the electron microscopy and to Messrs. D. K. Acheampong and P. C. Aculey for technical assistance.

Accepted for publication 14 May 1974.

ABSTRACT

Cocoa necrosis virus (CNV) was isolated from infected cocoa (*Theobroma cacao* L.) leaves by precipitation of virus with 10% polyethylene glycol 6000 (PEG 6000), chromatography on Celite 545 using decreasing concns of polyethylene glycol as eluent, or by purification of resuspended PEG-precipitated virus by differential centrifugation. The problem of effectively removing host-plant materials from the mucilaginous extracts was overcome by the chromatography on Celite or by treating the crude extracts with 0.2% pectinase prior to precipitation of the virus with PEG. Both methods enabled further purification of virus by gel-filtration, but recovery of virus was better with the differential centrifugation procedure than with chromatography on Celite.

Gel filtration with Sephadex G200 and Sepharose 4B (4% agarose) greatly enhanced the infectivity of preparations made from source material consisting largely of moderately necrotic leaves; infectivities of preparations made from highly necrotic leaves were not affected. This is attributed to removal of a high molecular wt inhibitor which presumably becomes degraded in the highly necrotic tissues. Purified preparations contained numerous isometric particles, 25-28 nm in diam. Some in vitro properties of the virus are described.

Phytopathology 64:1325-1330.

The abundance of tannins and mucilage in cocoa (*Theobroma cacao* L.) leaves makes the isolation of cocoa viruses from them a difficult task (5). There are no suitable alternative hosts from which to purify most of these viruses. In some cases (for example, cocoa swollen shoot virus) the problem is compounded by the low virus content in the leaves. An isolate of cocoa necrosis virus (CNV) recently described from Ghana (8) has been found to reach a high concn in cocoa leaves, thus offering an opportunity for the purification and characterization of the virus. Accordingly, new methods of purification were sought and the success achieved is reported in this paper together with some properties of the virus. While this work was in progress Kenten (6) purified CNV from *Phaseolus vulgaris*, L. and showed it to be a serotype of tomato black ring virus.

MATERIALS AND METHODS.—*Virus isolate and culture.*—The CNV isolate obtained from naturally infected cocoa in the Brong Ahafo region of Ghana (8) was propagated in the gauze-house by graft-inoculation of 6-12 mo-old Amelonado cocoa seedlings. Infected flush leaves were collected during the 2nd and 3rd wk after graft-inoculation. Large leaf samples were extracted immediately; small samples were kept at 0-4 C for several days during which time sufficient leaf material was collected.

Preparation of sap.—Virus was extracted from infected cocoa leaves as follows: after removal of midribs, leaves were homogenized (30 g/400 ml fluid) for 2 min in cold 0.05 M sodium phosphate buffer (pH 6.5) containing 0.005 M sodium diethyldithiocarbamate (DIECA), 0.005 M β-mercaptoethanol and 2% (w/v) hide powder (2). After storage at 0-4 C for 15-18 h the mucilaginous slurry was squeezed through cotton cloth and centrifuged at 10,000 *g* for 15 min. Aliquots were dialyzed against 10 volumes of 0.05 M sodium phosphate buffer (pH 6.5) containing 0.005 M mercaptoethanol (buffer A) for 8-15 h at 0-4 C for infectivity assays.

In earlier experiments 0.005 M thioglycollic acid was used instead of β-mercaptoethanol. The latter was employed in subsequent experiments to avoid orange discoloration of extracts which was attributed to thioglycollic acid.

Virus purification.—Polyethylene glycol (PEG 6000, molecular wt 6,000) was added in small amounts to the extracted sap to a concn of 10% (w/v) and the extract kept at 0-4 C for 15-20 h. The presence of PEG at this concn, and of the mucilage in cocoa leaves made the preparation highly viscous so that centrifugation at 65,000 *g* for 30 min was required to remove the precipitate. After the supernatant was thoroughly removed, the pellet was suspended in 5-10 ml of buffer A. Celite 545 (Koch-Light Laboratories Ltd., England) (about 1.5 g) was added to the suspended pellet and thoroughly mixed. Sodium phosphate buffer (0.05 M, pH 6.5) containing 0.2 M NaCl and 10% (w/v) PEG was added to the slurry to bring the final PEG concn to 7%, and the slurry transferred onto a 2-cm-high Celite 545 bed in a 1 × 50-cm glass column. The column was washed successively with equal volumes of the above buffer system containing 5, 3, 1, and 0% (w/v) PEG (3). For infectivity tests, the fractions were dialyzed for 8-12 h against 10 volumes of 0.05 M sodium phosphate buffer (pH 7.2) containing 0.005 M mercaptoethanol (buffer B). The infective fractions (1% and 0% PEG eluates, see below) were bulked and the virus was sedimented at 90,000 *g* for 90 min and suspended in 2-4 ml of buffer B.

Alternatively, after precipitation with 10% PEG and resuspension in buffer A, virus was purified by three cycles of differential centrifugation (alternately at 10,000 *g* for 20 min and 90,000 *g* for 90 min) at 0-4 C. The final pellet was resuspended in 2.0 ml of buffer A.

Clarification of sap with pectinase.—As leaves could not be successfully vacuum-infiltrated with 0.2% (w/v)

solution of pectinase (Koch-Light Laboratories Ltd., England) (in 0.05 M citrate buffer, pH 6.1) at 25 C for 2 h, advantage was taken of the stability of the virus at room temp (see below) to degrade mucilage in the extracted sap with pectinase prior to PEG precipitation. Extracted sap was incubated with pectinase at 25 C for 20 h and the insoluble material was removed by centrifugation at 10,000 *g* for 10 min at 25 C. Virus was then precipitated with 10% PEG and further purified by differential centrifugation as described above.

Further purification by gel filtration.—Sephadex G200 was used in conjunction with the purification procedure involving chromatography on Celite 545. It was replaced in later experiments with Sepharose 4B (4% agarose) because of the superior flow rates on the latter. About 1.0 ml of virus suspension (products of Celite chromatography or differential centrifugation) was introduced onto a 1 × 12-15 cm column of gel previously saturated with buffer A at 0-4 C. The column was then washed with about 2.5 bed volumes of the buffer and the filtrate concd by ultracentrifugation.

Electron microscopy.—Virus preparations were examined in the Hitachi Universal Model 11E-1 electron microscope after mixing with neutral (2%, w/v) phosphotungstate on a collodion-coated 158 meshes/cm grid.

Infectivity tests.—Amelonado cocoa beans were inoculated with virus preparations (0.8 ml virus suspension/20 beans) as described previously (8). The number of seedlings showing symptoms 3-4 wk after sowing was taken as an estimate of the infectivity.

RESULTS.—*Properties of CNV in sap.*—Sap extracted as above was infective at a dilution of 10^{-2} and occasionally at 10^{-3}; after storage at room temp (25-29 C) for 3 days, but not 4 days; after storage at 0-4 C for 10 days, but not 17 days; and after heating at 55 C for 10 min, but not at 60 C. Freezing overnight destroyed infectivity.

Clarification with n-butanol.—Attempts to purify the virus using *n*-butanol as a clarifying agent were not successful. CNV was not affected by 8.5% (v/v) *n*-butanol, but higher concns were required to coagulate chloroplasts. At 9.5%, where coagulation of chloroplasts was effective, the virus was almost completely inactivated after 60-80 min at 25 C or 15 h at 0-4 C.

Precipitation of virus by PEG and inactivation by NaCl-PEG.—Results of preliminary experiments showed that more than 90% of the virus precipitated at 10% PEG concn, but little or none at 5% or 3%. When virus was precipitated from crude extracts with 7-8% PEG in the presence of 0.2 M or 0.4 M NaCl followed by filtration through Celite 545, all infectivity was lost. Precipitation with PEG-NaCl was characterized by voluminous, highly viscous precipitates due to the precipitation of large amounts of mucilage.

The effect of precipitation with PEG-NaCl on yield of infective virus was investigated further in two experiments in which infectivities of virus precipitated from equal portions of crude extracts by 7% PEG with or without 0.4 M NaCl were compared. The results are shown in Table 1. In experiment 1, 6.3 g of infected leaves were extracted with 300 ml extracting fluid. In experiment 2, to increase the mucilage content, 5.3 g of virus-free flush leaves were extracted together with 2.3 g of infected leaves in 200 ml of extracting fluid. After centrifugation at low speed, equal portions of the crude extracts (120 ml and 75 ml, respectively, for experiments 1 and 2) were treated as described already with PEG (7%, w/v) or PEG plus NaCl (0.4 M). The resuspended pellets were centrifuged at low speed to remove insoluble material, dialysed, and assayed for infectivity together with the crude extracts which had been kept at 0-4 C over the period of experiment and dialysed. In experiment 1, the number of infections produced by the PEG-NaCl preparation was more than half the infections produced by the control (without NaCl) whilst in experiment 2 the infections produced by the PEG-NaCl preparation were fewer than half the number produced by the control. In one experiment, treatment of crude sap with NaCl (0.4 M) at 0-4 C for 15 h did not cause any appreciable loss of infectivity.

In contrast to the total loss of infectivity in the Celite-filtered preparations (see above) the infectivities of the PEG-NaCl-precipitated virus suspension (Table 1) were estimated to be greater than the corresponding crude extracts, which suggests that losses due to adsorption on Celite may be the cause for the total loss encountered with the PEG-NaCl-precipitated, Celite-filtered preparations.

Recovery of virus in Celite column chromatography with decreasing PEG concentrations.—Infectivity was eluted from the Celite 545 column mainly in the 1% and 0% PEG fractions in approximately equal amounts when the column was washed with 1.5 to 2.5 bed volumes at each PEG concn. The 5% and 3% PEG eluates, or the pellets obtained from them by ultracentrifugation were

TABLE 1. The effect of co-precipitation of mucilage with cocoa necrosis virus (CNV) by PEG-NaCl on yield of infective virus

Experiment	Volume of virus preparation (ml)	Infectivity[a]	
		PEG only	PEG-NaCl
1.			
Untreated sap	245	3/40	3/40
Suspended pellet, nondiluted	10	34/40	24/40
10^{-1} dilution	...	20/40	15/40
10^{-2} dilution	...	5/40	3/40
2.			
Untreated sap	156	1/40	1/40
Suspended pellet, nondiluted	5	26/40	14/40
10^{-2} dilution	...	7/40	3/40

[a]The numerator represents number of infected seedlings; and the denominator, the number of beans inoculated.

TABLE 2. Infectivity of PEG-precipitated cocoa necrosis virus (CNV) after chromatography on Celite 545 and Sephadex G200 filtration

Virus preparations[a]	Volume (ml)	Infectivities at various dilutions			
		Non-dil.	10^{-1}	10^{-2}	10^{-3}
1.					
7% PEG eluate	8	2/30[b]	...	...	...
5% PEG eluate	8	0/30	...	...	...
3% PEG eluate	8	0/30	...	...	...
1% PEG eluate	8	29/30	...	...	...
0% PEG eluate	8	30/30	...	...	...
2.					
7% PEG eluate	15	0/40	...	...	...
5% PEG eluate	15	0/40	...	...	...
3% PEG eluate	15	0/40	...	...	...
1% PEG eluate	15	40/40	32/40	4/40	
0% PEG eluate	15	40/40	17/40	0/40	
Combined 0% and 1% fractions after filtration through Sephadex and concn by ultracentrifugation[c]	12	38/40	20/40	2/40	
3.					
7% PEG eluate	20	...	...	...	...
5% PEG eluate	20	0/20	...	...	...
3% PEG eluate	20	0/20	...	...	...
1% PEG eluate	20	3/20	...	...	...
0% PEG eluate	20	3/20	...	...	...
Combined concd 1% and 0% fractions before Sephadex gel filtration	36	3/20	...	...	...
Sephadex filtrate before concn by centrifugation	31	20/20	...	...	...
Sephadex filtrate after concn	3	...	20/20	5/20	1/20

[a]Preparations 1, 2, and 3 were from 360 g, 60 g, and 92 g of infected leaf materials, respectively. Preparations 1 and 2 were made from highly necrotic leaf material; preparation 3 was from leaves which were mostly at the initial stages of infection.
[b]The numerator represents number of infected seedlings; and the denominator, the number of beans inoculated.
[c]Ten ml each of the 0% and 1% PEG fractions were concentrated to a small volume prior to the gel filtration.

TABLE 3. Infectivity of cocoa necrosis virus (CNV) at some stages during purification by PEG-precipitation, differential centrifugation, and gel-filtration

Virus preparations	Volume (ml)	Infectivities at various dilutions				
		Non-dil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}
1.						
i. Clarified sap after treatment with pectinase (0.2%, w/v) for 24 h at 25 C	245	25/40[a]	...	...	...	...
ii. (i) after precipitation with PEG and one cycle of differential centrifugation	2.5	...	40/40	22/40	...	...
2.						
i. Extracted sap before treatment with pectinase		36/40	14/40	...	...	...
ii. Sap after clarification with pectinase for 20 h at 25 C	335	28/40	28/40	6/40	...	...
iii. (ii) after precipitation with PEG and two cycles of differential centrifugation	3	...	40/40	38/40	31/40	17/40
iv. 1.2 ml of (iii) after Sepharose gel-filtration and concn by ultracentrifugation	1.2	...	40/40	40/40	40/40	40/40

[a]The numerator represents number of infected seedlings; and the denominator, the number of beans inoculated.

not infective. Highly infective preparations were obtained from the 1% and 0% eluates when source leaves showed extensive necrotic symptoms (i.e., leaves at advanced stage of infection) (Table 2, preparations 1 and 2). Whether filtration was done at 25 C or 0-4 C, the combined 0% and 1% PEG fractions had only about 50%

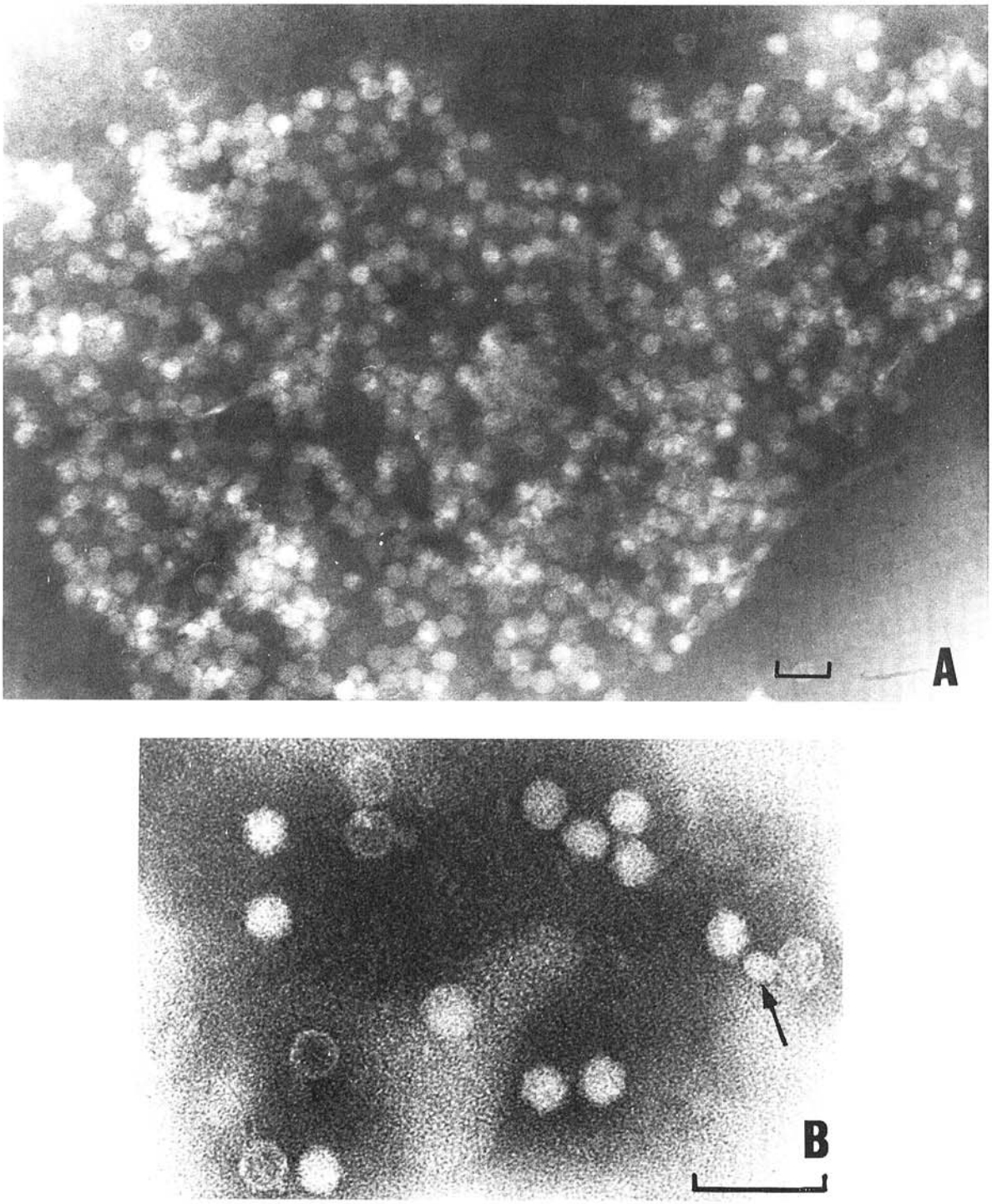

Fig. 1-(A, B). Electronmicrograph of purified cocoa necrosis virus (CNV). **A)** A large aggregate of CNV particles. **B)** CNV particles in a less dense area of the same preparation as **A)**. Arrow shows one of the smaller 11-12 nm particles described in the text. The preparation was made from 20.6 g of infected leaves by clarification of sap with pectinase and two cycles of differential centrifugation. The bars represent 100 nm.

of the infectivity of the crude sap. Elution with excess buffer after the 0% PEG fraction gave no infective virus.

The chromatography on Celite 545 was found to remove practically all coloring matter and the concd 1% and 0% fractions were clear and nonviscous. However, electron microscopy showed that the preparations made by this method were more dilute than those obtained by differential centrifugation of PEG-precipitated virus from equivalent amounts of source material.

Effect of clarifying the sap with pectinase on purification by differential centrifugation.—Incubation of extracted sap with pectinase followed by precipitation of virus with PEG and one to two cycles of differential centrifugation yielded clear, nonviscous preparations. Without the pectinase treatment, some mucilage persisted in the pellets after three cycles of differential centrifugation. Possible loss of virus by the pectinase treatment was investigated by comparing the infectivities of preparations incubated with the enzyme with those of untreated aliquots stored at 0-4 C over the experimental period. The results were erratic. For example, two pectinase-treated extracts showed similar infectivities as the untreated aliquots (e.g., Table 3, prep. 2). In another experiment the untreated and pectinase-treated aliquots produced, respectively, 33 and 10 infections out of 40 beans inoculated. Similarly, the infectivities for the untreated and pectinase-treated aliquots in one experiment were, respectively, 26 and 14 (out of 40 inoculated) but when virus was subsequently precipitated with PEG and then purified and concd by differential centrifugation, there was no clear difference in the infectivities (34 and 38, respectively) of the two preparations. The results suggest that some virus may be lost by incubation with pectinase, but this is probably compensated for by removal of mucilage.

Results of two experiments in which clarification of crude sap with pectinase was employed in the purification procedure are shown in Table 3. In the first experiment, the purified preparation diluted 100-fold was as infective as the pectinase-clarified sap. In experiment 2, the 3 ml preparation obtained after two cycles of differential centrifugation, when diluted a 1,000-fold infected 38 of the 40 beans inoculated as compared with 14 out of 40 for the 10-fold diluted crude sap.

Effect of gel filtration.—Gel filtration was found to greatly improve the infectivity of some virus preparations, particularly those obtained from source leaves mostly at the less-advanced stage of infection. In Table 2, preparation 3, the 1% and 0% PEG fractions were only weakly infective (three infections) but its Sephadex G200 filtrate, at approximately the same dilution, infected all 20 beans inoculated; and when the concd filtrate was tested at a concn seven times more dilute than the original volume (i.e., at 10^{-2}), its infectivity was equal to that prior to the gel filtration. A similar result was obtained when a preparation made by differential centrifugation was filtered through Sepharose 4 B (Table 3, preparation 2). In this experiment, 1.2 ml of a 3 ml virus suspension was filtered through the gel, followed by sedimentation of virus by ultracentrifugation and concn to 1.2 ml. At a dilution of 10^{-4}, the Sepharose product infected all 40 beans inoculated, whilst the untreated aliquot at the same dilution infected only 17 beans. Tests were not made beyond the 10^{-4} dilution, but it is estimated that the Sepharose gel filtration enhanced the infectivity by at least a factor of 10. The enhancement of infectivity by gel filtration was observed only with preparations made from source material with a greater proportion of leaves at the less-advanced stage of infection and was not observed with preparations from leaves consisting largely of highly necrotic leaves. In the only comparison made, electron microscopic examination of the preparation before and after Sepharose-gel filtration [Table 3, preparation 2 (iv)] showed that the concd gel-filtered product contained relatively fewer particles than the untreated aliquot [preparation 2, (iii)], suggesting that some particles were retained in the column. This was not observed with the Sephadex G200 filtrates.

Electron microscopy.—All preparations when mixed with neutral phosphotungstate contained isometric particles with a hexagonal outline which were 25-28 nm in diam. The particles were more numerous (and frequently in aggregates, Fig. 1-A) in highly infective preparations than in less infective ones.

In one preparation a few particles, 11-12 nm diam and similar to those described by Kenten (6) were found (Fig. 1-B).

Ultraviolet absorption spectrum.—Purified preparations showed an absorption peak at 260 nm and a minimum near 244 nm. The 260/280 absorption ratios for the virus preparations described in Table 3, preparations 1 (ii) and 2 (ii) were 1.35 and 1.44, respectively. After gel filtration the ratio for the second preparation, i.e., 2 (iv) was 1.53.

DISCUSSION.—The precipitation of polyionic polymers (e.g., dextran sulphate and methyl cellulose) at high salt concns (9) is exemplified here by the finding that PEG alone precipitated virus with only small amounts of mucilage, whilst the presence of 0.2 to 0.4 M NaCl caused large amounts of mucilage to be precipitated. Tannins and mucilage have been identified as important components of cocoa and other plants which cause inactivation of viruses during their isolation (5, 7). Mucilage, at high concns, causes loss of infectivity. Thus, dilute sap is highly infective, but considerable amounts of infectivity are lost when virus is precipitated under conditions where large amounts of mucilage are also precipitated. This exposure to the highly viscous environment probably causes virus inactivation. In the experiments described here, precipitated virus was always removed by centrifugation prior to the assays. The possibility that the loss was due to trapping of virus particles by precipitated mucilage cannot, therefore, be ruled out. Fractionation with $(NH_4)_2SO_4$ (in the absence of PEG) gave fractions with increasing infectivity up to the 60% (w/v) $(NH_4)_2SO_4$ which was tried (1). Polyethylene glycol and NaCl mixtures are widely used for the precipitation of viruses, and in the present work NaCl at 0.4 M had no adverse effect on CNV. It is, therefore, unlikely that the loss of infectivity was caused by exposure of virus to high ionic concns or by sensitivity of the virus to NaCl.

Whereas substantial losses occurred in the chromatography on Celite, the differential centrifugation method, with or without the pectinase treatment, gave a good recovery of infective virus. However, the effective removal of host plant materials by Celite due, possibly, to

its ability to act both as a filter and an adsorbent, (4, 10) would make it useful for the preparation of clear virus suspensions for serological and other studies.

The use of pectinase as a clarifying agent is essential for obtaining clean preparations by a few cycles of differential centrifugation. This treatment also facilitated further purification by gel filtration which, otherwise, was not possible because of the persistence of mucilage in preparations obtained by differential centrifugation. It is possible also that recovery of virus during chromatography on Celite would be better with pectinase-clarified extracts than with untreated ones. It seems likely that chromatography on Celite 545 with PEG, and digestion with pectinase, may both enable clean preparations of other cocoa viruses to be made from cocoa leaves, the latter procedure being suitable for the stable viruses only.

The increased infectivity of CNV preparations following the gel filtration may be attributed to removal of host inhibitors. The enhancement of virus infectivity by gel filtration, due to removal of virus inhibitors, has been observed also for other fruit-tree viruses (10). Aliquots for infectivity tests were always dialyzed prior to the comparison with the gel-filtered aliquots, and as the enhanced infectivity was observed only with preparations from leaves showing relatively less necrotic damage, it may be assumed that the inhibitor is a nondialysable, biologically active, polymer which probably becomes degraded in the highly necrotic tissues.

LITERATURE CITED

1. ADOMAKO, D., and G. K. OWUSU. 1973. Purification of cocoa necrosis virus (CNV) from cocoa leaves. Pages 224-228 *in* Rep. Cocoa Res. Inst. Ghana. 1971-1972.
2. BRUNT, A. A., and R. H. KENTEN. 1963. The use of protein in the extraction of cocoa swollen shoot virus from cocoa leaves. Virology 19:388-392.
3. CLARK, M. F. 1968. Purification and fractionation of alfafa mosaic virus with polyethylene glycol. J. Gen. Virol. 3:427-432.
4. FRANCKI, R. I. B. 1972. Purification of viruses. Pages 295-335 in C. I. Kado and H. O. Agrawal, eds. Principles and techniques in plant virology, Van Nostrand-Reinhold, New York. 688 p.
5. FULTON, R. W. 1966. Mechanical transmission of virus from woody plants. Annu. Rev. Phytopathol. 4:79-102.
6. KENTEN, R. H. 1972. The purification and some properties of cocoa necrosis virus, a serotype of tomato black ring virus. Ann. Appl. Biol. 71:119-126.
7. MATTHEWS, R. E. F. 1970. Plant virology. Academic Press, New York. 778 p.
8. OWUSU, G. K. 1971. Cocoa necrosis virus in Ghana. Trop. Agric. Trin. 48:133-139.
9. PHILIPSON, L., P. A. ALBERTSON, and G. FRICK. 1960. The purification of viruses by aqueous polymer phase systems. Virology 11:533-571.
10. VENEKAMP, J. H. 1972. Chromatographic purification of plant viruses. Pages 369-389 *in* C. I. Kado and H. O. Agrawal, eds. Principles and techniques in plant virology, Van Nostrand-Reinhold, New York. 688 p.