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IDENTIFICATION OF COWPEA MOSAIC
VIRUS ISOLATES

HARI OM AGRAWAL

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DER
LANDBOUWHOGESCHOOL
WAGENINGEN

IDENTIFICATION OF COWPEA MOSAIC VIRUS ISOLATES

(MET EEN SAMENVATTING IN HET NEDERLANDS)

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE RECTOR MAGNIFICUS IR. W. F. EIJSVOOGEL,
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DE WEG- EN WATERBOUWKUNDE EN DE BOSBOUWARCHITECTUUR,
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VAN EEN COMMISSIE UIT DE SENAAT
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN
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HARI OM AGRAWAL



IDENTIFICATION OF COWPEA MOSAIC VIRUS ISOLATES

A DISSERTATION

SUBMITTED TO
THE STATE AGRICULTURAL UNIVERSITY
WAGENINGEN, THE NETHERLANDS, IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF AGRICULTURAL SCIENCES
ON FRIDAY MAY 1, 1964 AT 4 P.M.

BY

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DER
LANDBOU'WHOGESCHOOL
WAGENINGEN

H. VEENMAN & ZONEN N.V. - WAGENINGEN - 1964

STELLINGEN

Propositions

I

A reliable identification of a plant virus should include not only host range, properties of the virus in plant sap and transmission studies but also serology, electron microscopy, biophysical and biochemical characteristics. In places where facilities do not exist for work on all these aspects mutual co-operation and collaboration among research workers should be sought.

II

In the light of the recent knowledge on the structure of viruses the term 'spherical viruses' should be abandoned.

III

A classification of viruses can possibly be based on serological characteristics, amino acid composition of the viral protein, base ratios of the nucleic acid and on the morphology including the physical architecture of the virus particles.

IV

The evidence that the *corpus allatum* produces two different hormones namely a 'gonadotropic hormone' and the 'juvenile hormone' is not conclusive.

H. SÄGESSER, J. Ins. Physiol. 5: 264-285. 1960.

M. LÜSCHER, and A. SPRINGHETTI, J. Ins. Physiol. 5: 190-212. 1960.

V. B. WIGGLESWORTH, J. Ins. Physiol. 9: 105-119. 1963.

P. KARLSON, Insect Hormones, in Comprehensive Biochemistry, Vol. 2. Elsevier Publishing Co., 1963.

V

Lincoln et al.'s evidence that the flowering stimulus of *Xanthium* has been isolated is not convincing.

LINCOLN et al., Science 133: 756, 1961.

LINCOLN et al., Nature 195: 918, 1962.

VI

MCDONNELL'S conclusion that in *Fusarium oxysporum* f. *lycopersici* diseased tomatoes, pectolytic enzymes should not play any important role is inadequately supported by experimental data.

K. MCDONNELL, Trans. Brit. mycol. Soc. 45: 55-62. 1962.

VII

Foreign aid to improve agriculture cannot be based only on 'know-how' acquired in other countries but should in principle be directed to promote research for knowledge adapted to local conditions.

VIII

The migration of scientists is a world-wide phenomenon and denotes a world-wide change for the betterment of the social, political, economic, and scientific climate.

STEVAN DEDIJER, *Nature* 201: 964-967, 1964.

IX

In the interest of public health, smoking in public should be prohibited.

Knowledge is limitless and so also the application of truth. Every day we add to our knowledge of the power of the Atman¹, and we shall keep on doing ever the same. New experience will teach us new duties, but truth shall ever be the same. Who has ever known it in its entirety?

MAHATMA GANDHI

*to my parents and
to all my friends over the globe,
striving for a better mutual understanding,
with affection and regard.*

¹ The Godhead that is within every being.

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CHAPTER 1

INTRODUCTION

Several viruses of tropical legumes are reported in the literature. Unfortunately, many of them are incompletely studied and described, quite often making a comparison not only misleading but almost impossible. Viruses affecting cowpea, *Vigna unguiculata* (L.) Walp.¹, are no exception to this observation and can provide a starting example.

Cowpeas are commonly grown throughout the tropics and subtropics and probably constitute one of the most ancient vegetable sources of human food. They provide food for millions of people and feed for a vast number of livestock and are produced on a large-scale mainly in the south of the United States, Australia, the Mediterranean region, Southern Rhodesia, and South Africa (SELLSCHOP, 1962). Cowpeas are also produced on a commercial scale in parts of Northern Nigeria. In South America, cowpeas are grown to a limited extent in northern Argentina, Paraguay and Venezuela. In Africa cowpea is one of the most important leguminous crops in terms of production and its use as a food crop. Not only the dry seeds but, also depending on the variety, green pods and seeds or young leaves may be eaten. Specially selected edible varieties provide a popular food in some of the southern states of the U.S.A.

Among other legume virus diseases a mosaic disease of cowpea was observed in fields around Paramaribo in Surinam (South America) during the year 1959. The material was sent to Wageningen for diagnosis, and BOS (personal communication) made preliminary studies in an attempt to identify the virus. No definite conclusions on the identity of the virus could be made with these results and the descriptions in the literature. In the meantime VAN HOOFF (1962a) was able to transmit the virus by means of beetles, suggesting a possible similarity with a cowpea mosaic virus described by DALE (1949).

A review of the literature indicates that quite similar symptoms can be produced by different viruses on the same host and also that slight differences in symptoms do not necessarily mean that they should be produced by different viruses. There has been a tendency to describe a virus as 'new' wherever slight differences in host range, thermal inactivation point, dilution end point, and longevity in vitro of the virus in plant sap, could be found, thus adding to the already existing confusion. Such complications have been partly due to differences in procedures, host plants, varietal differences, and strain differences, in different countries, often unrealized. A contribution to standardizing the procedures for international identification of legume viruses was made by BOS,

¹ Apparently there seems to be some confusion on the taxonomy of this species. The names *Vigna sinensis* Endl., *Vigna sinensis* Savi, and *Vigna unguiculata* (L.) Walp. have been used interchangeably for cowpeas by different authors. On the basis of all the literature available, SELLSCHOP (1962) concluded that *Vigna unguiculata* (L.) Walp. is the valid name for cowpeas. He obtained further confirmation on the correctness of this name for the cultivated cowpea by the authorities at Royal Botanic Gardens, Kew. Hence this name is accepted by the present author.

HAGEDORN and QUANTZ (1960) and a further step has been taken in the formation of an international working group on legume viruses. It has also been realized that more and more work on the intrinsic properties of the viruses themselves should be done for a definite identification of the disease, the causal agent and subsequent control.

Indications of differences in host range and properties of the virus in plant sap from the earlier descriptions, the existing confusion in the identity of cowpea mosaic virus and absence of a complete description, led the present author to make an attempt to fill this gap. Hence the present investigation was taken up to make a detailed study of the cowpea mosaic virus isolates obtained from Surinam for providing a reliable identification and characterization of this virus with the help of all conventional and possible modern techniques of virus study. An effort was also made to identify the cowpea mosaic viruses reported by other workers. The relationship of the present virus with other viruses is discussed. Emphasis has been laid on biophysical, biochemical, and immunological characteristics of the virus without undermining the value of host range and other older criteria.

REVIEW OF LITERATURE

Cowpea mosaic transmitted by bean leaf beetle (*Cerotoma trifurcata* Först.) was reported as early as 1924 by SMITH from the United States. The disease was known to be present in the states of Louisiana, Arkansas, and Indiana, and caused mottling, crinkling and distortion of the cowpea leaves. The virus could also be mechanically transmitted. No more details on this virus are available and no further studies have been made. It seems possible that this virus still exists in cowpea plants in the United States, may be in complex with other viruses and has escaped for some reason detection and isolation by later workers.

DALE (1949, 1953) reported a cowpea mosaic transmitted by leaf beetle, *Cerotoma ruficornis* (Oliv.) from Trinidad. The virus was found to be seed-borne in *Vigna unguiculata* and was not transmitted by aphids. CHANT (1959, 1960, 1961) reported a beetle (*Oothecha mutabilis* Sahlb.) transmitted cowpea mosaic virus from Nigeria. He proposed a new name 'cowpea yellow mosaic virus' for it, since it produced a yellow mosaic on cowpea and showed certain other differences. He further reported that the virus in Nigeria is particularly severe in its effects on leaf area, flower production, and yield. CHANT (1962) in further studies on the Trinidad virus suggested, on the basis of similarities in host range, that this virus and the Nigerian virus may be related.

Beetle transmission of the Surinam isolates was studied by VAN HOOFF (1962a, 1962b). The isolates Vu and Vs (see virus and plant material) were transmitted by leaf beetles *Cerotoma variegata* F., *Diabrotica* sp. (probably *laeta* F.), and *Diphaulaca* sp. (probably *meridae* Barber). The Sb isolate was transmitted only by *Cerotoma variegata* F. VAN HOOFF (1962b) also found wild plants of *Vigna vexillata* (L.) Benthham and an undetermined *Vigna* species infected with the same virus in Surinam.

In addition to the above beetle-transmitted cowpea mosaic diseases, several other mosaic diseases of *Vigna* species have been reported from different countries including China (YU, 1946), India (VASUDEVA, 1942; CAPOOR, VARMA and UPPAL, 1947; CAPOOR and VARMA, 1956; NARIANI and KANDASWAMY, 1961), Indonesia (HARJONO, 1959), Japan (HINO, 1960), New Guinea (VAN VELSEN, 1962), Portugal (OLIVEIRA, 1947), South Africa (KLESSER, 1960), United States of America (MCLEAN, 1941; SNYDER, 1942; WARID and PLAKIDAS, 1950, 1952; ANDERSON, 1955 a, b, c, d, 1957, 1959), and U.S.S.R.-Central Asia (VLASOV, 1960). Any comparison and conclusions on the identity of these viruses must await their complete description.

VIDANO (1959) in his investigations on withering of cowpea in Italy mentions the presence of a 'cowpea mosaic virus complex' transmissible by several insect species and suggests its possible similarity to the Trinidad cowpea mosaic virus of DALE. It is impossible to conclude on the identity of virus(es) involved in this complex with VIDANO'S data, although it seems probable on the basis of symp-

toms described in this publication that a virus similar or related to the beetle-transmissible cowpea mosaic might have been present.

Strains of several earlier identified viruses are also known to infect and produce a mosaic disease in cowpea. ANDERSON (1955b) and KLESSER (1960) isolated a strain of cucumber mosaic virus from naturally infected cowpeas showing mosaic symptoms. LISTER and THRESH (1955), and BAWDEN (1956, 1958) reported the occurrence of a strain of tobacco mosaic virus causing a mosaic disease of cowpea. SHEPHERD and FULTON (1962) reported a strain of southern bean mosaic virus seed-borne and producing a mosaic in cowpea. CORBETT (1957, 1962 personal communication) identified ANDERSON's cowpea mosaic virus nothing but a strain of bean yellow mosaic virus. BRIERLEY and SMITH (1962) isolated what they called a cowpea strain of bean yellow mosaic virus from three gladiolus cultivars.

Note added in proof: BRANDES (1964) in his publication "Identifizierung von gestreckten pflanzenpathogenen Viren auf morphologischer Grundlage", published as "Mitteilungen (Heft 110) aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, Berlin-Dahlem", used the name "cowpea mosaic virus" for an elongated virus, which he reported to be also serologically related to bean common mosaic virus. Since his virus is an elongated virus, quite distinct from the cowpea mosaic virus (SMITH, 1924; DALE, 1949; CHANT, 1959, 1962) which is polyhedral in shape and is beetle-transmissible, the name "cowpea mosaic virus" for his virus is not justified.

CHAPTER 3

VIRUS AND PLANT MATERIAL

The following isolates of cowpea mosaic virus were used in this investigation:

1. Cowpea yellow mosaic from Nigeria
2. Cowpea mosaic from Trinidad
3. Vu isolate of cowpea mosaic from Surinam, South America
4. Vs isolate of cowpea mosaic from Surinam
5. Sb isolate of cowpea mosaic from Surinam

The names Vu, Vs, and Sb for the above 3 isolates have been given just for convenience by the present author and are not intended to be descriptive. The Nigerian and the Trinidad isolates were kindly supplied by Dr. A. J. GIBBS, Rothamsted Experimental Station, Harpenden, England; the Vu and Vs isolates by Dr. L. BOS, Wageningen, and the Sb isolate was kindly provided by Dr. H. A. VAN HOOF, Wageningen.

Single lesion cultures from *Phaseolus vulgaris* L. var. 'Pinto' were used and the virus isolates were maintained on cowpea by mechanical inoculation. Periodical checks to ensure consistent identity of the isolates were made on *Phaseolus vulgaris* varieties 'Pinto' and 'Beka', and *Chenopodium amaranticolor* Coste and Reyn. which were found to be good local lesion and/or systemic hosts. All inoculations were made using the fore-finger and employing Carborundum (500 mesh) as abrasive. After inoculation the leaves were rinsed in a stream of water. Two to three week old cowpea plants were used in all cases. The bean (*P. vulgaris*) plants were most susceptible and gave uniform results when the two primary leaves were well expanded and the *Chenopodium amaranticolor* plants when they had 6-8 leaves; hence in all experiments only such plants were used. For all the other species fairly young plants were used although the age was found not to be so critical. Crude juice diluted in 0.01 M phosphate buffer pH 7.0 was routinely used as inoculum for maintaining the cultures. All plants were raised and kept in the greenhouse at a temperature of about 20°C supplemented with artificial (fluorescent) light in winter when the light conditions were rather poor. Sometimes in summer the temperature was slightly higher for short periods. The temperature and light conditions, in general, were found to have profound effect on the growth of the plants and on the appearance of virus symptoms.

General procedures essentially as suggested by Bos et al. (1960) were employed. The details of the procedures in individual cases, any modifications and new methods employed, are given and discussed at pertinent places in the individual chapters.

CHAPTER 4

HOST PLANT REACTIONS

4.1. HOST RANGE AND SYMPTOMATOLOGY

Several species of plants were inoculated with Vu and Vs isolates separately. The Sb isolate was tested only on species which were found to be diagnostically important for the identification of the virus. The reactions, in general, of the Nigerian and Trinidad isolates reported earlier (DALE, 1949; CHANT, 1959, 1962) on several hosts were confirmed and used for comparison with the other isolates. The experiments were repeated at different times of the year to provide for any seasonal variations and 6–10 plants of each species were used each time. The results were generally recorded 2–4 weeks after inoculations. Back inoculations were made from all those plants which did not show any symptoms to check if virus could be recovered from them and if any of them were symptomless carrier of the virus. The results are summarized in Table 1 and the symptoms produced by several of the isolates on some of the species are shown in Photos 1–8. A comparison of the symptoms produced by Vu and Sb isolates on *Phaseolus vulgaris* var. 'Beka' and *Chenopodium amaranticolor* is presented in Photo 9.

It is evident from Table 1 that the symptoms produced by Vu and Vs isolates on different species are essentially similar although there are some minor differences. The Vs isolate, as indicated in the table, produced less severe symptoms than the Vu isolate under identical conditions. On the other hand the Sb isolate shows marked differences from the Vu and Vs isolates.

The species *Chenopodium amaranticolor*, *Chenopodium quinoa*, *Glycine max*, *Phaseolus aureus*, *Phaseolus lunatus*, *Phaseolus mungo*, *Phaseolus vulgaris*, *Pisum sativum*, and *Vigna unguiculata* have been found to be good hosts for the virus while *Amaranthus caudatus*, *Capsicum annum*, *Cucumis sativus*, *Datura stramonium*, *Lycopersicum esculentum*, *Medicago sativa*, *Trifolium incarnatum*, *Trifolium pratense*, and *Vicia faba* did not react at all and no virus could be recovered. Several species gave local reaction which was not very distinct.

In cowpea (*Vigna unguiculata*) the Vu, Vs, and Trinidad isolates produced a severe mosaic with light and dark green patches and prominent blister-like areas on the trifoliolate leaves in contrast to the Nigerian and Sb isolates which gave mainly a bright yellow mosaic and vein-clearing. The Sb and Nigerian isolates also produced severe systemic symptoms on *Chenopodium amaranticolor* in addition to local lesions caused by all the isolates. Similarly the reaction on 'Beka' beans is very marked in that the Vu, Vs, and Trinidad viruses produced severe apical and systemic necrosis in addition to mosaic symptoms, and the plants mostly collapsed within a week after inoculation while the Nigerian and Sb isolates rarely gave any necrosis. The reaction on peas (*Pisum sativum*) showed a very interesting phenomenon (Photo 4). The symptoms consisted of a diffuse mottling, slight vein-banding, bright yellow areas, and systemic mosaic. The plants appeared to have recovered, the young apical leaves quite often were

symptomless for sometime, while the sprouting side shoots showed bright mosaic symptoms. The reason for this sort of recovery and cyclic appearance of symptoms is not clear at the moment.

4.2. CYTOLOGY

Examinations of epidermal strips were made to check for inclusion bodies following the method used by RUBIO-HUERTOS (1962). The strips were stained with 1 % phloxin without previous fixation, mounted in water and observed under the light microscope. The amorphous inclusions and the nucleoli were stained very bright red while the nuclei were stained pink. Chloroplasts and plastids remained unstained. This greatly facilitated the identification of the inclusion bodies which was kindly confirmed by Dr. RUBIO-HUERTOS.

Out of all the plants infected by cowpea mosaic virus, peas and cowpeas were found to be the best material for such studies. The concentration of the inclusion bodies was about the best in peas, since most of the epidermal cells in this host were found to contain one. These inclusions were amorphous and mostly vacuolar (Photo 10). Their shape varied but was generally ovoid and they were larger than the nuclei. Some cells also showed granular inclusions (Photo 10B, and E). The granular type could possibly be a stage in the formation of the former. No such structures were ever seen in the strips from healthy plants. The presence of these bodies and their morphology might help in the identification of the virus and a more detailed study probably would indicate the relationship of such intracellular structures to the development and formation of virus particles.

4.3. VARIETAL REACTION

4.3.1. *On Phaseolus vulgaris*

Twelve American and Dutch *P. vulgaris* varieties, including some of those commonly used for virus testing, were inoculated for their reaction to Vu and Vs isolates. The results were recorded 7-10 days after inoculations and are summarized in Table 2.

It is evident from Table 2 that the reaction of individual virus isolates is different on different varieties. The isolate Vu produces very severe systemic symptoms on var. 'Beka'; only local symptoms on var. 'Pinto'; local symptoms only on var. 'Bountiful', but virus recoverable from systemically invaded symptomless leaves; and no symptoms on and no virus recoverable from var. 'Processor'. It is also evident from the table that symptoms produced by the Vs isolate resemble in general the symptoms produced by the Vu isolate, although there are slight differences in some cases.

4.3.2. *On Vigna unguiculata*

Fourteen cowpea varieties, some of which selected on the basis of their reaction to Nigerian cowpea yellow mosaic (WELLS and DEBA, 1961), were tested against Vu and Vs isolates. The variety 'Monarch Blackeye' was kindly supplied by Mr. H. H. FISHER of the New Crops Research Branch, U.S.D.A., A.R.S., Beltsville, Maryland, U.S.A.; varieties 'Brabham K 892', 'Victor K 798', 'Jackson Alabama', 'Brabham Victor', 'Arlington', 'New Era', and 'Groit', were ob-

TABLE 1. Host range and symptoms of cowpea mosaic virus (isolates Vu, Vs, and Sb).

Plant species	Isolate		
	Vu	Vs	Sb
1. <i>Amaranthus caudatus</i> L.	-	-	•••
2. <i>Arachis hypogaea</i> L.	-	LL (ca. 0.5 mm), no sys. reaction	•••
3. <i>Capsicum annuum</i> L.	-	•••	•••
4. <i>Cassia tora</i> L.	-	•••	•••
5. <i>Chenopodium amaranticolor</i> Coste & Reyn.	LL (ca. 0.5 mm), no sys. reaction dark LL (0.5-1 mm), no sys. symptoms but virus recovered from young leaves on back inoculation LL (very minute, 0.5-1 mm in diameter), no sys. reaction	LL (ca. 0.5 mm), no sys. reaction LL (very minute, 0.5-1 mm in diameter), sys. reaction?	chlorotic LL (0.5-1 mm) later becoming necrotic, severe sys. reaction (mosaic and chlorotic spots), severe leaf distortion, buckling and puckering
6. <i>Chenopodium quinoa</i> Willd.	distinct LL (1-2 mm in diameter) sys. mosaic, severe distortion of leaves chlorotic LL (1-2 mm), no sys. reaction	distinct LL (1-2 mm in diameter), sys. mosaic	••• •••
7. <i>Crotalaria striata</i> DC.	LL (1-1.5 mm), no sys. reaction	•••	•••
8. <i>Crotalaria usaramoensis</i> Bak. f.	-	•••	•••
9. <i>Cucumis sativus</i> L.	-	-	•••
10. <i>Datura stramonium</i> L.	sys. mosaic	sys. mosaic	•••
11. <i>Dalichos lablab</i> L.	yellow spots on the inoc. and sys. leaves, sys. yellow mosaic, slight leaf deformation	yellow spots on the inoc. and sys. leaves, sys. yellow mosaic, slight deformation	sys. yellow mosaic, and vein clearing
12. <i>Glycine max</i> (L.) Merr.	LL (irregular in shape, few and large, 2-6 mm), sys. mottling	LL (irregular in shape, few and about 1.5-3 mm) sys. mottling	LL (few and irregular in shape), no sys. reaction
13. <i>Gomphrena globosa</i> L.	-	-	•••
14. <i>Lycopersicon esculentum</i> Mill.	-	-	•••
15. <i>Medicago sativa</i> L.	-	-	•••
16. <i>Nicotiana glutinosa</i> L.	chlorotic LL, no sys. reaction	chlorotic LL, no sys. reaction	-
17. <i>Nicotiana tabacum</i> L. var. 'White Burley'	chlorotic LL, no sys. reaction	chlorotic LL, no sys. reaction	-
18. <i>Petunia hybrida</i> Vilm.	no distinct symptoms, but virus recovered on back inoculation LL (1-2 mm), no sys. reaction	no distinct symptoms, but virus covered on back inoculation few LL (chlorotic and necrotic) severe sys. nec., apical necrosis, sys. mosaic and sys. necrotic spots on the trifoliolate leaves	•••
19. <i>Phaseolus abortivus</i> Burkart	sys. bright mosaic, yellow spots on trifoliolate, green and small yellow patches on the leaves, severe veinal necrosis and nec. spots primary leaves full of chlorotic	sys. yellow mosaic, green areas lining the veins, necrosis not so severe as with Vu	chlorotic LL, sys. mosaic, deforma-
20. <i>Phaseolus aureus</i> Roxb.	-	-	•••
21. <i>Phaseolus lunatus</i> L.	-	-	•••

22. <i>Phaseolus mungo</i> L.	lesions, some becoming necrotic; puckering, deformation and veining, necrotic and yellow spots on the trifoliolate. Nec. in stem, petiole and apex. Sys. mosaic	same symptoms as with Vu	tion of the trifoliolate leaves and yellow spots, very little necrosis
23. <i>Phaseolus vulgaris</i> L. var. 'Beka'	veinclearing, blistering on the trifoliolate, severe sys. mosaic	LL, no sys. reaction	nec. LL, very severe apical necrosis, plants collapse
24. <i>Phaseolus vulgaris</i> L. var. 'Pinto'	LL, severe mosaic, distortion of lamina, necrotic and yellow spots on the primary leaves, epinasty in the trifoliolate, puckering on the leaves, venal, petiole, and apical necrosis, mottling on the pods, plants collapse	LL, no sys. reaction	reaction similar to Vu, but much less severe and no apical necrosis
25. <i>Pisum sativum</i> L. var. 'Eroica'	LL very distinct (2-4 mm), no sys. reaction	same symptoms as with Vu	LL (1-2 mm) not very distinct, no sys. reaction
26. <i>Pisum sativum</i> L. var. 'Mansholt's Pluk'	vein clearing, sys. mosaic, yellow spots and some fine necrotic dots on young leaves	same symptoms as with Vu	vein clearing and sys. mosaic
27. <i>Trifolium incarnatum</i> L.	vein clearing, slight leaf curling and sys. mosaic	same symptoms as with Vu	-
28. <i>Trifolium pratense</i> L.	-	-	...
29. <i>Vicia faba</i> L.	-	-	-
30. <i>Vigna unguiculata</i> (L.) Walp. var. 'Monarch Blackeye' (U.S.A.)	chlorotic and some necrotic spots on the inoc. leaves, bright mosaic, severe leaf deformation, vein clearing, puckering, few nec. spots on trifoliolate, nec. in stem, petiole and apex, some blister-like areas on the trifoliolate, plants collapse	same symptoms as with Vu but not so severe	bright yellow mosaic, vein clearing leaf distortion and reduction in size, some blister-like areas, on the trifoliolate leaves
31. <i>Vigna unguiculata</i> (L.) Walp. var. 'Blackeye' (Surinam)	symptoms as above, necrosis still severe	symptoms as above, plants collapse	bright yellow mosaic and vein clearing
32. <i>Zinnia elegans</i> Jacq.	sys. yellow spots and mottling	sys. yellow spots and mottling	...
LL = local lesions	Sys. = Systemic ... = plants not inoculated	Nec. = Necrosis or necrotic	-- = no reaction and no virus recoverable

TABLE 2. Reaction of some bean (*Phaseolus vulgaris*) varieties¹ inoculated with Vu and Vs isolates of cowpea mosaic virus.

variety	Vu isolate	Vs isolate
'Beka'	indistinct chlorotic and necrotic local lesions, systemic bright mosaic, puckering and necrotic spots on trifoliolate leaves, epinasty, severe veinal, petiole and apical necrosis, plants collapse	indistinct chlorotic local lesions, severe systemic necrosis, puckering, and systemic bright mosaic
'Pinto 111'	very distinct LL (2-3 mm), no systemic reaction	chlorotic LL, occasionally necrotic, no systemic reaction
'White Seeded Tender Green'	LL (0.5-3 mm) indistinct, systemic mosaic with severe necrosis, occasional apical necrosis	as for Vu, but no necrosis
'Cornell 49-242'	LL chlorotic (0.4-0.6 mm), chlorotic yellow spots on trifoliolate leaves	as for Vu
'Processor'	no symptoms	no symptoms
'Sanilac'	LL (0.5-2 mm), no systemic reaction	few chlorotic and some necrotic local lesions, no systemic reaction
'Red Mexican 34'	LL (1-2 mm), some systemic necrotic lesions on trifoliolate leaves, no systemic mosaic	few chlorotic ringspots on inoculated leaves, no systemic reaction
'Metis'	LL (0.5-1 mm), no systemic reaction	LL (0.5-1 mm) indistinct, no systemic reaction
'Kievit Koekoek'	LL (1-2 mm) indistinct merging into veinal necrosis, no systemic reaction	as for Vu
'Red Kidney'	LL, no systemic reaction	LL, no systemic reaction
'Kentucky Wonder Wax'	no local reaction, vein clearing in trifoliolate leaves and diffuse mosaic	no local reaction, diffuse mosaic
'Bountiful'	LL (2-3 mm), no definite systemic reaction, but virus recovered on back inoculation	LL, no systemic reaction.

LL = local lesions

¹ The seeds were kindly supplied by Mr. N. HUBBELING of the Institute of Phytopathological Research (I.P.O.), Wageningen.

tained from USDA Regional Plant Introduction Centre, Experiment Station, Georgia, U.S.A. (through Dr. D. G. WELLS). All the other varieties were kindly supplied by Dr. J. F. WIENK of the Tropical Agriculture Laboratory, Wageningen.

The results are summarized in Table 3. It is clear from the table that all the cowpea varieties tested were susceptible to Vu and Vs isolates although the severity of symptoms varied a great deal. Further, the varieties reported to be resistant to the Nigerian cowpea yellow mosaic were also found to be resistant to the Sb isolate while they were susceptible to the Vu and Vs isolates. This indicated a similarity between the Nigerian and Sb isolates on one hand, and Vu and Vs isolates on the other.

TABLE 3. Reaction of some cowpea (*Vigna unguiculata*) varieties inoculated with Vu and Vs¹ isolates of cowpea mosaic virus.

variety	symptoms on inoculated (primary) leaves	systemic symptoms
'Monarch Blackeye'	chlorotic and necrotic lesions	vein clearing, distortion and deformation of the lamina, necrosis in stem and petiole, bright mosaic with puckering and blister-like areas; often severe apical necrosis killing the plant
'Brabham K 892'	idem	idem
'Victor K 798'	idem	vein clearing and necrotic spots on trifoliolate leaves, necrosis in stem, apical necrosis or systemic mosaic
'Jackson Alabama'	very distinct chlorotic lesions	vein clearing, bright systemic mosaic, very little necrosis
'Brabham Victor'	chlorotic and necrotic lesions	vein clearing and necrotic spots on trifoliolate leaves, necrosis in stem quite severe, apical necrosis or systemic mosaic
'Arlington'	dark reddish necrotic lesions	very severe apical necrosis, necrosis in stem, <i>plants collapsed</i>
'New Era'	chlorotic lesions	bright systemic mosaic, blistering and vein clearing, <i>no necrosis</i>
'Groit'	chlorotic and necrotic lesions	as for 'Monarch Blackeye', apical necrosis very severe, mosaic not so bright
'Early red'	irregular necrotic lesions	systemic mosaic and vein clearing
'FC 31705'	idem	idem
'Negro'	idem	idem
'PI 221731'	idem	as for 'Monarch Blackeye', quite some apical necrosis
'L 742'	idem	systemic mosaic
'Delhi Local'	necrotic lesions	systemic necrotic spots, leaf deformation, systemic mosaic

¹ The Vs isolate in general produced milder symptoms than the Vu isolate.

CHAPTER 5

PROPERTIES OF THE VIRUS IN PLANT SAP

The term 'Physical Properties' in the past has been commonly used by virologists to include the thermal inactivation point, dilution end point, and aging in vitro of the virus in plant sap. Bos et al. (1960) used the term 'Physicochemical properties in plant sap' to include these characteristics. These terms seem to be misleading in the light of all the recent knowledge on the properties of the viruses themselves (Bos, personal discussion). Hence the more precise and simplified term 'Properties of the virus in plant sap' is used here to include the properties in question.

Cowpea plants (var. 'Monarch Blackeye') were inoculated, when the primary leaves were well expanded, and the sap extracted after two weeks. Young 'Pinto bean' plants with the two primary leaves were used as local lesion host and procedures as laid by Bos et al. (1960) were employed. Phosphate buffer (0.01 M) pH 7.0 was used for making dilutions. Same number of leaves (8-10) were used for all the treatments and lesions were counted after 10 days in all cases.

In view of the restricted importance of these criteria, as discussed later (Pages 38-39), no detailed data are provided here.

5.1. THERMAL INACTIVATION

In a number of tests the virus (Vu isolate) in the sap was inactivated after being heated for 10 minutes at temperatures between 65-70°C.

5.2. TOLERANCE TO DILUTION

The virus (Vu isolate) in the sap was still infective at a dilution of 1:10,000 but no lesions were formed at 1:100,000.

5.3. AGING IN VITRO

The virus (Vu isolate) in the crude sap diluted 1:10 in 0.01 M phosphate buffer pH 7.0, stored at room temperature ($23^{\circ}\text{C} \pm 4$), was found to have a longevity in vitro of 3-5 days.

CHAPTER 6

PURIFICATION

The virus isolates were purified by a modification of the butanol-chloroform procedure (STEBRE, 1956). The procedure is outlined in Fig. 1. Cowpea (mostly var. 'Monarch Blackeye' or var. 'FC 31705') leaves infected for 2 weeks showing distinct symptoms were stored in a deep freeze for 2-3 days, thawed, and disinte-

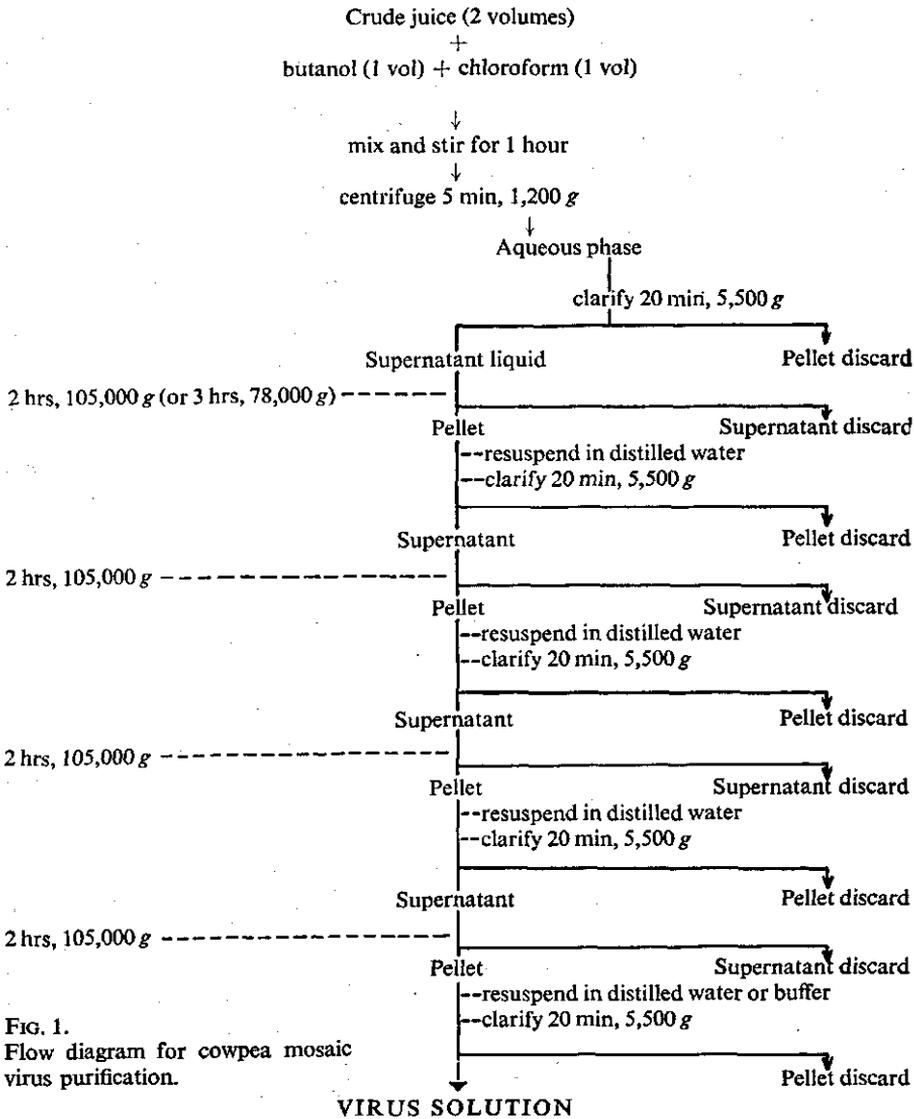


FIG. 1.
Flow diagram for cowpea mosaic virus purification.

grated in a Waring blender in 2 ml 0.1 M (cold) phosphate buffer pH 7.0 per gram of tissue. The juice was extracted through cheesecloth. All further steps were carried out in the cold room at a temperature of about 2°C. One volume of butanol and 1 volume of chloroform were mixed with 2 volumes of crude juice with continuous stirring. The mixture was stirred for 1 hour and then centrifuged in a Servall Type SS-1 superspeed centrifuge for 5 minutes at 1,200 g. The top layer consisting of the aqueous phase and containing the virus was removed and pooled together. This was clarified by centrifuging for 20 minutes at 5,500 g. The clarified suspension was now centrifuged in Spinco model L preparative ultracentrifuge for 2 hours at 105,000 g to sediment the virus (for large quantities this first high speed centrifugation can be substituted with 78,000 g for 3 hours with some loss of virus in the supernatant). The supernatant was discarded and the pellet was resuspended in distilled water. The suspension was given a low speed centrifugation for 20 minutes at 5,500 g. Three more cycles of differential centrifugation were given to get rid of any contaminants. The final pellet was resuspended in distilled water or buffer, and this procedure gave extremely pure preparations. Almost no infectivity was present in the discarded fractions at different stages. No final pellet was obtained when healthy material was treated in a similar manner.

The purified preparations were stored in the cold room (ca. 2°C) and generally retained infectivity for more than a month under these conditions. After this period some disintegration of the particles was observed, especially when the preparation was in water. Such material under the electron microscope revealed many broken and disintegrated particles. This happened to a certain extent even when the preparations were in 0.1 M phosphate buffer pH 7.0. Preparations stored for longer periods were kept frozen since only little disintegration of the particles was observed under these conditions.

6.1. CONCENTRATION OF THE VIRUS IN THE COWPEA PLANT

Several purification runs were made with certain quantities of 2 week, and 4 week old materials infected with the Vu isolate and treated in the same manner to check if there were significant differences in the yield of virus. Both the materials gave about the same yield. Hence, for all purification work only 2 week old material was used. The yields generally were in the range of 80–100 mg of nucleoprotein for 200 g tissue.

6.2. SPECTROPHOTOMETRIC AND ANALYTICAL ULTRACENTRIFUGE STUDIES

The purified virus preparations on examination in a Beckman DU model UV spectrophotometer gave a spectrum typical of nucleoprotein. Such a spectrum for Vu isolate is shown in Fig. 2. It had maximum UV absorption at 259 m μ and minimum at 240 m μ . Values of $E_{\max}/E_{\min} = 1.43$ and $E_{260}/E_{280} = 1.69$ were calculated for this isolate. Similar values for the other isolates were: Vs isolate, $E_{\max}/E_{\min} = 1.43$ and $E_{260}/E_{280} = 1.61$; Trinidad isolate, $E_{\max}/E_{\min} = 1.42$ and $E_{260}/E_{280} = 1.61$; Nigerian isolate, $E_{\max}/E_{\min} = 1.48$ and $E_{260}/E_{280} = 1.62$; and Sb isolate, $E_{\max}/E_{\min} = 1.43$ and $E_{260}/E_{280} = 1.53$.

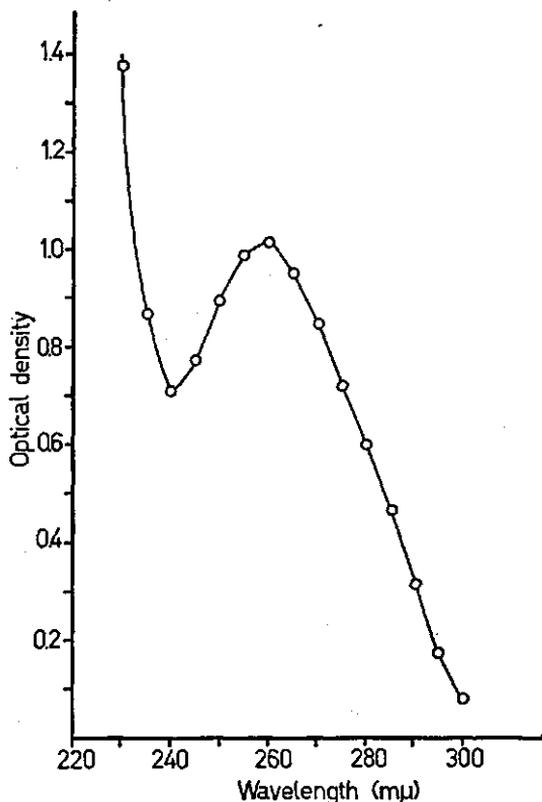


FIG. 2
Ultraviolet absorption spectrum
of a purified preparation of cow-
pea mosaic virus (Vu isolate).

Sedimentation experiments in a Spinco analytical ultracentrifuge with purified virus preparations obtained from 2 week old material, as used above, revealed mostly 3 schlieren peaks, sometimes 4 (Fig. 3, 4, 5 and 6). Sedimentation coefficients ($S_{20,w}$) for the different peaks calculated according to the method of MARKHAM (1960), at infinite dilution were found to be 54, 84, and 104 S , for the Vu isolate; 52, 83, and 101 S for the Vs isolate; and 55, 86, and 103 S for the Sb isolate. Similar determinations for the Trinidad cowpea mosaic and Nigerian cowpea yellow mosaic viruses gave values of 53, 67, 84, 103 S , and 56, 77, 83, 106 S , for the different peaks respectively. Preparations mostly in water and sometimes in 0.1 M phosphate buffer pH 7.0 were used for these measurements. The final values in the two cases gave very slight differences.

The relative proportion of the different peaks varied for different isolates as seen in the ultracentrifugation patterns cited above. Preliminary experiments indicated that the proportion of the peaks might also vary depending on the duration of infection period and by a change in the light and temperature conditions under which the plants were grown. The presence of 4 peaks was not restricted to Nigerian (Fig. 6) or Trinidad isolates since preparations of all isolates sometimes showed a similar pattern. The absence of the additional peak (Fig. 6, 2nd peak) in Figures 3, 4, and 5 may be due to a very small proportion of this com-

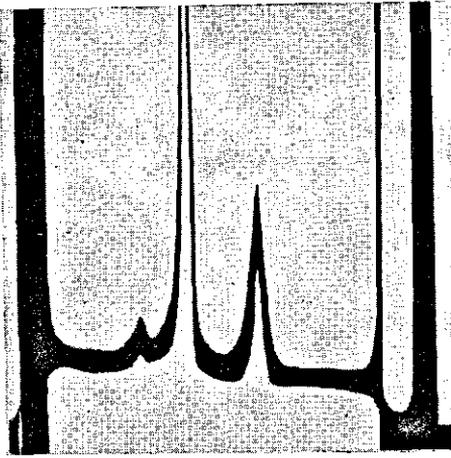


FIG. 3.
 Ultracentrifugation schlieren pattern of an unfractionated preparation of cowpea mosaic virus (Vu isolate) in water. Sedimentation is from right to left, temperature = 20.4°C. Photo after centrifugation at 31,410 rev/min for 20 minutes. $S_{20,w}$ extrapolated to infinite dilution: 1st peak (right) = 54 S, 2nd peak (middle) = 84 S, and 3rd peak (extreme left) = 104 S.

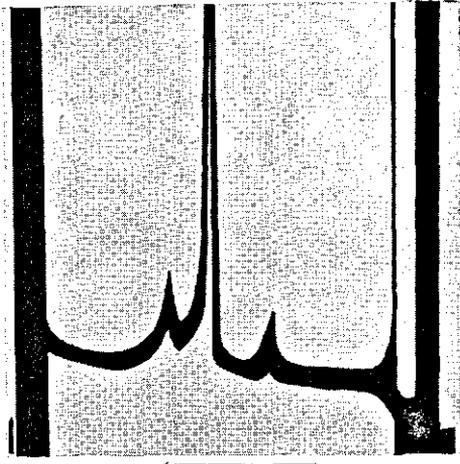


Fig. 4.
 Ultracentrifugation schlieren pattern of an unfractionated preparation of cowpea mosaic virus (Vs isolate) in water. Sedimentation is from right to left, temperature = 16.0°C. Photo after centrifugation at 31,410 rev/min for 20 minutes. $S_{20,w}$ extrapolated to infinite dilution: 1st peak (right) = 52 S, 2nd peak (middle) = 83 S, and 3rd peak (extreme left) = 101 S.

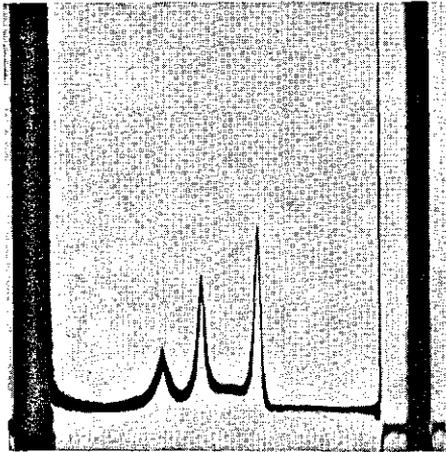


FIG. 5.
 Ultracentrifugation schlieren pattern of an unfractionated preparation of cowpea mosaic virus (Sb isolate) in water. Sedimentation is from right to left, temperature = 20.1°C. Photo after centrifugation at 31,410 rev/min for 20 minutes. $S_{20,w}$ extrapolated to infinite dilution: 1st peak (right) = 55 S, 2nd peak (middle) = 86 S, and 3rd peak (extreme left) = 103 S.

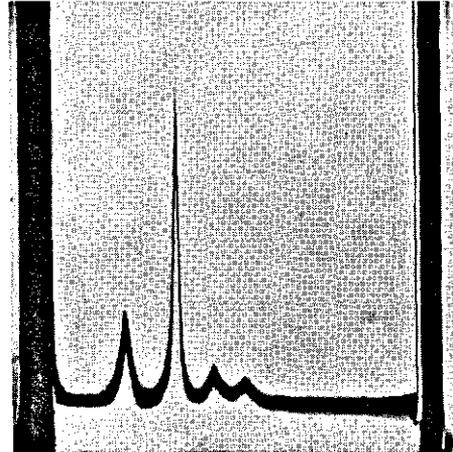


FIG. 6.
 Ultracentrifugation schlieren pattern of an unfractionated preparation of cowpea mosaic virus (Nigerian isolate) in water. Sedimentation is from right to left, temperature = 20.5°C. Photo after centrifugation at 31,410 rev/min for 24 minutes. $S_{20,w}$ extrapolated to infinite dilution: 1st peak (extreme right) = 56 S, 2nd peak = 77 S, 3rd peak = 83 S, and 4th peak (extreme left) = 106 S.

ponent in the preparations. The relative quantity of the 55 *S* peak (Fig. 5) was maximum for the Sb isolate.

Since the ultracentrifugal analyses showed that the purified preparations consisted of mixtures of sedimenting components and since the calculations of sedimentation coefficients were made from such analyses, the values cannot be very precise and are only relative. The 3 components (components with values around 54, 84, and 104 *S*) were separated (Page 33) and sedimentation coefficient measurements from the separated components gave $S_{20,w}$ values of 58, 100, and 119 *S* for the different peaks respectively. These values did not show any significant difference for the different isolates. The differences between the values of the separated components and when the same components were in mixture, are possibly due to the effect of one component on the sedimentation coefficient of another. These effects may be discussed in terms of an interaction between the relative concentration of the different components, backward flow and effective viscosity (SCHACHMAN, 1959).

6.3. ELECTROPHORETIC ANALYSIS

A Perkin-Elmer model 38-A electrophoresis apparatus with 2 ml Tiselius cell was used for these experiments. All the samples were dialyzed in the corresponding buffer for at least 48 hours before running electrophoresis. The preparations showed considerable heterogeneity depending on the pH of the buffer used. One distinct peak (Fig. 7) was obtained for the Vu isolate in 0.1 M phosphate buffer pH 7.0, although there was some indication of heterogeneity. Apparently, this heterogeneity was caused by a slower moving component which was in extremely small quantity (Fig. 7B). A mobility of 4.0×10^{-5} cm/sec/volt/cm was calculated for the Vu isolate; whereas the Vs isolate gave a mobility of 3.8×10^{-5} cm/sec/volt/cm. Under similar conditions the Sb isolate showed two distinct peaks (Fig. 8) indicating two components, the fast moving component with a mobility of 4.25×10^{-5} cm/sec/volt/cm (54.6 %) and a slow moving component with a mobility of 2.80×10^{-5} cm/sec/volt/cm (44.8 %). The percentages were calculated from the dimensions of the respective peaks. Measurements for the Trinidad (Fig. 9) and Nigerian (Fig. 10) viruses gave values of 3.55×10^{-5} (6.4% component), and 2.90×10^{-5} (83.9 % component); and 4.05×10^{-5} (44.5 % component) and 2.66×10^{-5} (55.5 % component) cm/sec/volt/cm respectively. The exact nature of the components is not known at present since they have not yet been separated. The results of immunoelectrophoresis presented in the following pages do indicate that these components are closely related particles for the individual virus isolate, and are not normal plant constituents.

Electrophoretic runs were also made with the Vu isolate in 0.1 M phosphate buffer at pH 5.1, 5.5, 6.5, and 7.5. The preparations showed definite heterogeneity at these pH. Fig. 11 shows the picture obtained at pH 5.5, where the two definite peaks were better separated. The effect of pH on the electrophoretic pattern can be clearly seen by comparing Figs. 7 A and 11. The mobility measurements as indicated in these figures showed a lowering with pH and the patterns indicated an increased heterogeneity with better separation at pH 5.5 than at pH 7.0.

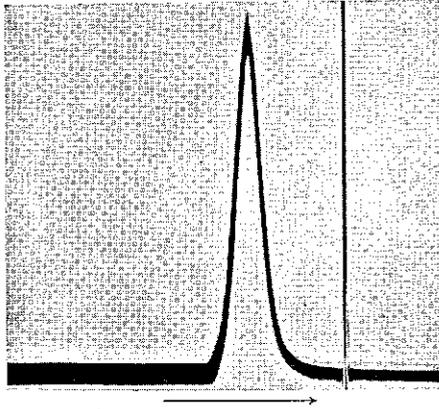


FIG. 7A.
Electrophoretic pattern (ascending limb) of a purified preparation of Vu isolate of cowpea mosaic virus in 0.1 M phosphate buffer pH 7.0 (current = 15 ma). Photo taken 100 minutes after final boundary formation. The vertical line is the reference line. The electrophoretic mobility was 4.0×10^{-5} cm/sec/volt/cm.

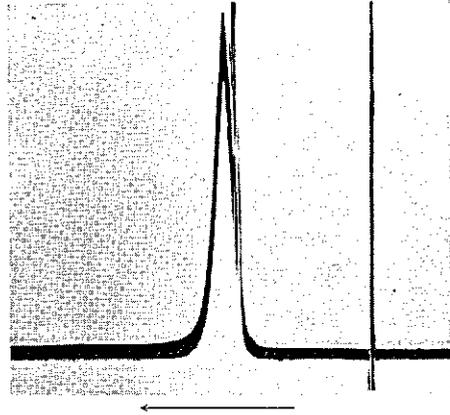


FIG. 7B.
Electrophoretic pattern (descending limb) of a purified preparation of Vu isolate of cowpea mosaic virus in 0.1 M phosphate buffer pH 7.0 (current = 15 ma). Photo taken 85 minutes after final boundary formation. The vertical line is the reference line. The electrophoretic mobility was 4.0×10^{-5} cm/sec/volt/cm. The indistinct peak on the right indicates heterogeneity of the preparation.

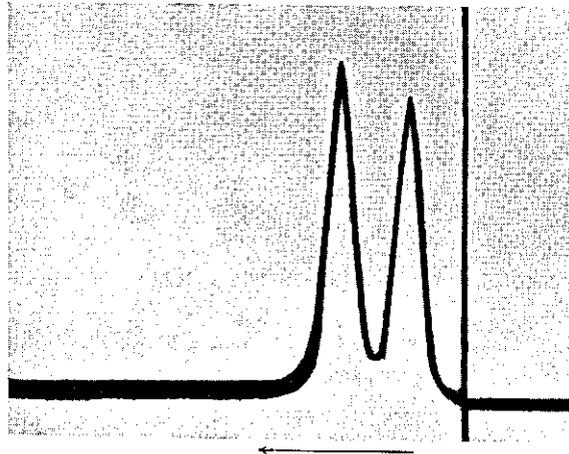


FIG. 8.
Electrophoretic pattern (descending limb) of a purified preparation of Sb isolate of cowpea mosaic virus in 0.1 M phosphate buffer pH 7.0 (current = 14 ma). Photo taken 70 minutes after final boundary formation. The vertical line is the reference line. The electrophoretic mobility of the peak on the left (54.6% component) was 4.25×10^{-5} and for the one on the right (44.8% component) was 2.80×10^{-5} cm/sec/volt/cm.

FIG. 9.

Electrophoretic pattern (descending limb) of a purified preparation of Trinidad cowpea mosaic virus in 0.1 M phosphate buffer pH 7.0 (current = 14 ma). Photo taken 120 minutes after final boundary formation. The vertical line is the reference line. The electrophoretic mobility of the small peak on the left (6.4% component) was 3.55×10^{-5} and for the large peak on the right (83.9% component) was 2.90×10^{-5} cm/sec/volt/cm.

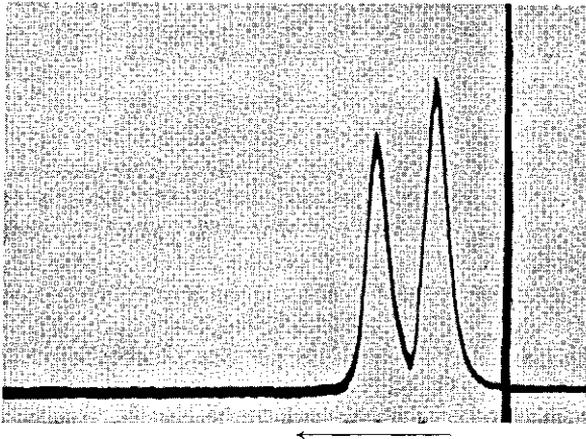
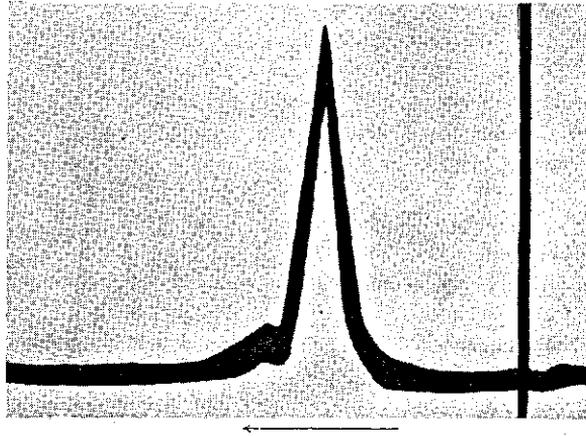


FIG. 10.

Electrophoretic pattern (descending limb) of a purified preparation of Nigerian cowpea yellow mosaic virus in 0.1 M phosphate buffer pH 7.0 (current = 10 ma). Photo taken 90 minutes after final boundary formation. The vertical line is the reference line. The electrophoretic mobility of the peak on the left (44.5% component) was 4.05×10^{-5} and for the one on the right (55.5% component) was 2.66×10^{-5} cm/sec/volt/cm.

The isoelectric point for the Vu isolate was calculated from the mobility measurements of the rapidly migrating component at the different pH, and was found to be 3.4 (Fig. 12) at 0.1 ionic strength. The virus could be precipitated from the solution at this pH for all the isolates, thus confirming the value obtained by the electrophoretic measurements.

The slow movement at pH 5.5 can be explained on the basis of the fact that this pH is closer to the isoelectric point (Fig. 12) resulting in the particles having a greater net (negative) charge at pH 7.0 than at pH 5.5, and hence a greater mobility at the higher pH. The increased heterogeneity at the lower pH may be due to particles having slight differences in the amount of net charge and such differences apparently becoming clearer when closer to the isoelectric point. Since the pattern obtained in the analytical ultracentrifuge at pH 5.5 was essentially similar to that at pH 7.0, the difference in separation of the peaks at these two pH in electrophoresis possibly could not be due to polymerization at the lower pH.

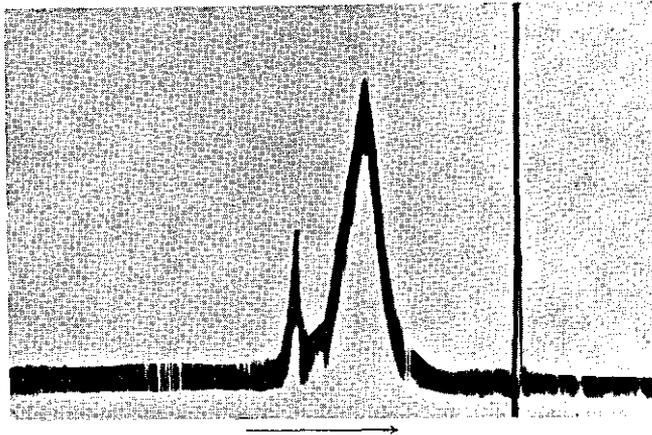


FIG. 11. Electrophoretic pattern (ascending limb) of a purified preparation of Vu isolate of cowpea mosaic virus in 0.1 M phosphate buffer pH 5.5 (current = 13 ma). Photo taken 118 minutes after final boundary formation. The vertical line is the reference line. The electrophoretic mobility of the large and small peak was 2.70×10^{-5} and 2.35×10^{-5} cm/sec/volt/cm respectively.

Electrophoretic mobility has been found to be constant for a particular strain of a virus at a given pH and ionic strength but usually varies from strain to strain (BRINTON and LAUFFER, 1959). KNIGHT and LAUFFER (1942) showed that the ordinary and rib grass strains of tobacco mosaic virus (TMV) had different mobilities under the same conditions. However, they found that the proteins isolated from the two strains by alkali treatment had the same mobilities, even though their amino acid analyses were considerably different. SIEGEL and WILDMAN (1954) used electrophoretic mobility as one of the physico-chemical criteria in their classification of 8 strains of TMV. Such a comparison of the mobilities among the cowpea mosaic virus isolates seems difficult in view of the heterogeneity of the preparations. However, a comparison of the mobility of the rapidly

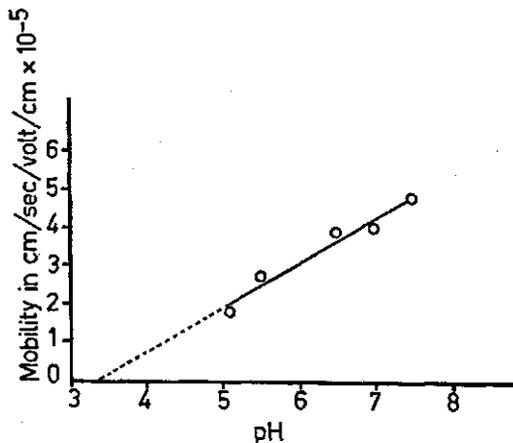


FIG. 12. pH-Mobility curve for cowpea mosaic virus (Vu isolate).

migrating component of the isolates shows that the value for the Sb and the Nigerian isolates is slightly higher than the corresponding values for the Vu, Vs, and Trinidad isolates. It might be also possible to use this difference in distinguishing the two groups. On the basis of the heterogeneity and the proportion of the different peaks, the Nigerian and the Sb isolates can be put together in one group, while the Vu and Vs in another group. The Trinidad isolate however, does not exactly fit in either of these groups.

BAWDEN (1958) found electrophoretic heterogeneity in preparations of a strain of TMV from leguminous plants. The two components, called major and minor components, were similar in their biological and serological properties. However, no information is given on their sedimentation behaviour. BANCROFT (1962) reported an ultracentrifugal and electrophoretic heterogeneity with purified preparations of bean pod mottle virus, a situation more or less similar to cowpea mosaic virus. His results showed that single electrophoretic components were centrifugally heterogeneous and that single centrifugal components were electrophoretically heterogeneous. He suggested that differences in nonantigenic groupings or in protein folding could account for the observed electrophoretic heterogeneity.

SCHWERDT and SCHAFFER (1956) used moving boundary electrophoresis as a step in a purification procedure for the crystallizable poliomyelitis virus from tissue culture fluid. Electrophoresis of a partially purified preparation of the MEF-1 strain consistently showed four components. Infectivity was associated with the component of lowest mobility.

HARRIS and KNIGHT (1955) found that by treating TMV with the enzyme carboxypeptidase, threonine residues were released. The biological properties, size, shape, and density of the treated virus remained identical with the untreated virus while the electrophoretic mobility and serological properties were altered. Since this enzyme occurs naturally in the plant it may effect cowpea mosaic virus and other viruses similarly during extraction (MARKHAM, personal communication, 1963).

In the presence of these instances, it is really impossible to provide an explanation for the electrophoretic heterogeneity of cowpea mosaic virus preparations at this stage. It seems that this heterogeneity is a property of the isolate or the particular strain and is not caused only by enzymatic actions, since all the isolates were multiplied in the same host under identical conditions. It may be possible as well that the amount of nucleic acid in the virus particle has some influence on its net charge and hence on the electrophoretic behaviour.

6.4. ISOLATION AND INFECTIVITY OF RIBONUCLEIC ACID

Ribonucleic acid (RNA) was isolated using essentially the phenol extraction method of GIERER and SCHRAMM (1956) as modified by HALL and DOTY (1959). To a 10 ml solution of the virus in 0.01 M phosphate buffer pH 7.0 at a concentration of approximately 10 mg/ml, at room temperature was added 0.2 ml of a 6% solution of sodium lauryl sulphate. The mixture was stirred for 5 minutes and then placed in an ice bath. All subsequent steps were carried out in a cold

room (ca. 2°C). The solution was shaken for 10 min with 10 ml of water-saturated phenol (freshly redistilled) in a long-necked flask on a wrist-action shaker. The phenol and aqueous phases were separated by centrifugation for 5 min at 3,000 rev/min in a low speed centrifuge. The RNA-containing aqueous layer was pipetted out and shaken two more times with 10 ml phenol-water, followed in each case by centrifugation as before. A final extraction was done with 5 ml phenol-water and stirring for 5 minutes, followed by centrifugation. The solution was extracted three times with 40 ml ether. Practically all the phenol was removed after this treatment. The RNA was precipitated by addition of alcohol and separated by centrifugation. The pellet (RNA) was resuspended in distilled water and made up to the original volume of the virus solution.

The RNA extracted as indicated above, gave a spectrum as shown in Fig. 13, with an absorption maximum at 257 m μ and minimum at 232 m μ . Infectivity tests made on 'Pinto' beans, 'Beka' beans, *Chenopodium amaranticolor*, and cowpea gave positive results. The extracted RNA solution treated with pancreatic ribonuclease (5.4 μ g/ml), did not give any infectivity while the controls were infective. This indicated that the infectious nucleic acid was in fact RNA.

A comparison of the relative infectivity of the dilutions of nucleic acid preparations in water and the parent virus solutions containing equivalent amounts of nucleic acid is presented in Tables 4 and 5. For all quantitative measurements *Chenopodium amaranticolor* plants were used; since they were easy to raise,

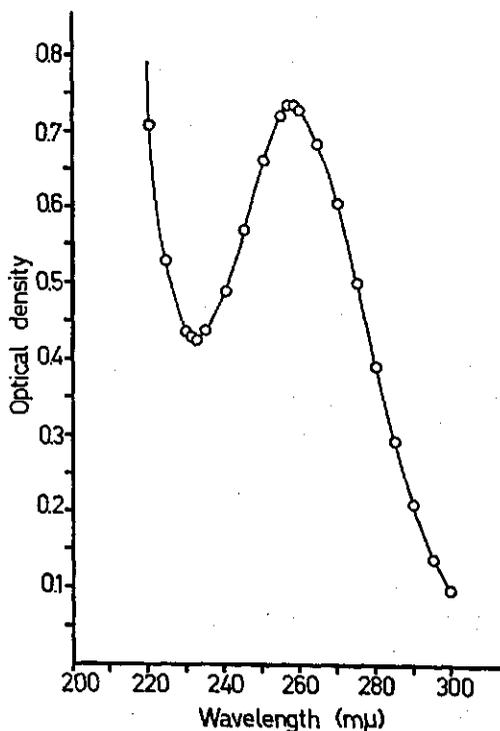


FIG. 13.
Ultraviolet absorption spectrum of nucleic acid isolated from a cowpea mosaic virus preparation (Vu isolate). Water was used as the solvent.

retained susceptibility for longer periods when compared to beans and were found to be, in general, more sensitive to infection. Each preparation was inoculated to at least eight leaves. The local lesion counts in all cases were made 10 days after inoculation.

It is evident from Table 4 that a nucleic acid preparation diluted four times gave about as many lesions as the parent virus solution diluted thousand times. Hence the infectivity of the nucleic acid for the Vu isolate was found to be 0.4 % that of intact virus containing an equivalent amount of nucleic acid. Similar comparisons for a nucleic acid preparation of the Sb isolate (Table 5) gave an infectivity of 0.1 % of that of intact virus containing an equivalent amount of nucleic acid. Since the comparison was made on the basis of dilution and the yield of RNA after phenol extraction was not taken into account, the estimation of relative infectivity cannot be very precise.

TABLE 4. Comparison of the infectivity of cowpea mosaic virus (Vu isolate) and its nucleic acid.

Inoculum	Dilution (in distilled water)	Average number of lesions per leaf on <i>Chenopodium amaranticolor</i>
Nucleic acid	Undiluted	206
	1/2	70
	1/4	27
	1/6	8
	1/10	2
Cowpea mosaic virus (Vu isolate)	1/10	287
	1/100	119
	1/500	45
	1/1000	24
	1/10000	1

TABLE 5. Comparison of the infectivity of cowpea mosaic virus (Sb isolate) and its nucleic acid.

Inoculum	Dilution (in distilled water)	Average number of lesions per leaf on <i>Chenopodium amaranticolor</i>
Nucleic acid	1/10	371
	1/50	43
	1/100	17
	1/1000	1
	1/10000	0
Cowpea mosaic virus (Sb isolate)	1/1000	497
	1/10000	255
	1/20000	177
	1/50000	99
	1/100000	19
	1/1000000	1

CHAPTER 7

ELECTRON MICROSCOPY

Purified virus preparations suspended in distilled water were sprayed on to formvar-coated electron microscope grids (silver plates), shadowed with palladium at an angle of 25° , and were examined in a Philips (EM 100) electron microscope. Spherical virus particles uniform in size and morphology were found (Photo 11A and B). The shadows showed that the particles were angular or polyhedral and not exactly spherical, which can be better seen in Photo 11C, taken under a Siemens Elmiskop I electron microscope.

Negatively stained preparations employing 2 % phosphotungstic acid (dissolved in distilled water) adjusted to different pH with potassium hydroxide, mixed with an equal volume of virus suspension and sprayed on to carbon-coated grids (BRENNER and HORNE, 1959) were made and examined under the Siemens microscope. Sometimes it was necessary to adjust the concentration and volume of potassium phosphotungstate depending on the concentration of the virus in the suspension. The electron micrographs obtained are shown in Photos 12 and 13. Some of the particles in the photographs which show more or less a ring-like structure are presumably empty particles whereas most of them represent apparently particles containing nucleic acid. The rings are believed to represent the protein coats or shells of the virus particles. This appearance of two types of particles is analogous to what has been found with several other polyhedral viruses. Potassium phosphotungstate used at pH 5.1, 6.6, and 7.0 did not seem to make much difference. The particles could not stand potassium phosphotungstate at much lower or higher pH, when they were found to be mostly broken and disintegrated.

7.1. SIZE DETERMINATION

The measurements of the particles for the Vu isolate were 270 Å (side to side) – 300 Å (between extreme points) for particles containing nucleic acid and 250 Å (side to side) – 270 Å (between extreme points) for empty particles. All the other isolates gave measurements of 230–250 Å (for empty particles) and 240–270 Å (for particles containing nucleic acid) with slight differences.

7.2. STRUCTURE

The general appearance of the preparations after negative staining is shown in Photos 12 and 13. Most of the particles in these micrographs, especially the ones containing presumably the nucleic acid (A) show a hexagonal profile while many of the empty particles (B) show a more circular or spherical pattern. Thus it is clear that the particles are not exactly spherical but polyhedral (icosahedral) in shape.

Some of the particles show evidence of substructure in an extremely good preparation (Photo 14) although it shows up with rather low contrast. The micrographs in this photo were made in the normal way but one of them was printed

in reversed contrast. This technique often helps the substructure of the virus particles to be seen more clearly. The exact appearance of any particular particle depends on its orientation and also to a certain extent on its degree of immersion in the sheet of phosphotungstic acid (HUXLEY and ZUBAY, 1960). An attempt has been made to derive from the electron micrographs an arrangement of the morphological (presumably protein) subunits on the surface of the particle, to account for the different appearances observed.

CRICK and WATSON (1956) discussed the structure of small viruses on the basis of the hypothesis that these viruses are built up of identical subunits packed together in a regular manner. They also discussed the reasons in support of the idea why many small spherical viruses should have cubic symmetry. This symmetry has already been demonstrated for bushy stunt virus (CASPAR, 1956) and for turnip yellow mosaic virus (KLUG, FINCH, and FRANKLIN, 1957). Assuming this symmetry for a spherical virus, CRICK and WATSON further discussed the possible combinations of symmetry elements, number and type of rotation axes, number of asymmetric units, and the corresponding solid structure. On the basis of these hypotheses and by analogy with the turnip yellow mosaic virus structure it seems that the cowpea mosaic virus also possesses a cubic symmetry and has a further combination of 5:3:2 (icosahedral) symmetry elements. This is also supported by the type of shadows obtained in a shadow-casted preparation and the hexagonal profile of the particles. On the basis of similar evidence HARRISON and NIXON (1960) concluded that tomato black ring, raspberry ring-spot and arabis mosaic viruses have an icosahedral structure.

MARKHAM et al. (1963) utilising the symmetry of the virus particle described a rotation technique to increase the amount of information obtainable from electron micrographs. This technique has also been employed here, and appears to be of considerable help. Any artifacts in the differentiation of the photographs by this technique can be avoided by careful interpretation.

The patterns have been used to construct a model of the structure and a comparison is presented with a similar model built of table tennis balls (Photo 15). The arrangement seems to resemble an icosahedral body having 60 subunits and 5:3:2 axial symmetry. A similar structure on the basis of X-ray diffraction studies has been suggested for poliomyelitis virus (FINCH and KLUG, 1959). The number of subunits which can be counted at the periphery of the particles mostly is 15 (Photo 15D, and Photo 17A) which is in agreement with the proposed model. A comparison of the Photos 16, 17A, and 17B where individual selected virus particles were rotated according to one of the symmetry elements of the model, shows a very good agreement in outline and number of subunits. The micrograph in Photo 16 shows a particle in threefold axis with its three subunits in the centre as also seen in the model. The subunits appear dark since the original photograph in this case was printed in reversed contrast. A micrograph of an empty particle or the 'core' in Photo 17A, in fivefold symmetry, shows a good agreement with the model in outline and in the number of subunits at the periphery. Fifteen subunits (Photo 17A) can be counted at the periphery as also seen in Photo 15D. It can also be noted that the same particle shows a circular or spher-

ical outline in fivefold symmetry while a hexagonal profile in threefold symmetry. Photo 17B shows a nucleic acid containing particle in fivefold symmetry (rotated $n = 5$) showing 5 subunits in the centre resembling the model. Particles in twofold symmetry i.e. 4 subunits in the centre suitable for the rotation technique could not be found.

A comparison was also made with the proposed model (rhombic triacontahedron and pentakis dodecahedron) of turnip yellow mosaic virus (TYMV) having 32 subunits (HUXLEY and ZUBAY, 1960; NIXON and GIBBS, 1960). But this model was found to be incompatible with the structure of the cowpea mosaic virus since it was impossible to count 15 subunits in the periphery of this model. Moreover, with the cowpea mosaic virus we could not see a pattern of 6 subunits with one in the centre, a clear pattern found in the TYMV structure. It seems that these two viruses are structurally different, at least in their arrangement of subunits, and this might also account for the fact that no serological relationship between them could be found (AGRAWAL and MAAT, 1964).

It may be emphasized here that in the absence of any X-ray diffraction data and since the exact nature, morphology, and chemical structure of the subunits is not known, the electron microscope observations should be interpreted with great caution.

CHAPTER 8

SEROLOGY

8.1. PREPARATION OF ANTISERA

Rabbits previously bled for normal serum were injected with purified virus. Three intramuscular injections of 1 ml each, containing equal quantity of Difco incomplete Freund's adjuvant were given 2 days apart; followed by a sub-cutaneous injection of 1 ml virus after 8 weeks. The rabbits were trial bled during this time to check the titer. After 2 weeks this was followed by 2 intravenous injections of 2 ml virus each in the ear veins at an interval of 3 days. The rabbits were finally bled after 2 weeks and the antisera tested and stored in deep freeze. Part of the antisera were freeze-dried in small ampoules containing 1 or 2 ml quantities each. Antisera prepared against Vu, Vs, and Sb isolates were found to have a titer of 1024 and they did not give any reaction with the normal plant constituents present in sap of healthy plants.

8.2. SEROLOGICAL TECHNIQUE

OUCHTERLONY agar double-diffusion test (1948, 1958, and 1962) as also outlined by VAN SLOGTEREN (1955) and by BALL (1961) was used for all the antigen-antibody reactions. Merthiolate (a concentration of 5 mg per 100 ml agar) was added to the agar as a preservative. Tests made with and without merthiolate did not show any difference in the reaction and no indication of any non-specific precipitation due to merthiolate was found. Crude or purified preparations were tested and the petri dishes were incubated at room temperature for several days after which the final results were recorded. Proper controls in each test were invariably included, to assure that the positive reactions were between virus and its antibody and did not involve any other constituents. Tests were repeated several times and different dilutions were used to ascertain the negative results. All dilutions were made in physiological saline, 0.85 % sodium chloride in distilled water.

8.3. SEROLOGICAL RELATIONSHIPS

All the 5 cowpea mosaic virus isolates were tested against the Vu, Vs, Sb, and Nigerian antisera. The antiserum against the Nigerian isolate was supplied by Dr. A. J. GIBBS. In addition to these antisera, the 5 virus isolates were also tested with antisera obtained against wild cucumber mosaic virus (supplied by Dr. D. J. HAGEDORN, Wisconsin), tobacco ringspot virus (supplied by Dr. H. A. SCOTT, Beltsville), tobacco necrosis virus (supplied by Mr. D. Z. MAAT, Wageningen), southern bean mosaic virus (supplied by Dr. SCOTT, Beltsville), bean pod mottle virus (supplied by Dr. J. B. BANCROFT, Purdue; and Dr. SCOTT, Beltsville), alfalfa mosaic virus (supplied by Mr. MAAT, Wageningen), cowpea strain of southern bean mosaic virus (supplied by Dr. R. J. SHEPHERD, California), and red clover mottle virus Dutch isolate (supplied by Mr. MAAT, Wageningen). Some of the results are represented in Table 6 and Photos 18 and 19.

TABLE 6. Serological relationships among the five cowpea mosaic virus isolates and bean pod mottle and

antigen	antiserum ¹		Vu isolate			Vs isolate			Sb isolate			Nigerian isolate				
	dilutions	0	$\frac{1}{4}$	$\frac{1}{16}$	$\frac{1}{64}$	0	$\frac{1}{4}$	$\frac{1}{16}$	$\frac{1}{64}$	0	$\frac{1}{4}$	$\frac{1}{16}$	$\frac{1}{64}$	0	$\frac{1}{4}$	$\frac{1}{16}$
Vu, crude sap		++	++	++	++	++	++	++	++	++	+	-	++	++	+	-
Vu, purified		++	++	++	++	++	++	++	++	++	+	-	++	++	+	-
Vs, crude sap		++	++	++	++	++	++	++	++	++	+	-	++	++	+	-
Trinidad, crude sap		++	++	++	++	++	++	++	++	++	+	-	++	++	+	-
Trinidad, purified		++	++	++	++	++	++	++	++	++	+	-	++	++	+	-
Sb, crude sap		+	±	-	-	+	±	-	-	++	++	++	++	++	++	++
Nigerian, crude sap		+	±	-	-	+	±	-	-	++	++	++	++	++	++	++
RCMV, Dutch isolate, purified		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
sap from healthy plant		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

++ = strong precipitin line; + = faint precipitin line; ± = very faint precipitin line; - = no reaction

¹ Dilutions 1:256 and 1:1024 were also used in the tests. These data are omitted from the present table among the different isolates.

The Vu, Vs, and Trinidad viruses gave strong reactions with Vu and Vs antisera while very faint reactions with Sb and Nigerian antisera (Table 6) indicating that the 3 viruses are either serologically identical or very closely related. Similarly the Nigerian and Sb viruses gave a strong reaction with their homologous and heterologous antisera, but a faint reaction with Vu and Vs antisera, also indicating that these 2 viruses are either serologically identical or very closely related.

The cowpea mosaic virus (CPMV) isolates were found to be distantly related to bean pod mottle virus and a distant relationship was also found between these isolates and red clover mottle virus (RCMV; SINHA, 1960) Dutch isolate (Photo 19). Positive reaction between CPMV strains and RCMV (Dutch isolate) antiserum was obtained, but not the reverse. This presumably is due to the low titer of the antisera. The Vu, Vs, and Trinidad isolates gave positive reactions with Scott's bean pod mottle virus (BPMV) antiserum but not with BPMV (BANCROFT). No reactions were obtained with any of the 5 cowpea mosaic virus isolates and the other antisera in the present tests. It may, however, be possible to find serological relationships among some of these and other viruses when sera of high titer are available (AGRAWAL and MAAT, 1964).

red clover mottle viruses as determined by agar-gel diffusion tests.

RCMV, Dutch isolate				BPMV, from Scott				BPMV, from Bancroft				Normal serum				antiserum ¹	
0	$\frac{1}{4}$	$\frac{1}{16}$	$\frac{1}{64}$	0	$\frac{1}{4}$	$\frac{1}{16}$	$\frac{1}{64}$	0	$\frac{1}{4}$	$\frac{1}{16}$	$\frac{1}{64}$	0	$\frac{1}{4}$	$\frac{1}{16}$	$\frac{1}{64}$	dilutions	antigen
++	+	-	-	+	±	-	-	-	-	-	-	-	-	-	-	-	
++	+	-	-	+	±	-	-	-	-	-	-	-	-	-	-	-	Vu, purified
+	±	-	-	+	±	-	-	-	-	-	-	-	-	-	-	-	Vs, crude sap
+	±	-	-	+	±	-	-	-	-	-	-	-	-	-	-	-	Trinidad, crude sap
++	+	-	-	+	±	-	-	-	-	-	-	-	-	-	-	-	Trinidad, purified
+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Sb, crude sap
+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Nigerian, crude sap
++	++	++	++	-	-	-	-	-	-	-	-	-	-	-	-	-	RCMV, Dutch isolate, purified
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	sap from healthy plant

RCMV = red clover mottle virus; BPMV = bean pod mottle virus

since they did not provide any additional information for a comparison of the serological relationships

Serological tests made with the asparagus-bean mosaic virus described by HINO (1960) and the Vu and Vs antisera made by the present author gave negative results (HINO, personal communication 1963). This indicates that the virus described by HINO from Japan is different from the cowpea mosaic virus.

SHEPHERD (1963) isolated a mosaic virus from naturally infected cowpea from Arkansas. The Arkansas virus has been reported by him to be closely related to the Trinidad virus and both these viruses reacted with antiserum to pod mottle virus and Nigerian virus. The Vu antiserum reacted with all these viruses in tests kindly performed by Dr. SHEPHERD (personal communication). It is probable, as also suggested by Dr. SHEPHERD, that the Arkansas virus is the same or closely related to the one with which SMITH worked in 1924, and which was not reisolated since then (see review of literature).

8.4. IMMUNOELECTROPHORESIS

In all the OUCHTERLONY agar-gel diffusion tests the antisera obtained gave only one precipitation line with their homologous and heterologous (closely related antigen. Since the purified virus preparations invariably showed electrophoretic and ultracentrifugal heterogeneity, presumably due to particles differ-

ing in their nucleic acid content; an immunoelectrophoretic study of the purified virus preparations was made to check it further.

The micro-method of SCHEIDEGGER (1955) as modified by PEETOOM (1961) was used. Standard 76 × 26 mm glass microscope slides or larger glass plates were coated with 2–3 drops of warm 3% Bacto agar in distilled water. The slides were dried at 100°C for 5 minutes after which 2–3 ml warm 1.3% agar in either 0.05 M veronal buffer pH 8.6 or in 0.1 M phosphate buffer pH 5.2 was pipetted to make an agar bed about 2 mm deep. Circular wells, 2 or 3 mm in diameter were cut to contain the antigen (Vu isolate) solution. It was not necessary to seal the bottoms of the wells with agar in this case since the diameter was so small and no diffusion of the antigen under the agar was found. Electrophoresis was carried out at room temperature. The buffers used were 0.05 M veronal pH 8.6 and 0.1 M phosphate pH 5.2. The buffer used as solvent for the agar was the same as that used for electrophoresis except that the agar solvent contained traces of sodium merthiolate. The duration of electrophoresis was 1 hour with a current of about 10 ma and 60 volts per slide (6 volt/cm). After electrophoresis had been concluded, a trench (2 mm wide) was cut in the agar bed parallel to the direction of current flow and filled with undiluted antiserum. The plates were incubated at 16°C overnight in a humidity chamber, repeatedly washed in physiological saline for 3 days and finally stained with amido black.

In tests made with the Vu isolate, one sharp precipitation line appeared after immunoelectrophoresis at pH 8.6 and the pattern of the lines suggested electrophoresis towards the positive pole (Photo 20). The antigen well on top of the picture in Photo 20 was especially made larger (7 mm diameter) than the other ones of the normal size (2–3 mm diameter) to check if the quantity of the antigen affected the pattern of the lines. This, however, did not create any significant difference in the pattern of the lines. A diffuse extension of the line towards the positive pole and appearance of another fine line coinciding for most part with the sharp line indicated electrophoretic heterogeneity of the preparations. The pattern was clearer at pH 5.2 where the lines were sharper and indicated at least 2 and possibly 3 immunologically similar or closely related components differing in their electrophoretic mobilities (Photo 21). The exact nature of these lines is not clear at the moment.

These results of immunoelectrophoresis are in agreement with those of Tiselius electrophoresis. A comparison between Photos 20, 21 and Figs. 7A and 11 shows a great effect of the pH on the pattern. The movement is less apparent at pH 5.2 (Photo 21) than at pH 8.6 (Photo 20). This can be easily explained since the isoelectric point of the Vu isolate was found to be 3.4 (Fig. 12). The mobility decreases as the pH approaches the isoelectric point and hence a slower movement in immunoelectrophoresis can also be expected at pH 5.2 than at 8.6. The Sb isolate also showed a similar heterogeneity as the Vu isolate, at these two pH.

SEPARATION OF THE DIFFERENT COMPONENTS

A consistent heterogeneity of the purified virus preparations in the analytical ultracentrifuge and electrophoresis indicated the presence of more than one component. Electron micrographs from such preparations shadow-casted with palladium did not show any apparent heterogeneity. However, marked differences were found when preparations were negatively stained with phosphotungstic acid, indicating possibly the presence of particles varying in density as shown in the electron micrographs earlier. An effort was made to separate these fractions by employing centrifugation in a dense caesium chloride solution. Density gradient centrifugation in strong CsCl solutions for the fractionation of macromolecules was first used by MESELSON et al. (1957). By employing this procedure, particles with different densities can be made to sediment as separate zones in the CsCl gradient of appropriate density. The density of the CsCl solution employed would naturally vary depending on the densities of the particles. Centrifugation of a dense CsCl solution for extended periods at suitable centrifugal force establishes a gradient through the tube and the layered sample sediments at corresponding density in this gradient. Such CsCl gradients have been used by different workers (MATTHEWS, 1959a, 1959b, and 1960; LEVINTOW and DARNELL, 1960; AGRAWAL et al., 1962; BREEDIS et al., 1962) in the purification and study of several viruses.

Different densities of CsCl in solution were tried; a solution containing 7.13 g CsCl per 10 ml water and having a density of about 1.45 was found to give a very good separation of the zones with this virus and was used for all further experiments. About 10–15 mg of virus preparation in a volume of 1 ml water was layered over 3 ml of CsCl solution of density 1.45, and overlaid with 1 ml of mineral oil in a 5-ml lusteroid tube and centrifuged for 24 hrs at 35,000 rev/min in the SW39 rotor of the Spinco model L ultracentrifuge. The rotor, tubes, and all materials were pre-cooled to a temperature of 0–4°C and all manipulations were carried out in the cold room at this temperature to avoid any anomalous effects due to the strong salt solutions. A photograph of a tube after such a run with the Sb isolate is shown in Photo 22A. Several opalescent bands may be seen in this photograph. All further studies on the separation of the components were done employing the Sb isolate since the relative proportion of the top component was much higher (30–40%) with this isolate when compared to the Vu isolate. The infectivity tests were made on *Chenopodium amaranticolor* due to reasons mentioned earlier (Page 24).

The components were withdrawn by means of a needle bent twice at right angles and attached to a hypodermic syringe. This enabled to take the zones out while being able to see their movement precisely and allowed only a minimum of intermixing. The components were centrifuged separately in the Spinco model L ultracentrifuge at high speed (105,000 g) to sediment the particles, resuspended in distilled water and layered on CsCl gradients as before. By repeating this

procedure three times the components could be obtained in pure form. The methods for testing the purity of the fractions included visual observation by scattered light of a single band and checking in the analytical ultracentrifuge for a single peak. The components as they appeared during this procedure are shown in Photo 22B, C, and D. The three components were now tested for their UV absorption, infectivity, serological behaviour, and by means of negative staining under the electron microscope. The UV spectra are shown in Fig. 14. The optical density of the middle and bottom components at 260 m μ was adjusted to the same value for a valid comparison of the two spectra. The middle component gave a minimum at 242 m μ and maximum at 259 m μ with $E_{\max}/E_{\min} = 1.41$ and $E_{260}/E_{280} = 1.62$. The bottom component gave a minimum at 240 m μ and maximum at 260 m μ with $E_{\max}/E_{\min} = 1.44$ and $E_{260}/E_{280} = 1.71$. The top component absorbed maximally at 280 m μ and gave a minimum at 250 m μ . These spectra indicate that both middle and bottom components are nucleoprotein while the top component was apparently a protein with very little or no nucleic acid as found for the top component of turnip yellow mosaic virus (MARKHAM and SMITH, 1949). However, it is not possible on the basis of these spectra to find real quantitative differences in the nucleic acid content of the middle and bottom components. Since the two components exhibited definite differences in their density and since no apparent difference was found in the protein coat as revealed by serological tests (Table 7), it is presumed that the

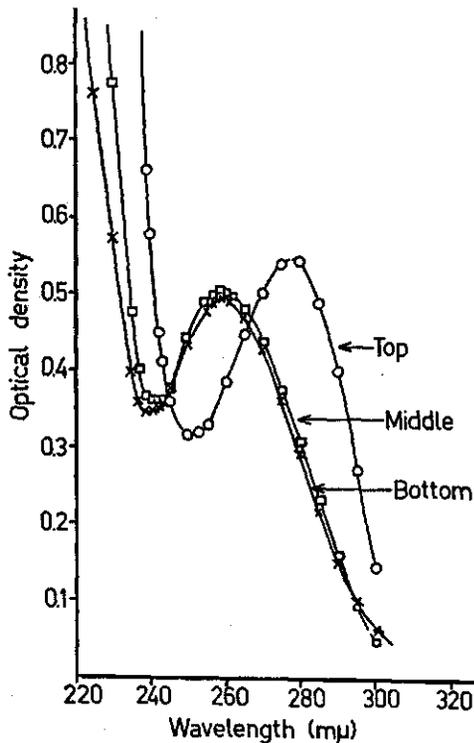


FIG. 14.
Ultraviolet absorption spectra of the three separated components of cowpea mosaic virus (Sb isolate).

differences are due to varying amounts of nucleic acid. The small differences between the two spectra of the middle and bottom component, and the bottom component having a minimum at lower wavelength and slightly higher E_{\max}/E_{\min} and E_{260}/E_{280} , may point in the same direction.

TABLE 7. The serological relationships among the three centrifugal components of cowpea mosaic virus (Sb isolate) as determined by agar-gel diffusion tests.

antiserum ¹ antigen	dilutions											
	0	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{8}$	$\frac{1}{16}$	$\frac{1}{32}$	$\frac{1}{64}$	$\frac{1}{128}$	$\frac{1}{256}$	$\frac{1}{512}$	$\frac{1}{1024}$	$\frac{1}{2048}$
Sb isolate, unfractionated virus	++	++	++	++	++	++	++	++	++	++	++	--
Top component	++ + ²	++ + ²	++	++	++	++	++	++	++	++	+	--
Middle ³ component	++	++	++	++	++	++	++	++	++	++	++	--
Bottom ³ component	++	++	++	++	++	++	++	++	++	++	++	--

¹ = the antigen used to induce antibody production contained all three centrifugal components of the Sb isolate. The antiserum titer was 1024.

² = indication of a second line due to a probable disintegration of particles

³ = unfractionated virus, as well as the middle and bottom components were used at 0.09 mg/ml. The top component was used at about that concentration.

The plus signs indicate the intensity of reaction.

Runs made in the analytical ultracentrifuge with the separated components gave sedimentation coefficients ($S_{20,w}$) of 58, 100, and 119 *S* extrapolated to infinite dilution, which were higher than the values obtained for the different peaks in the unfractionated preparations (Page 17).

Negative staining and electron microscopy of the top component predominantly showed what are called 'empty particles' or the 'core' in the micrographs earlier, while similar preparations with the middle and bottom components showed predominantly particles with nucleic acid. No apparent distinction between the middle and bottom components could be made after negative staining under the electron microscope.

Serological tests made employing agar-gel diffusion method and antiserum against unfractionated virus revealed no difference in the titer with the different components (Table 7). This suggests that possibly there are no differences in the different components as far their antigenic properties are concerned, meaning in turn that they have a similar surface structure and protein coat. This further suggests that the differences might lie in the nucleic acid content or composition or both.

Infectivity tests revealed that the top component did not possess any infectivity, while most of the infectivity was associated with the middle component. The number of lesions per leaf, produced by the middle and bottom components adjusted to the same concentration was 354 and 19 respectively, while the top component did not give any lesions. The infectivity comparisons of the middle and

bottom components were made after adjusting their optical density at 260 m μ to the same value. So the bottom component gave only about 5% infectivity when compared to the main and the middle component. It may be pointed out here that this low infectivity of the most dense component (bottom) is unique; since in all cases known so far with different components, it is the bottom component which is the only or the most infectious component. The reason for this difference in the infectivity of the middle and bottom components and their relationship is not understood at the moment. The possibility of contaminating infectivity in the bottom component is not completely excluded at this stage. There was, however, no evidence for dimerization of the particles. The separated zones on rerunning in CsCl solution sedimented reproducibly on exactly the same place in the gradient. Moreover, the sedimentation coefficient of the bottom component was less than what would be expected for such a dimer. According to MARKHAM (1962), the observed values for the ratios of the sedimentation coefficients of monomers and dimers of spherical viruses are 1:1.4 approximately. The sedimentation coefficient values of the separated middle and bottom components for the cowpea mosaic virus isolates being 100 and 119 *S* gave a ratio of approximately 1:1.2.

In another run in CsCl, made by using a partially purified virus preparation obtained by the initial low speed centrifugation (without any chloroform and butanol treatment of the crude sap followed by a cycle of low and high speed centrifugation, 3 zones were obtained. The zones were taken out as described earlier and rerun in CsCl. They corresponded to the zones obtained in the chloroform-butanol treated material. The ribosomes present in such preparations mostly disintegrated in the CsCl, formed a separate layer on the top and could be separated easily. The result of this experiment indicates that the zones were not produced as an artifact of the chloroform-butanol treatment.

Purified virus preparations of the other isolates (Vu, Vs, Trinidad, and Nigerian) were also given similar centrifugations in CsCl and several bands were observed in all cases. The relative proportions of the different components varied depending on the virus isolate. The quantity of the top component with the Vu, Vs, and Trinidad isolates was invariably very small when compared to the Sb and Nigerian isolates.

Evidence presented in the foregoing pages on a consistent heterogeneity of the purified virus preparations, leads to the idea that particles with different nucleic acid content are present in the preparations. It seems unlikely that these particles are artifacts of purification procedures since evidence has been found for their presence in preparations obtained by different methods. This is further supported by the fact that it is possible to isolate the different components and since each one of them after final purification, on rerunning in CsCl did not produce more than one zone, indicating that the zones are not produced during the run.

DISCUSSION AND CONCLUSIONS

The identity of the several different cowpea mosaic virus diseases and their causal viruses is not well understood, since the descriptions in most cases are not adequate and are mainly based on host range, thermal inactivation point, dilution end point, longevity in vitro, and transmission studies. With increasing knowledge of the morphological, biophysical, biochemical, and serological characteristics of the viruses, the earlier criteria are of limited importance now. The problem of virus identification and characterization is further complicated due to host-variety and strain differences and their geographical distribution which can never be predicted. This at times leads in designating the different strains of the same virus showing some variations as separate viruses. Hence, a full and complete description of any particular virus becomes all the more important. No one single criterion can be underestimated, how restricted its application in an individual case might be. Such studies are not only a pre-requisite for a classification of viruses but also a necessity in any breeding program for resistance against a particular virus and in understanding the very nature of viruses themselves. The present investigation should furnish an example in this regard.

The knowledge concerning 'cowpea mosaic virus' has been very arbitrary and incomplete. Symptoms produced on cowpea by tobacco mosaic virus, cucumber mosaic virus, southern bean mosaic virus, bean yellow mosaic virus and even by several other unclassified viruses have been supposed to be due to cowpea mosaic virus. Preliminary studies indicated that the group of beetle-transmitted cowpea mosaic virus isolates and related strains is different from hitherto described viruses causing mosaic disease in cowpea. Hence the name cowpea mosaic virus and the abbreviation CPMV is restricted only to the beetle-transmitted cowpea mosaic virus and its strains.

Vu, Vs, and Sb isolates from Surinam (Table 1) and the Trinidad and Nigerian isolates (DALE, 1949; CHANT, 1959, 1961, and 1962) were used in this investigation. Since single lesion cultures were used for all the present experiments and reactions of different hosts were periodically checked during the course of work it can be safely presumed that the different isolates represented pure cultures where no signs of any mutation occurred. A study of the host range of these isolates shows many similarities. The isolates Vu, Vs and Trinidad produce a severe mosaic with light and dark green patches and prominent blister-like areas on the trifoliate leaf in *Vigna unguiculata* while the Sb and Nigerian isolates produce mainly a bright yellow mosaic with distinct vein-clearing on all the varieties tested. The differences amongst the isolates become well marked on *Chenopodium amaranticolor* (Photo 9). The Vu, Vs, and Trinidad isolates produce only local lesions while the Sb and Nigerian isolates also produce severe systemic symptoms in addition to local lesions. The symptoms produced by Trinidad and Nigerian viruses on this host are in agreement with those reported by CHANT (1961, 1962). The reaction on 'Beka' bean also shows a clear difference since the

plants on inoculation with Vu, Vs, and Trinidad isolates completely collapse, and in some cases those surviving show systemic necrosis and necrotic spots in addition to severe mosaic; on the other hand, plants inoculated with Sb and Nigerian isolates rarely exhibit any necrosis. The diagnostic value of these hosts in distinguishing the two groups is apparent (Photo 9). It is interesting to note that only the Vu and Vs isolates produced local symptoms on *Nicotiana tabacum* and *Nicotiana glutinosa*. The symptoms produced by the Trinidad and Nigerian isolates on the hosts tested in the present studies are in conformity with those reported by DALE (1949) and CHANT (1959, and 1962) earlier.

The Vu isolate produces quite different symptoms on different bean (*Phaseolus vulgaris*) varieties and the Vs isolate gives almost a similar reaction as Vu (Table 2). The other isolates were tested only on varieties 'Beka' and 'Pinto' which were subsequently used for further experiments. CHANT (1959) reported that the Nigerian virus produced only diffuse chlorotic local lesions on bean varieties 'Prince', 'Canadian Wonder', and 'Long Tom'. In the present investigation with this virus the variety 'Pinto' gave diffuse necrotic local lesions and no systemic reaction while the variety 'Beka' showed a prominent systemic mosaic in addition to local symptoms. DALE (1949) reported that the Trinidad virus did not produce any infection on *P. vulgaris*, while CHANT (1962) on further studies with the same virus obtained local lesions on variety 'Comtesse de Chambord' but no systemic reaction. In the present experiments the Trinidad virus produced not only local lesions on varieties 'Pinto' and 'Beka' but also systemic symptoms on the latter. This points to the fact that any interpretations and comparisons on the susceptibility of a species to different viruses or related strains can only be made after testing several different standard varieties. A similar conclusion can be drawn on the basis of Table 3, on the susceptibility of cowpea (*V. unguiculata*) varieties. The varieties 'Arlington', 'Jackson Alabama', 'Victor K 798', 'Groit', 'Brahman Victor', and 'Brahman K 892' were reported to be resistant to the Nigerian cowpea yellow mosaic virus (WELLS and DEBA, 1961) after testing 116 cowpea varieties introduced from America. This was found in an attempt to breed resistant varieties against this virus in Nigeria. In the present investigation all these varieties were found to be highly susceptible to the Vu and Vs isolates, while they were apparently resistant to the Sb isolate (a reaction similar to the Nigerian virus). This again pointed out a difference between the Vu, Vs, and Sb isolates, and the similarity of the Nigerian virus to the Sb isolate; and in turn towards the possibility of these being strains of the same virus.

Examinations of epidermal strips from leaves and petioles of inoculated plants showed the presence of amorphous and mostly vacuolar (Photo 10) inclusion bodies. They were variable in shape but generally ovoid, and were larger than the nuclei. Granular inclusions were also seen in some of the cells, and are regarded a possible stage in the formation of the vacuolar type.

The thermal inactivation and the dilution end points of the virus in plant sap for the Vu isolate, in general, agreed to those reported by DALE (1949) and CHANT (1962) for the Trinidad virus. The longevity in vitro, however, was different for the Vu isolate than that reported by these authors for the Trinidad virus. Such

differences may possibly be due to the assay plant, variations in storage temperatures, the buffer used for dilution, and whether the crude sap is diluted before storage, since the stability of viruses in various buffers at different pH and temperatures may vary. No exact comparisons are possible unless these conditions are precisely reported and are identical. This suggests that much emphasis cannot be laid on such criteria especially for the identification of the viruses.

Virus isolates purified by a slightly modified chloroform-butanol procedure (STEERE, 1956) showed a consistent heterogeneity in analytical ultracentrifuge and electrophoresis. The fact that this heterogeneity was not very clear with some isolates at pH 7.0 in electrophoresis while very distinct at lower pH, points out the importance of checking the purified preparations at different pH before concluding on their purity especially when such precise information is necessary for biophysical and biochemical studies. Such preparations after negative staining with phosphotungstic acid showed under the electron microscope virus particles presumably with varying amounts of nucleic acid, although they cannot be distinguished easily, in a mixed preparation. More or less similar observations on the presence of different components (non-infective and infective) have been recorded for several spherical (polyhedral) viruses including turnip yellow mosaic virus (MARKHAM and SMITH, 1949; MARKHAM, 1951; MATTHEWS, 1958, 1959a, b, 1960), squash mosaic virus (RICE et al., 1955), wild cucumber mosaic virus (SINCLAIR et al., 1957; YAMAZAKI and KAESBERG, 1961), and bean pod mottle virus (BANCROFT, 1962). In these earlier instances the preparations were heterogeneous only in the ultracentrifuge except in case of bean pod mottle virus where they also showed an electrophoretic heterogeneity. Evidence has been presented in several of these cases that the particles were different in their nucleic acid content. On the basis of the data presented on cowpea mosaic virus isolates in the present work, a similar situation seems to exist. ARONSON and BANCROFT (1962) reported density heterogeneity in purified preparations of broad bean mottle virus. HAMILTON (1961) presented some evidence that the non-infectious particles associated with extracts from plants infected with brome mosaic virus were primarily derived from dissociation of the virus. The presence of empty and full particles has also been recorded for several animal viruses including SHOPE papilloma virus (BREEDIS et al., 1962), and polyoma virus (WINCOUR, 1963). Incomplete forms of influenza virus have been reported by VON MAGNUS (1954).

The sedimentation coefficients of the components from the different isolates did not show any marked differences although there were slight differences when the components were in a mixture. This is also an indication that all the isolates belong to the same virus. Slight differences obtained in sedimentation values in mixtures may possibly be due to variations in the relative quantities and their constituting more than one strain.

The electrophoretic mobilities of the components from the virus isolates in 0.1 M phosphate buffer pH 7.0 indicate some difference between isolates. The Sb and Nigerian isolates have slightly higher values for the faster components than the other three isolates. The two groups constituting the two strains may

also probably be distinguished on the basis of the heterogeneity and the proportion of the different components. An absolute comparison of these values, however, seems difficult due to the heterogeneity of the preparations since it is not known if the mobility of one component has any effect on the other.

Measurements of the particle dimensions of the negatively stained preparations of all the isolates show that invariably the empty particles are slightly smaller than the full particles. It is possible that the effect of drying is more pronounced on the empty particles and/or these differences in size are due to differences in hydration of the different type of particles (LAUFFER and BENDET, 1954). Distinct differences can also be seen in the side to side measurements and measurements between the extreme points, since the particles are polyhedral, appearing mostly hexagonal, and not exactly spherical. All the isolates have approximately similar dimensions except the Vu isolate which is somewhat larger in size. CHANT (1962) reported that the Trinidad virus consisted of polyhedral particles with a mean diameter of 25 $m\mu$ which is in agreement with the present measurements for this virus.

Observations on the structure of the virus particle indicate that it probably has an icosahedral (5:3:2) symmetry and possesses 60 subunits. Further investigations supported by X-ray diffraction data might give more information.

Serological tests with the different isolates and their antisera confirmed the earlier indications that all the isolates are related, belong to one virus, and fall into 2 groups. Hence they are designated as constituting 2 strains. The Vu, Vs, and Trinidad viruses comprise the 'Severe Strain' and the Sb and Nigerian viruses constitute the 'Yellow Mosaic Strain'. No serological relationship was found with any of the present isolates and other virus antisera tested, except with bean pod mottle virus and red clover mottle virus (Dutch isolate). It is interesting to point here that none of the present cowpea mosaic virus isolates infected red clover, the natural host of the red clover mottle virus. MACLEOD and MARKHAM (1963) reported serological relationship between turnip yellow mosaic and wild cucumber mosaic viruses, which are not known to have any common host plant. Since they did not include proper controls in their tests, this relationship was only probably due to plant antigens (VAN REGENMORTEL, 1963). However, in the presence of these instances it seems probable that serologically related groups among spherical viruses similar in physico-chemical and other properties can also be found as reported for elongated viruses (BRANDES and WETTER, 1959; and BRANDES, personal communication, 1962) on the basis of their morphology, serology and other characteristics.

It seems advantageous to employ immunoelectrophoresis in case of virus antigens with components differing in electrophoretic mobilities, since these components might not show up in regular serological tests. The technique should be of great help in identification of components in an electrophoretic heterogeneous preparation.

All the isolates on gradient centrifugation in dense CsCl solution showed several bands corresponding to the different components. Some of these components (from Vu and Sb isolates) could be separated and further purified. The

quantities obtained were not sufficient to permit detailed tests. Infectivity studies, serology, electron microscopy, and spectrophotometric analysis, were attempted. The top component consisting of the empty particles did not give any infectivity, while the middle component showed most of the infectivity. The bottom component was slightly infective. The empty particles constituting the top component were apparently devoid of nucleic acid or contained very little while the middle and bottom components possessed varying amounts of nucleic acid. The three components were indistinguishable serologically. The top component under the electron microscope showed what are believed to be the protein shells of the virus particles, while the middle and bottom components showed denser particles containing nucleic acid. No significant difference could be seen between the middle and bottom components under the electron microscope after negative staining. This agrees with the idea that they were possibly different only in their nucleic acid content. However, only a chemical analysis can decide these differences.

The various bands due to the different components were not produced by any action of isolation procedure or CsCl since they could be isolated by different purification procedures and when once isolated, sedimented reproducibly in dense CsCl solution. Preparations obtained by only differential centrifugation, without any chloroform-butanol treatment, layered on CsCl gradients gave several bands as in the usual preparations. There is no indication to favor the idea that any of them could represent contamination due to host material, since they all reacted with antiserum made against a mixture of all the components and this antiserum did not give any reaction with proteins from the healthy plants. Their invariable presence in all the isolates suggests that they are probably present in the plant as such. Some additional evidence was obtained by using untreated crude sap from infected plants after different periods of inoculation and running in the analytical ultracentrifuge. Several peaks due to the various components were observed. Healthy material similarly treated served as control.

There can be several explanations to account for the presence of these different components viz. empty particles, and particles with varying amounts of nucleic acid, apparently produced in the plant. The empty protein shell may be a precursor or a degradation product of the complete virus or a mixture of both; or it may be produced simultaneously and independently of the virus. Although some evidence for the validity of the precursor hypothesis was obtained for turnip yellow mosaic virus (JEENER, 1954; MATTHEWS, 1960), in the light of recent work this seems to be ruled out (MATTHEWS et al., 1963). Further work employing P³²-labelling of the nucleoprotein fractions and C¹⁴-labelled valine for the protein components should give more conclusive evidence on the origin of the different components, and this information might help in understanding the mechanism of biosynthesis in several of the polyhedral plant viruses, and in some animal and human viruses.

These results with the different components of cowpea mosaic virus seem quite unique when compared to other small viruses namely turnip yellow mosaic

virus, wild cucumber mosaic virus, and bean pod mottle virus, where the purified preparations also contained several components identical in protein content and composition but different in nucleic acid content. In the earlier instances it is only the bottom component which was infective while in case of cowpea mosaic virus most of the infectivity is associated with the middle component. No evidence was found for the possibility that the bottom component could represent a dimer of the middle component. The sedimentation values and the stability of the purified middle and bottom components for repeated sedimentation in CsCl as distinct bands, supported the idea that they were distinct and represented more than one infectious component. The absence of any serological difference among the components seem to rule out the possibility of any great differences in the protein coat of the virus particles. The question arises whether there is any difference in the RNA of the fractions and whether this difference is in size or composition or both. Studies on this line would indicate if there are any differences in molecular weight or chain length of infectious RNA. This together with a study of the interrelationship between the RNA of the different components might give an insight in the process of the synthesis of the virus.

SUMMARY

1. The beetle-transmitted cowpea mosaic is a severe disease in several tropical countries. A study comprising biological, biophysical, biochemical, and immunological characteristics of this virus was made for a definite identification and characterization.

2. Five isolates of cowpea mosaic virus were used for a comparative study: Vu, Vs, and Sb isolates from Surinam (South America), Nigerian cowpea yellow mosaic, and Trinidad cowpea mosaic. Single lesion isolations from *Phaseolus vulgaris* were used in all cases. The viruses were maintained on cowpea plants by mechanical inoculation.

3. The virus isolates infected *Arachis hypogaea*, *Cassia tora*, *Chenopodium amaranticolor*, *Chenopodium quinoa*, *Crotalaria striata*, *Crotalaria usaramoensis*, *Dolichos lablab*, *Glycine max*, *Gomphrena globosa*, *Phaseolus aureus*, *Phaseolus lunatus*, *Phaseolus mungo*, *Phaseolus vulgaris*, *Pisum sativum*, *Vigna unguiculata*, and *Zinnia elegans*. The symptoms produced by the isolates on these hosts are described. The Vu and Vs isolates also produced local symptoms on *Nicotiana glutinosa*, and *Nicotiana tabacum*. No symptoms were incited on *Amaranthus caudatus*, *Capsicum annuum*, *Cucumis sativus*, *Datura stramonium*, *Lycopersicon esculentum*, *Medicago sativa*, and *Vicia faba*, and no virus could be recovered from these plants.

4. Varieties of *Phaseolus vulgaris* reacted differently to the same virus isolate and also to the different isolates, pointing to the fact that any conclusions on the susceptibility of the species to this virus or to any other virus should be based only after testing several varieties. The variety 'Pinto' was found suitable for bioassay of the Vu, Vs, and Trinidad isolates, while it did not react so well with the Sb and Nigerian viruses.

5. All the cowpea varieties tested were found to be susceptible to Vu and Vs isolates, with differences in severity of symptoms. The varieties resistant to the Nigerian isolate also proved resistant to the Sb isolate, while they were found to be highly susceptible to the Vu and Vs isolates. This pointed to the possibility that strains of the virus exist, which will have to be taken into account in any breeding program for resistance against this virus.

6. The occurrence of amorphous and mostly vacuolar, sometimes also granular, inclusion bodies in the epidermal cells of cowpea and pea infected with cowpea mosaic virus is reported.

7. The Vu isolate was found to have a thermal inactivation point between 65–70°C, dilution end point between 10,000–100,000 and longevity in vitro of 3–5 days.

8. A purification procedure consisting essentially of chloroform-butanol treatment and differential centrifugation has been developed for cowpea mosaic virus. Purified preparations were highly infectious, gave a typical nucleoprotein spectrum in the UV spectrophotometer, and always showed ultracentrifugal and electrophoretic heterogeneity. No evidence was found that this heterogeneity was due to host material. Three peaks, sometimes four, were invariably found to

be associated with the purified virus preparations in the analytical ultracentrifuge examinations. The sedimentation coefficients (in a mixture of the components) extrapolated to infinite dilution in water at 20°C for the three peaks of the Vu isolate were found to be 54, 84, and 104 *S*; for the Vs isolate were 52, 83, and 101 *S*; and for the Sb isolate were 55, 86 and 103 *S*. Similar measurements for four peaks of the Nigerian and Trinidad viruses gave values of 56, 77, 83, 106 *S* and 53, 67, 84, 103 *S* respectively. The values for three peaks for all the isolates after separation, were found to be 58, 100, and 119 *S*.

9. The electrophoretic mobilities of the main peak for Vu and Vs isolates at pH 7.0 in 0.1 M phosphate buffer were found to be 4.0×10^{-5} cm/sec/volt/cm and 3.8×10^{-5} cm/sec/volt/cm respectively. The value for the Sb isolate under similar conditions was found to be 4.25×10^{-5} (54.6% component) and 2.80×10^{-5} (44.8% component) cm/sec/volt/cm. Measurements for the Trinidad and Nigerian viruses gave values of 3.55×10^{-5} (6.4% component), and 2.90×10^{-5} (83.9% component); and 4.05×10^{-5} (44.5% component) and 2.66×10^{-5} (55.5% component) cm/sec/volt/cm respectively. All preparations showed definite heterogeneity at pH below 7.0 in the same buffer.

10. The isoelectric point for the Vu isolate was calculated from the mobility measurements of the rapidly migrating component at different pH, and was found to be 3.4 at 0.1 ionic strength. The virus isolates could be precipitated from the solution at this pH.

11. Nucleic acid isolated from Vu and Sb isolates was found to be infective. The relative infectivities of the nucleic acid preparations for the Vu and Sb isolates were 0.4% and 0.1% respectively of that of intact virus containing an equivalent amount of nucleic acid.

12. Purified preparations of the viruses in all cases showed, by electron microscopy, more or less spherical or polyhedral particles. Negative staining of the preparations with phosphotungstate revealed the presence of empty particles, and particles apparently containing nucleic acid. The measurements for the Vu isolate gave a size of 270 Å (side to side) – 300 Å (between extreme points) for particles containing nucleic acid and 250 Å (side to side) – 270 Å (between extreme points) for empty particles. All the other isolates gave measurements of 230–250 Å (for empty particles) and 240–270 Å (for particles containing nucleic acid).

13. Observations on the structure of the virus particle as revealed by negative staining and electron microscopy are recorded. Different models to account for these observations were examined. An icosahedral model having 60 subunits and 5:3:2 axial symmetry seems to be most compatible with the virus structure.

14. Antisera obtained for the 5 isolates reacted with their own virus and with all the others showing that they were all serologically related. The Vu, Vs, and Trinidad viruses gave strong reactions with their homologous and heterologous antisera while a faint reaction with Sb and Nigerian antisera, indicating that the 3 viruses are either identical or very closely related. Similarly the Nigerian and Sb viruses gave a strong reaction with their homologous and heterologous antisera, but a faint reaction with Vu, Vs, and Trinidad antisera, also indicating that

these 2 viruses are either identical or very closely related. On the basis of serology and differences in reaction of certain hosts and physico-chemical properties the 5 virus isolates are grouped in 2 strains.

15. In immunoelectrophoresis only one distinct precipitation line was obtained with the Vu, and Vs viruses and their antisera at pH 8.6 although there was an indication of heterogeneity. The heterogeneity was more pronounced and the pattern was clearer at pH 5.2.

16. Cowpea mosaic virus isolates were found to be serologically related to bean pod mottle virus (BPMV, Scott) and a distant relationship was also found with the red clover mottle virus Dutch isolate. Serological tests with antisera against wild cucumber mosaic virus, tobacco ringspot virus, tobacco necrosis virus (bean stipple streak isolate), southern bean mosaic virus, alfalfa mosaic virus, cowpea strain of southern bean mosaic virus and the 5 virus isolates under study did not show any relationship.

17. The purified virus preparations exhibited several bands on density-gradient centrifugation in CsCl. The components could be purified by rerunning twice or thrice in CsCl gradient and the separated preparations gave single peaks in the analytical ultracentrifuge. The top or the lightest component was not infective while the middle component had the highest infectivity. All components were serologically indistinguishable.

18. On the basis of all evidence presented above, it is suggested that there are more than one type of particles present in the purified preparations (at least 3 components: empty particles, and particles with varying amounts of nucleic acid). No evidence was obtained that these components represented any artifacts since they were obtained by more than one purification procedure, and it is assumed that they are produced in the plant.

19. The data obtained for the three isolates of cowpea mosaic virus from Surinam (South America), studied alongwith 2 previously reported ones from Trinidad and Nigeria, revealed that they belong to the cowpea mosaic virus. A detailed description of the virus is given. The abbreviation CPMV is proposed to denote this virus to eliminate any possible confusion with CMV (cucumber mosaic virus). Two strains: (1) Severe Strain, designated as 'S' strain (including Vu and Vs isolates and Trinidad cowpea mosaic virus) and (2) Yellow mosaic strain, designated as 'Y' strain (including the Nigerian cowpea yellow mosaic and the Sb isolate), are distinguished on the basis of this study and these names are proposed for the identification and designation of the viruses in question.

SAMENVATTING

In verscheidene tropische landen komt een virus voor, dat bij *Vigna unguiculata* een ernstige ziekte teweegbrengt. Dit virus staat bekend onder de naam van 'cowpea mosaic virus' (CPMV). Het kan door bepaalde keversoorten worden overgebracht.

Dit geschrift geeft een verslag van onderzoeken naar biologische, biofysische, biochemische en immunologische eigenschappen van CPMV die kunnen dienen ter nadere identificering en karakterisering van dit virus.

Er werden vijf isolaties van CPMV vergeleken, nl. Vu, Vs en Sb uit Suriname, een isolatie uit Trinidad (verder aangeduid als 'Trinidad') en de als 'cowpea yellow mosaic virus' beschreven isolatie uit Nigeria (verder aangeduid als 'Nigeria'). Van iedere isolatie werd op *V. unguiculata* een cultuur in stand gehouden, die afkomstig was van een lokale lesie op *Phaseolus vulgaris*. De isolaties konden alle gemakkelijk met sap van plant op plant worden overgebracht.

Met de vijf isolaties konden behalve *V. unguiculata* de volgende plantesoorten worden geïnfecteerd: *Arachis hypogaea*, *Cassia tora*, *Chenopodium amaranticolor*, *Chenopodium quinoa*, *Crotalaria striata*, *Crotalaria usaramoensis*, *Dolichos lablab*, *Glycine max*, *Gomphrena globosa*, *Phaseolus aureus*, *Phaseolus lunatus*, *Phaseolus mungo*, *Phaseolus vulgaris*, *Pisum sativum*, en *Zinnia elegans*. Vu en Vs verwekten bovendien lokale symptomen op *Nicotiana glutinosa* en *Nicotiana tabacum*. Het virus kon niet worden overgebracht op *Amaranthus caudatus*, *Capsicum annum*, *Cucumis sativus*, *Datura stramonium*, *Lycopersicon esculentum*, *Medicago sativa* en *Vicia faba*. De desbetreffende gegevens benevens bijzonderheden over de symptomen op de vatbare soorten zijn in Tabel 1 samengevat. Foto's 1-9 geven een beeld van de ziekteverschijnselen bij enige plantesoorten.

Enige bonevariëteiten (*Phaseolus vulgaris*) reageerden op eenzelfde virusisolatie en ook op de verschillende isolaties verschillend (Tabel 2). De bonevariëteit 'Pinto' bleek geschikt voor de biologische bepaling van concentraties van Vu, Vs en 'Trinidad'. Ten aanzien van Sb en 'Nigeria' was deze variëteit voor dit doel minder geschikt.

Alle onderzochte variëteiten van *V. unguiculata* waren vatbaar voor Vu en Vs (Tabel 3). De variëteiten die resistent zijn tegen 'Nigeria' bleken eveneens resistent tegen Sb, terwijl deze rassen zeer vatbaar voor Vu en Vs waren.

In epidermiscellen van *Vigna*- en erwteplanten, besmet met CPMV werden amorfe insluitsels waargenomen (Foto 10). Deze lichaampjes worden van waarde geacht voor het stellen van de diagnose der ziekte.

Voor Vu werden de inactiveringstemperatuur, de verdunningsgrens en de houdbaarheid in vitro bepaald; deze werden respectievelijk vastgesteld op 65-70°C, 10.000-100.000 en 3-5 dagen.

CPMV werd uit sap van *V. unguiculata* gezuiverd door toepassing van een behandeling met chloroform en butanol, gevolgd door gedifferentieerd centrifugeren (Fig. 1). De aldus verkregen preparaten waren zeer infectieus en bezaten een absorptiespectrum, kenmerkend voor nucleoproteïnen (Fig. 2). In ultracentrifuge- en elektroforeseproeven bleken de viruspreparaten heterogeen te zijn (Fig.

3-11). Deze heterogeniteit was niet het gevolg van de aanwezigheid van normale in de plant voorkomende stoffen, doch moest worden toegeschreven aan verschillende componenten die met de virusinfectie samenhangen. Uit waarnemingen verricht op de mengsels der componenten, zoals zij in de gezuiverde preparaten van elk der virusisolaties voorkwamen, werden de sedimentatiecoëfficiënten der componenten door extrapolatie bij oneindige verdunning in water van 20°C berekend (Sectie 6.2).

In elektroforeseproeven werd het gedrag bepaald van de componenten zoals zij in de gezuiverde viruspreparaten aanwezig zijn (Sectie 6.3). Uit waarnemingen betreffende Vu werd het isoëlektrische punt van de snelst migrerende component berekend op pH 3,4 bij een ionenconcentratie van 0,1 (Fig. 12). CPMV bleek bij deze pH te worden geprecipiteerd.

Uit gezuiverde preparaten van Vu en Sb werd het nucleïnezuur door extractie met fenol geïsoleerd. Dit nucleïnezuur bleek besmettelijk te zijn in een mate van respectievelijk 0,4 en 0,1 % van het uitgangsmateriaal met een gelijk gehalte aan nucleïnezuur, berekend naar de aantallen vlekjes op *Chenopodium amaranticolor* (Tabellen 4 en 5). Daar de besmettelijkheid van het uit CPMV geïsoleerde nucleïnezuur na toediening van ribonuclease verdween, wordt geconcludeerd dat het virusnucleïnezuur van het RNA-type is.

Gezuiverde preparaten van de virusisolaties werden met de elektronenmicroscop bestudeerd. Ze bestonden uit min of meer bolvormige dan wel polyëdervormige deeltjes (Foto 11). In de met fosforwolframaat behandelde preparaten werden lege en gevulde deeltjes gezien (Foto's 12-14). In analogie met door anderen verrichte waarnemingen aan andere polyëdervormige virussen wordt aangenomen, dat de gevulde deeltjes bestaan uit een eiwitmantel, gevuld met nucleïnezuur en dat de lege deeltjes slechts uit een eiwitmantel bestaan. Gevulde deeltjes van Vu bleken 270 Å (gemeten tussen overliggende zijden) tot 300 Å (tussen overliggende hoeken) groot te zijn; voor lege deeltjes van dezelfde isolatie waren deze maten respectievelijk 250 en 270 Å. Voor de andere vier isolaties waren de afmetingen 230-250 Å (lege deeltjes) en 240-270 Å (gevulde deeltjes).

Op grond van de waarnemingen van de met fosforwolframaat behandelde viruspreparaten werd een model ontworpen van de eiwitmantel der deeltjes, nl. een icosaeëder bestaande uit 60 eenheden met een symmetrie van 5:3:2 (Foto's 15-17).

Tegen de vijf isolaties van CPMV werden antisera verkregen door inspuiting van konijnen met gezuiverde viruspreparaten. Vu, Vs en 'Trinidad' gaven duidelijke precipitatiereacties met hun homologe en heterologe antisera, doch slechts een zwakke reactie met antisera tegen Sb en 'Nigeria'. Hieruit volgt dat Vu, Vs en 'Trinidad' identiek of nauw verwant met elkaar zijn. Sb en 'Nigeria' gaven daarentegen sterke precipitatiereacties met hun homologe en heterologe antisera, doch een zwakke reactie met de antisera tegen Vu, Vs en 'Trinidad'. Hieruit kan worden geconcludeerd, dat Sb en 'Nigeria' identiek of nauw verwant aan elkaar zijn; tussen Vu, Vs en 'Trinidad' aan de ene kant en Sb en 'Nigeria' aan de andere kant bestaat slechts een verre verwantschap (Foto's 18, 19).

Immunoëlektroforeseproeven met Vu en Vs en hun antisera bij pH 8,6 gaven

een duidelijke precipitatielijntje te zien, hoewel er aanwijzingen voor heterogeniteit waren. Deze heterogeniteit kwam duidelijker tot uiting bij pH 5,2 (Foto's 20, 21).

De vijf isolaties van CPMV bleken serologisch verwant te zijn aan het 'bean pod mottle virus'. Een verre verwantschap met een Nederlandse isolatie van het 'red clover mottle virus' werd tevens vastgesteld (Tabel 6). Maar een serologische verwantschap kon niet worden geconstateerd tussen de vijf isolaties van CPMV en respectievelijk 'wild cucumber mosaic virus', 'tobacco ringspot virus', tabaksnecrosevirus (herkomst stippestreep van de boon), 'southern bean mosaic virus' en de 'cowpea strain' van dit virus en luzernemozaïekvirus.

Gezuiverde viruspreparaten werden gecentrifugeerd in een CsCl-medium met dichtheidsgradiënt (Foto 22). Aldus konden verschillende componenten worden gescheiden, die na twee- of driemaal in hetzelfde CsCl-medium te zijn gecentrifugeerd, in de analytische ultracentrifuge elk voor zich een karakteristiek sedimentatiepatroon met één piek gaven. De langzaamst sedimenterende component bleek niet infectieus te zijn. De sneller sedimenterende ('middelste') component was veel besmettelijker dan de snelst sedimenterende component. Dit verschijnsel verdient nadere studie. Serologisch gedroegen de drie componenten zich gelijk (Tabel 7).

Op grond van de thans verkregen gegevens wordt verondersteld, dat er tenminste drie soorten van deeltjes in gezuiverde CPMV-preparaten voorkomen, nl. lege deeltjes uitsluitend bestaande uit een eiwitmantel, en deeltjes waarvan de eiwitmantel verschillende hoeveelheden nucleïnezuur bevat. Aangenomen wordt, dat dit geen artefacten zijn, doch dat al deze deeltjes in de met CPMV besmette plant ontstaan.

De hier beschreven resultaten leiden tot de conclusie, dat de vijf onderzochte isolaties kunnen worden ondergebracht in twee stammen van CPMV, nl. 1. de 'severe strain' (aan te duiden met de letter 'S') waartoe Vu en Vs, alsmede het 'cowpea mosaic virus' van Trinidad kunnen worden gerekend en 2. de 'yellow mosaic strain' (aan te duiden met de letter 'Y') waartoe Sb en het 'cowpea yellow mosaic virus' van Nigeria behoren. Merkwaardig is, dat in Suriname beide stammen voorkomen.

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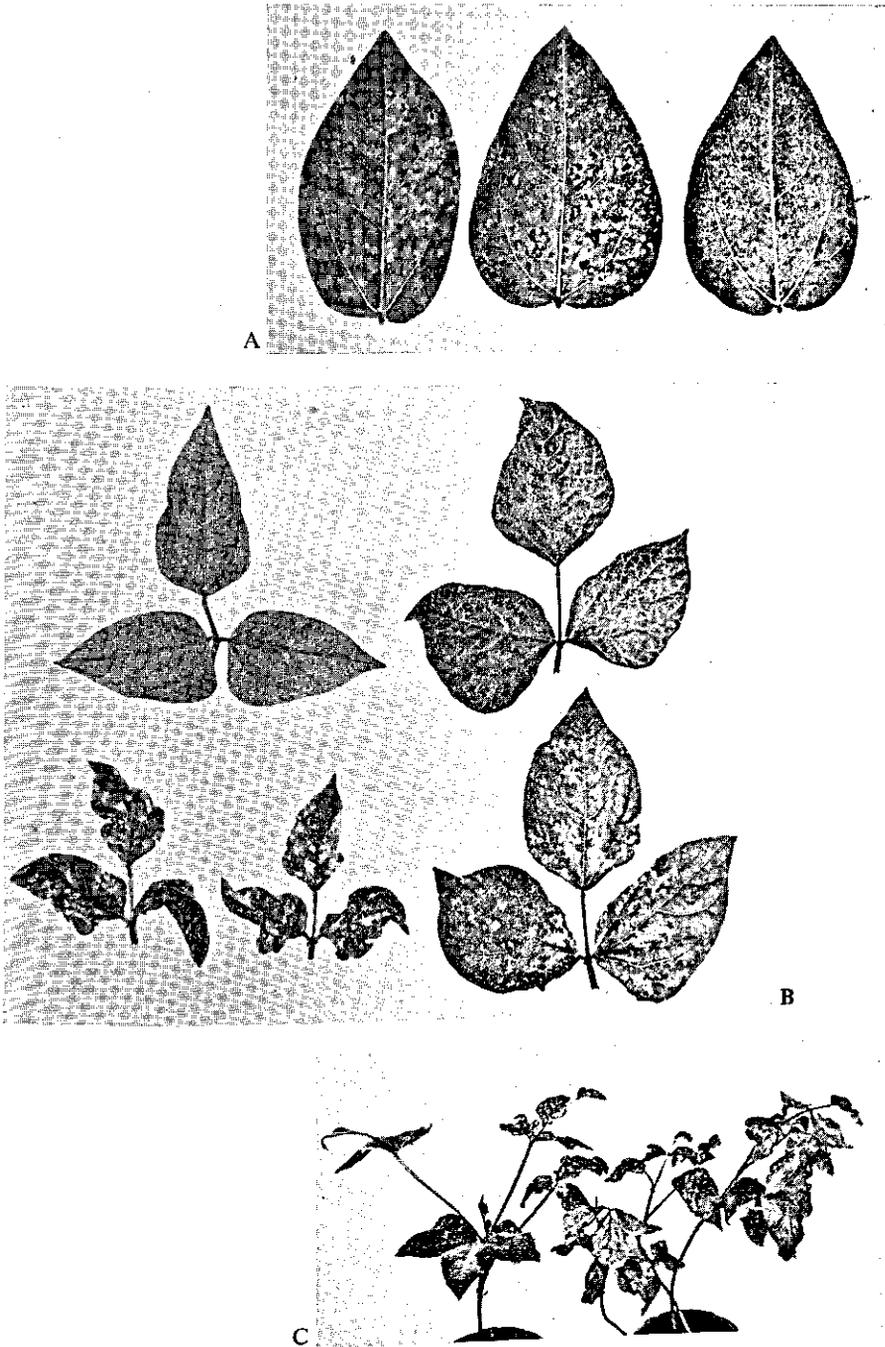


PHOTO 1. Cowpea, *Vigna unguiculata* (L.) Walp. var. 'FC 31705', inoculated with cowpea mosaic virus (Vu isolate). A, primary leaves showing lesions (right - healthy leaf); B, and C, showing systemic symptoms.

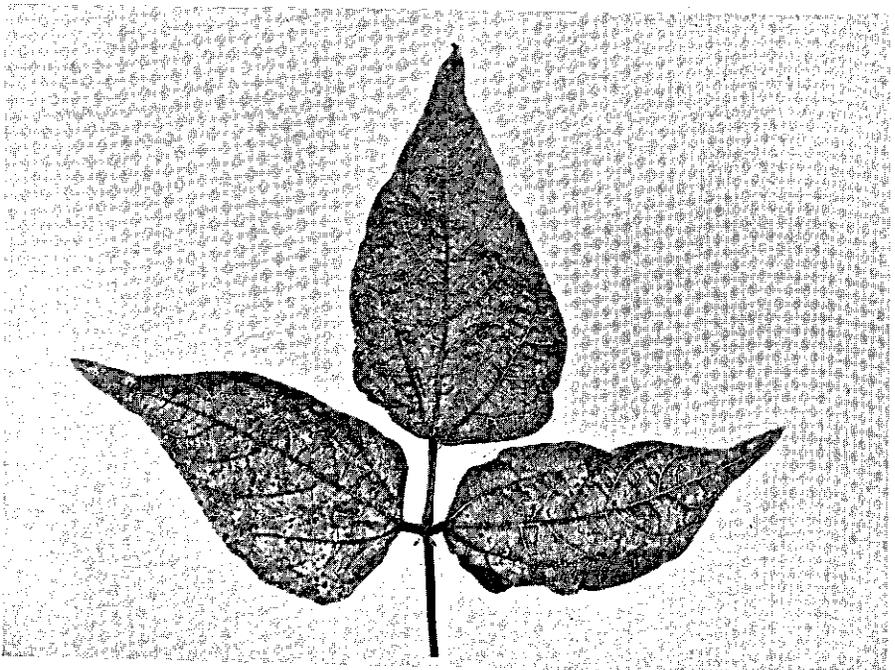
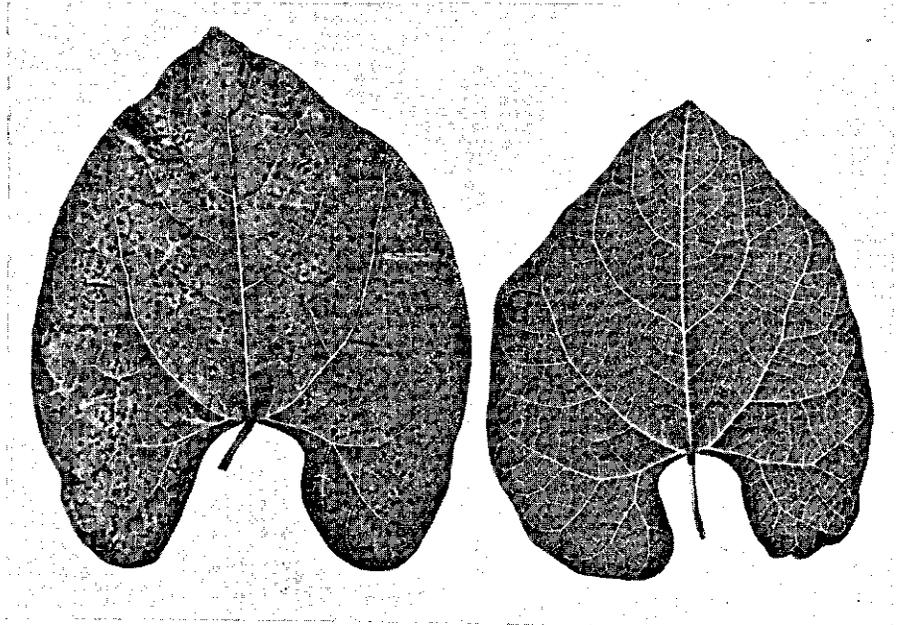
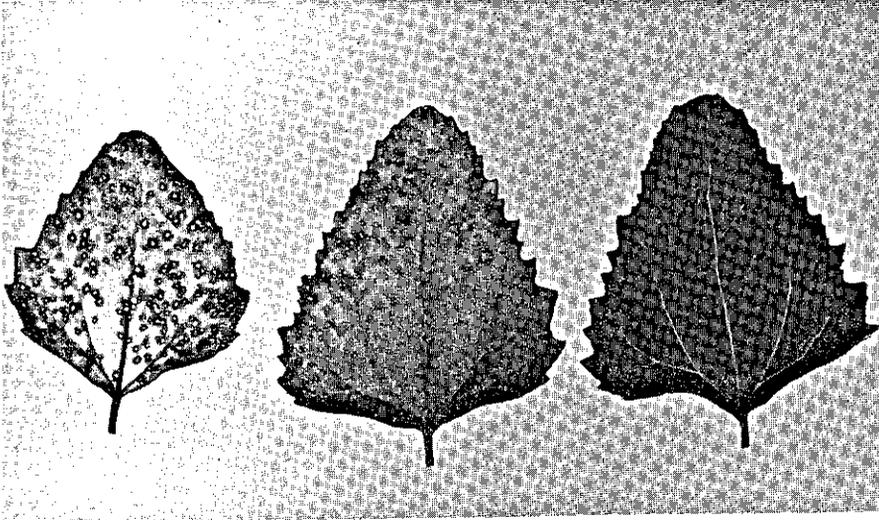


PHOTO 2. Leaves of *Phaseolus vulgaris* var. 'Beka' showing local (upper row, left) and systemic symptoms (lower row) due to cowpea mosaic virus (Vu isolate). Right (upper row), healthy leaf.



A

B

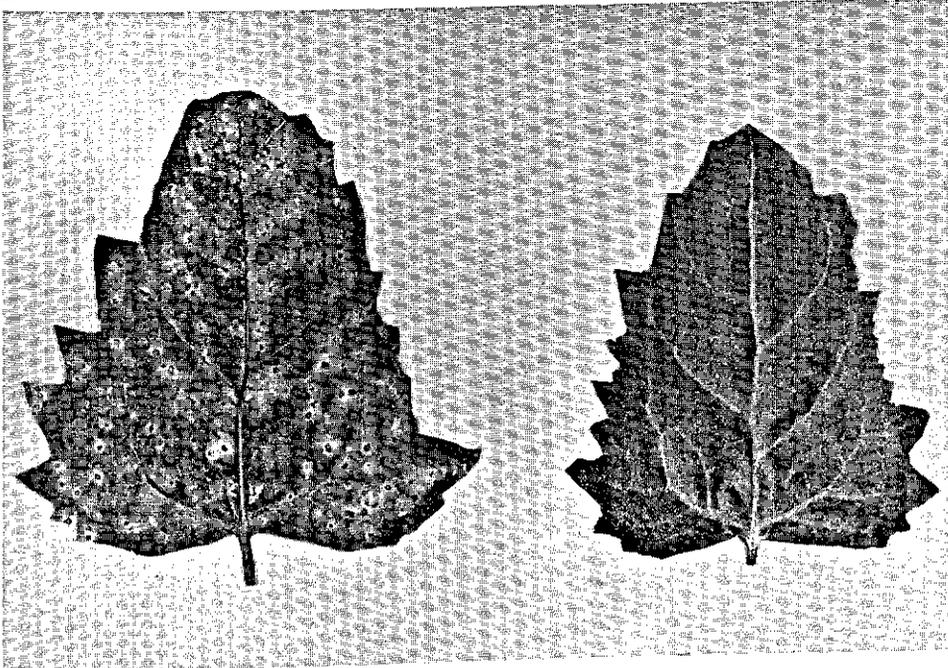


PHOTO 3. Leaves of *Chenopodium amaranticolor* Coste & Reyn. (A) and *Chenopodium quinoa* Willd. (B) showing local lesions due to cowpea mosaic virus (Vu isolate). Right - healthy leaves.

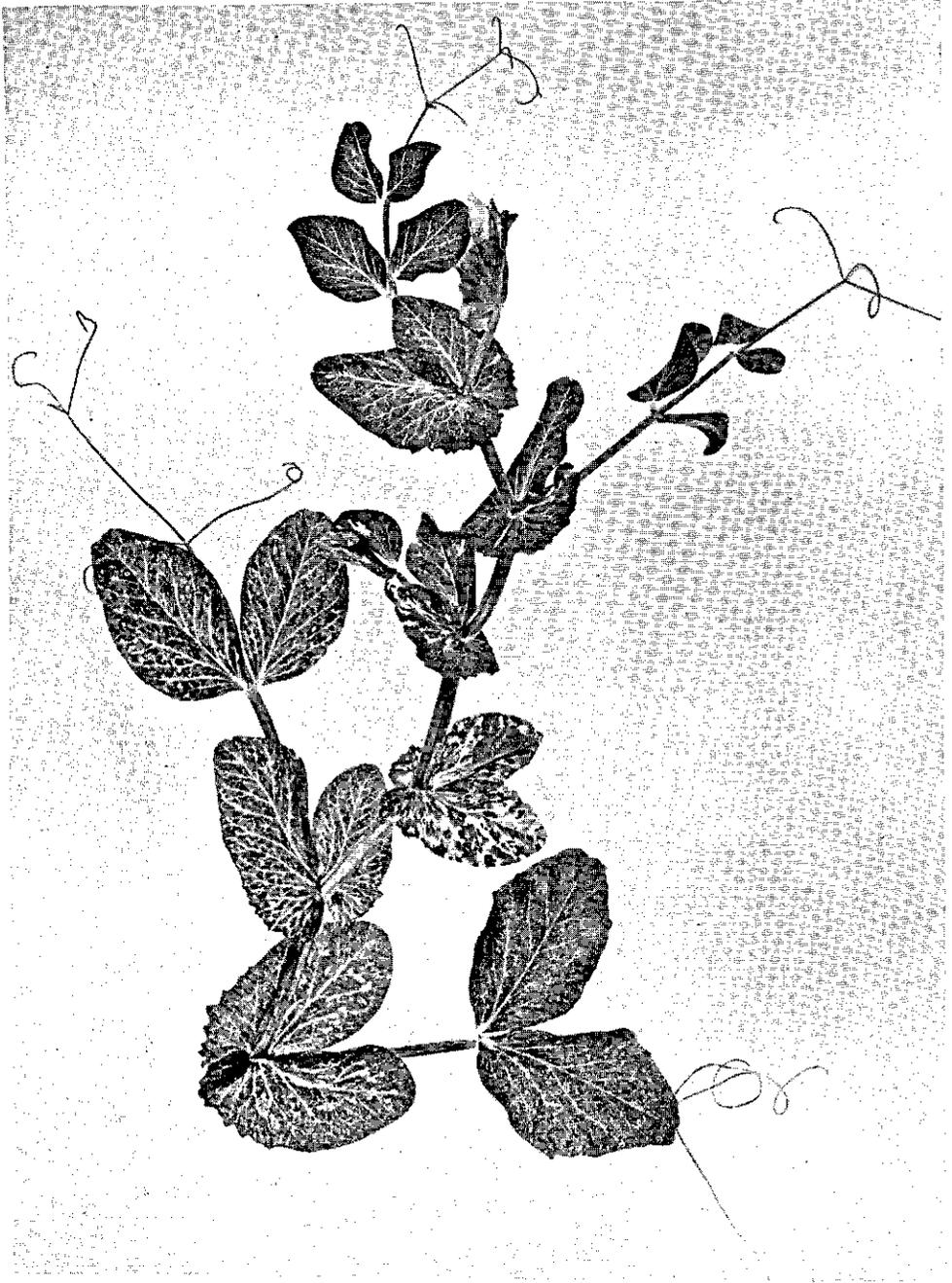


PHOTO 4. Systemic symptoms in pea (*Pisum sativum* L.) var. 'Eroica', inoculated with cowpea mosaic virus (Sb isolate). Diffuse mottling, systemic mosaic, slight vein-banding, bright yellow areas, and recovery in young leaves are typical symptoms on this host.

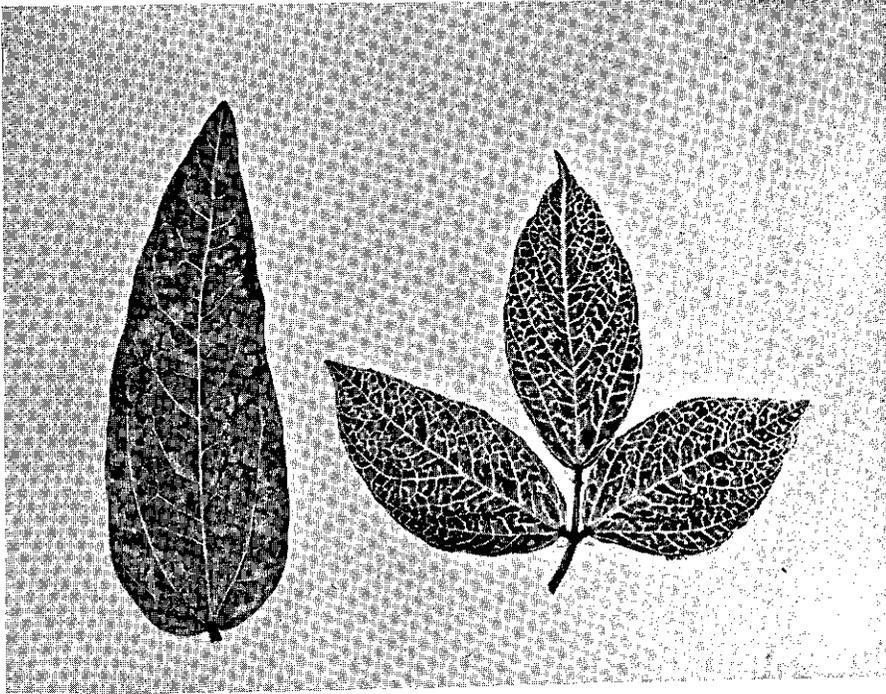


PHOTO 5. Leaves of *Phaseolus mungo* L. showing local (left) and systemic (right) symptoms due to cowpea mosaic virus (Vu isolate).

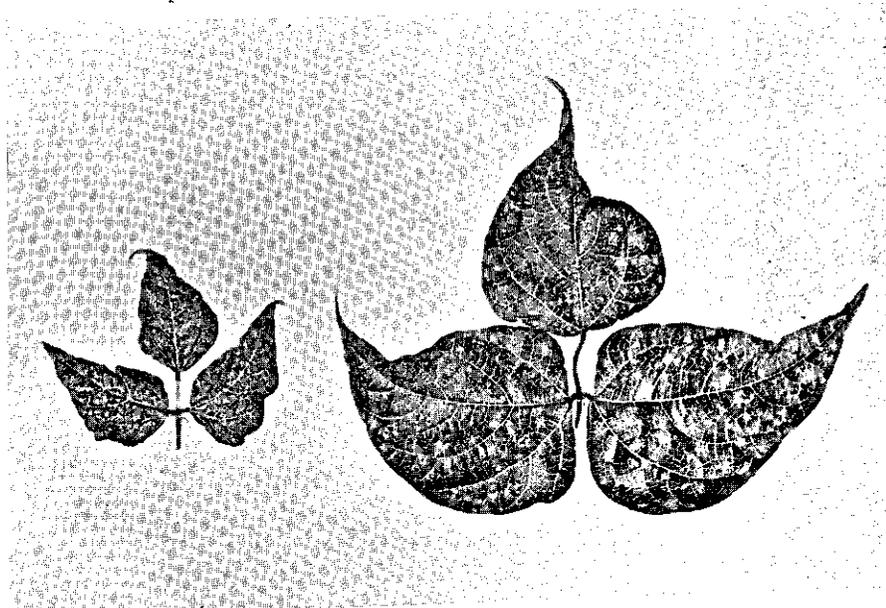


PHOTO 6. Trifoliate leaves of *Phaseolus lunatus* L. showing systemic symptoms due to cowpea mosaic virus (Sb isolate).

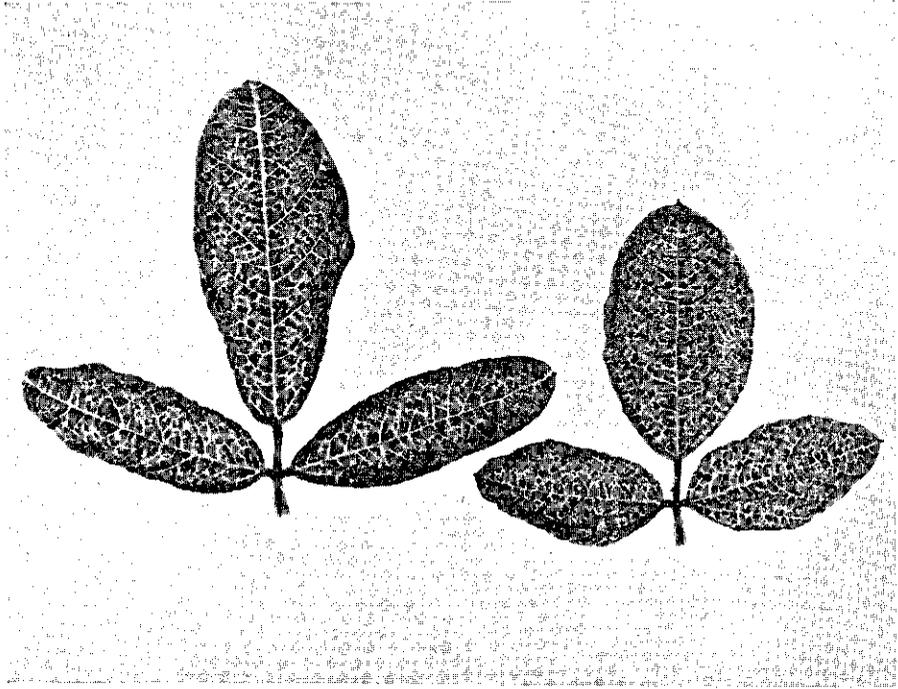


PHOTO 7. Leaves of *Glycine max* (L.) Merr. showing systemic symptoms due to cowpea mosaic virus (right – Nigerian isolate, left – Sb isolate).

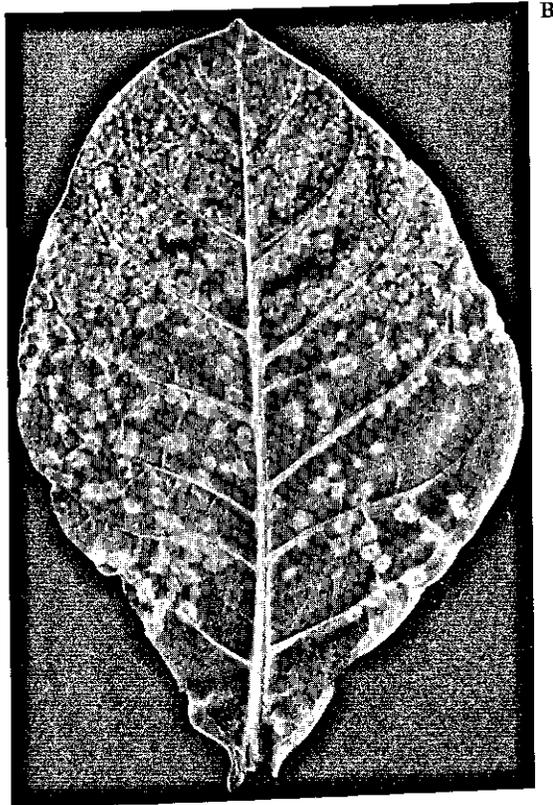
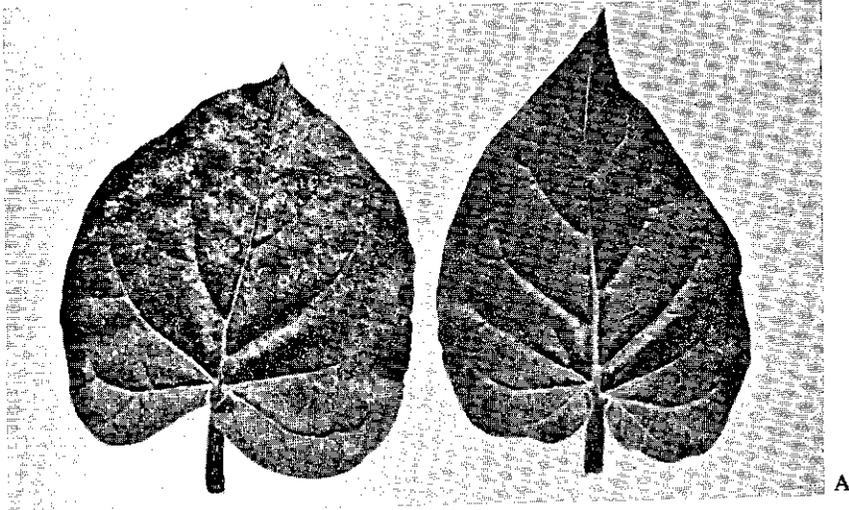


PHOTO 8. Leaves of *Nicotiana glutinosa* L. (A) and *Nicotiana tabacum* L. var. 'White Burley' (B) inoculated with the Vu isolate of cowpea mosaic virus, 14 days after inoculation, showing chlorotic lesions. Upper row, right: healthy leaf.

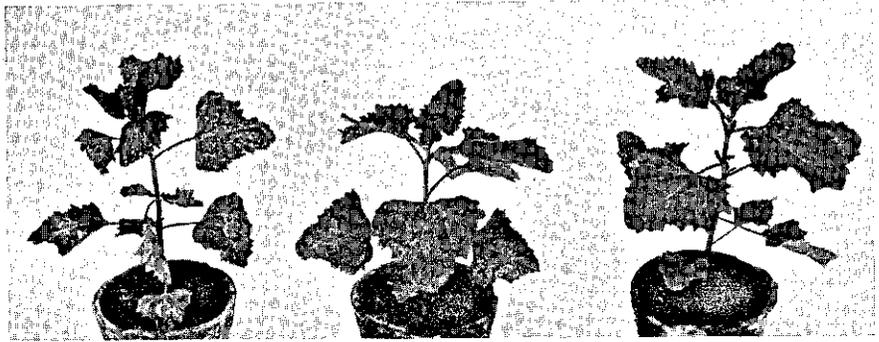


PHOTO 9. A comparison of the symptoms produced by Vu and Sb isolates of cowpea mosaic virus on *Phaseolus vulgaris* L. var. 'Beka' (lower row) and *Chenopodium amaranticolor* Coste & Reyn. (upper row). Plants on extreme left were inoculated with the Sb isolate and plants in the middle with the Vu isolate. Right, healthy plant. (Photo: I.P.O., Wageningen.)

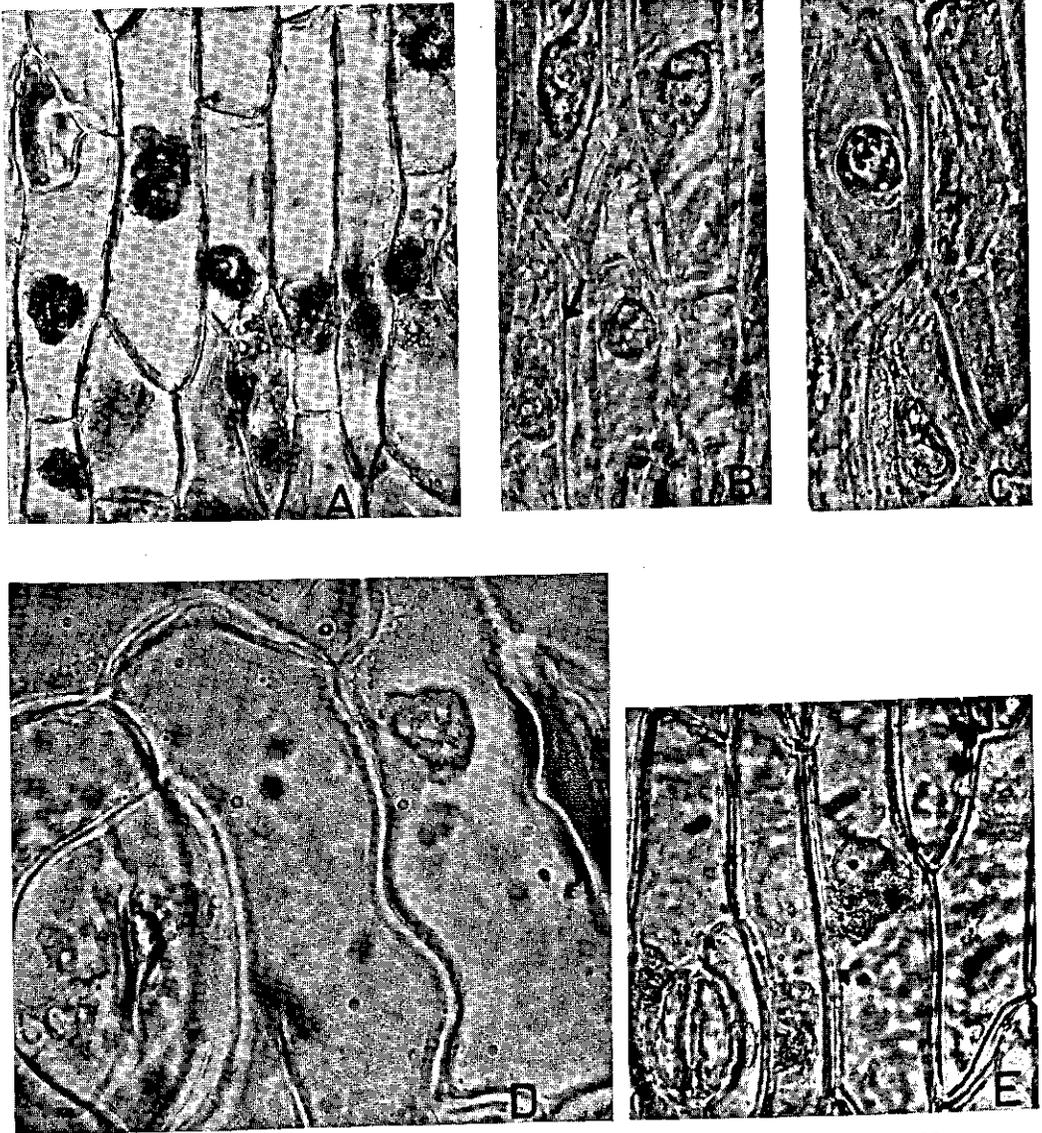


PHOTO 10. Photomicrographs of inclusion bodies in epidermal cells of peas and cowpeas 25 days after inoculation with cowpea mosaic virus. Unless indicated the inclusions are of vacuolar type. (A) Sb isolate, epidermal strip from pea petiole showing several inclusion bodies. Magnification: $\times 1000$ approximately. (Prep.: Rubio-Huertos; Photo: I.P.O., Wageningen). (B) same as (A) but a different preparation. The cell on the lower left marked with an arrow, shows a slightly granular inclusion. (C) Trinidad isolate, epidermal strip from pea petiole showing two inclusion bodies. Magnification: $\times 1000$. (D) Nigerian isolate, epidermal strip from cowpea primary leaf showing one inclusion body. Magnification: $\times 1700$. (E) Nigerian isolate, epidermal strip from cowpea petiole, showing a more or less granular inclusion. Magnification: $\times 1000$.

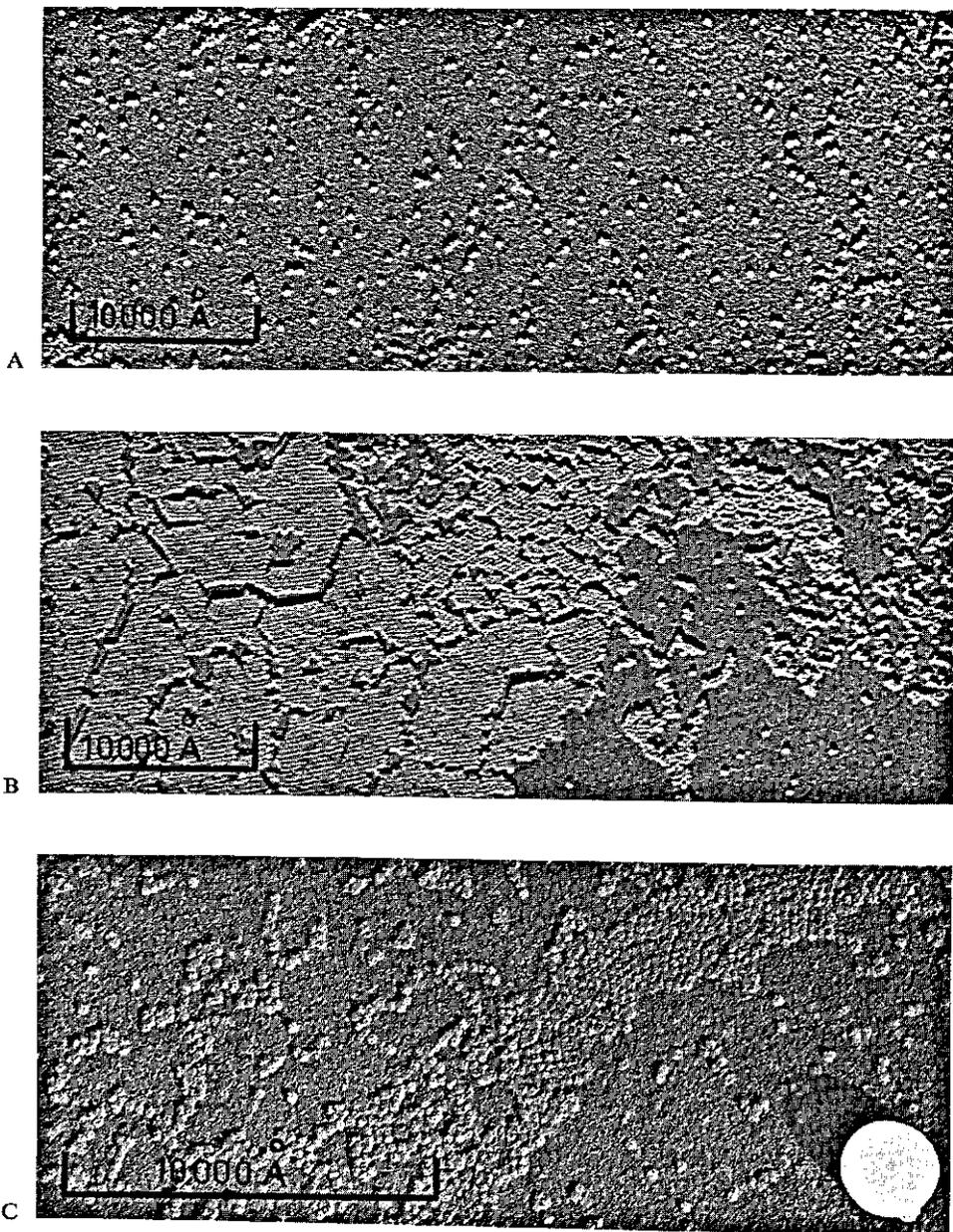


PHOTO 11. A. Electron micrograph of cowpea mosaic virus (Vs isolate). Magnification: $\times 24,000$. B. Electron micrograph of cowpea mosaic virus (Vu isolate). The preparation apparently showed a tendency to crystallize. Magnification: $\times 24,000$. C. Electron micrograph of cowpea mosaic virus (Vu isolate). Hexagonal contour, angularity of particles, and the angular shadows can be noted. Large white circle represents a particle of Dow polystyrene latex which has a diameter of $2,600 \text{ \AA}$. Magnification: $\times 48,000$.

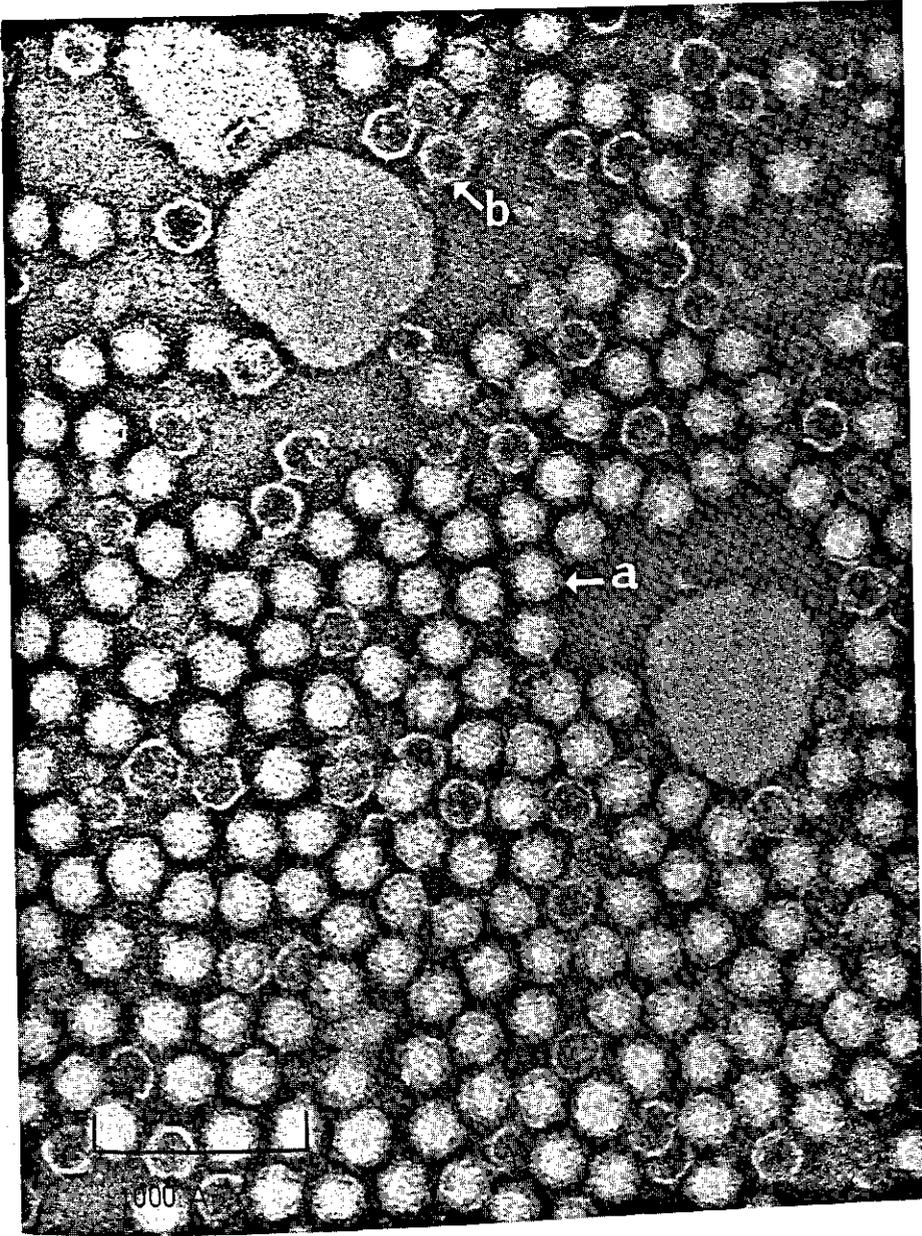


PHOTO 12. Electron micrograph of cowpea mosaic virus (Sb isolate), negatively stained with potassium phosphotungstate (pH 7.0). Magnification: $\times 270,000$. Particles with (a) and without (b) nucleic acid can be seen.

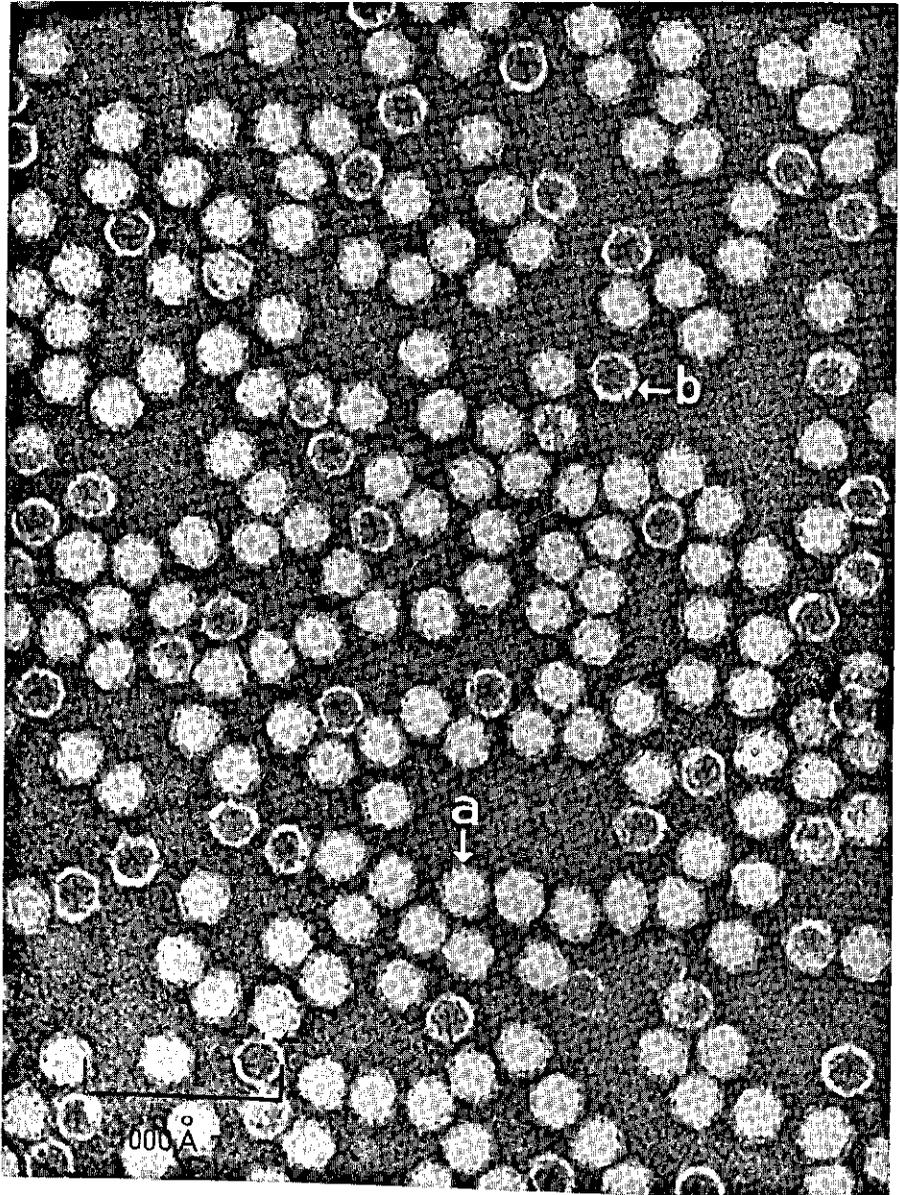
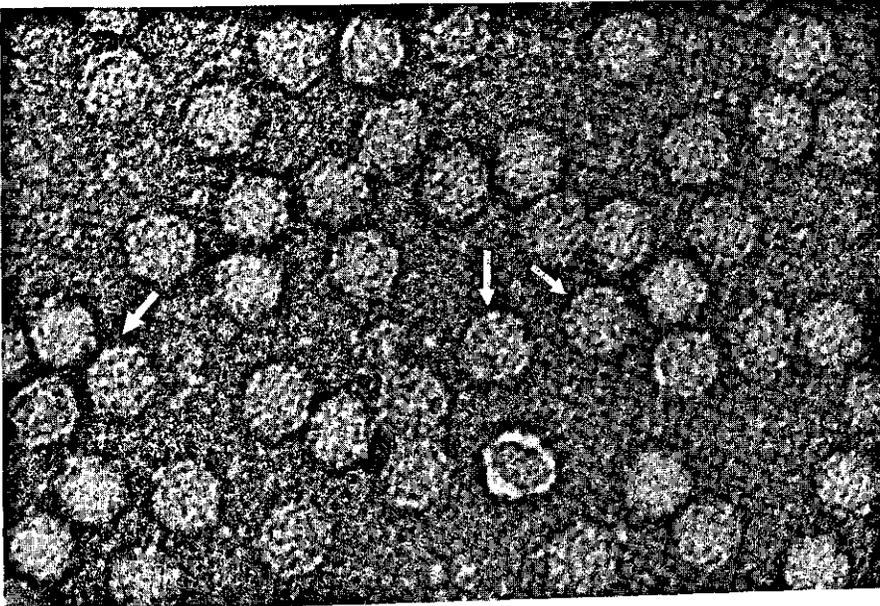


PHOTO 13. Electron micrograph of cowpea mosaic virus (Vu isolate), negatively stained with potassium phosphotungstate (pH 7.0). Magnification: $\times 270,000$. Particles with (a) and without (b) nucleic acid can be seen.



A

B

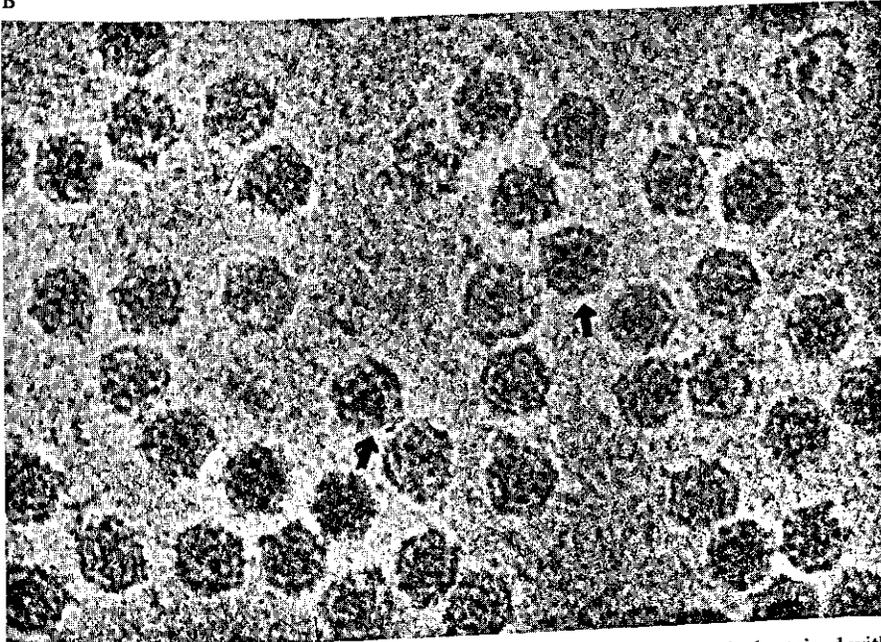


PHOTO 14. Electron micrographs of cowpea mosaic virus (Vu isolate), negatively stained with potassium phosphotungstate (pH 7.0). (A) printed in the normal way (B) printed in reversed contrast. Magnification: $\times 320,000$. The arrows indicate particles showing details of the substructure.

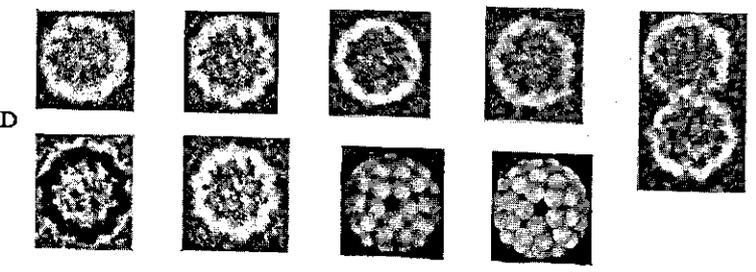
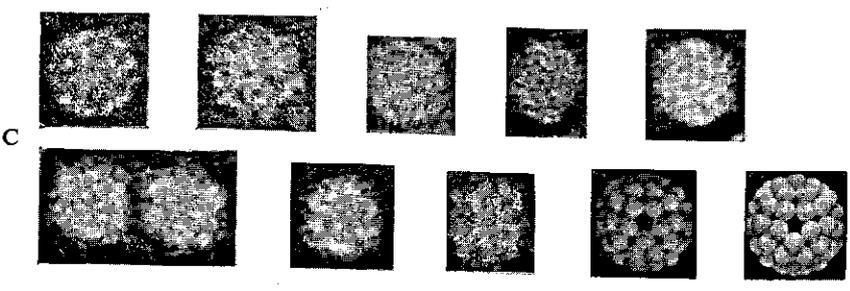
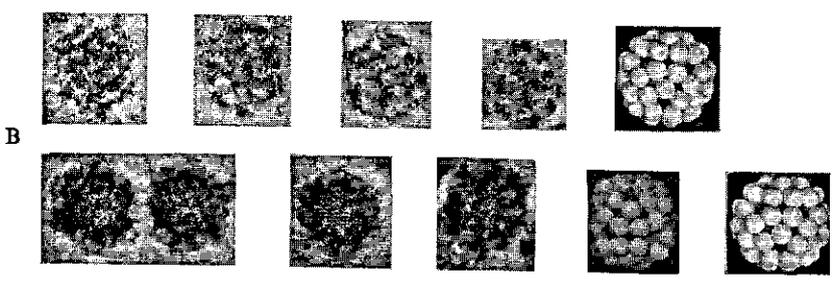
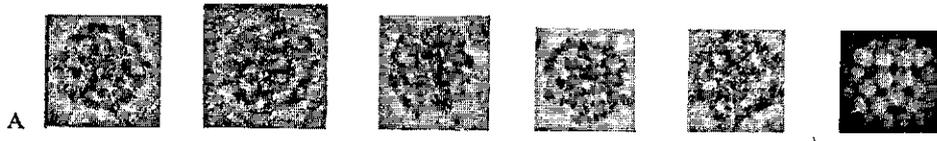


PHOTO 15. Selected cowpea mosaic virus particles (Vu isolate) in which some of the subunits can be seen. The micrographs are all reproduced at the same magnification, and any apparent differences in scale are due to their different degrees of immersion in the phosphotungstic acid layer and conditions of shrinkage and flattening. The particles were photographed in the normal way but those in A, B upper row, and D lower row, extreme left, were printed in reversed contrast. On the right are photographs of a model with 60 subunits represented by table tennis balls in an icosahedral body with 5:3:2 axial symmetry, and reproduced on approximately the same scale for comparison. Some of the photographs of the model are made out of focus to provide for a better comparison since it is not necessary at all that the morphology of the subunits is similar to the appearance of the table tennis balls in the model.

It can be seen that the model in fivefold symmetry axis (D) appears more or less spherical or somewhat pentagonal, agreeing with the profile of some of the particles, especially to the 'empty' ones. The same model in threefold symmetry (A, B) appears hexagonal resembling the profile of most of the nucleic acid containing particles.

The orientation of the different particles is different and not all the subunits can be seen in one and the same particle, hence a comparison can only be made by looking at the different particles in each row and comparing with parts of the model on the right.

Magnification: $\times 400,000$.

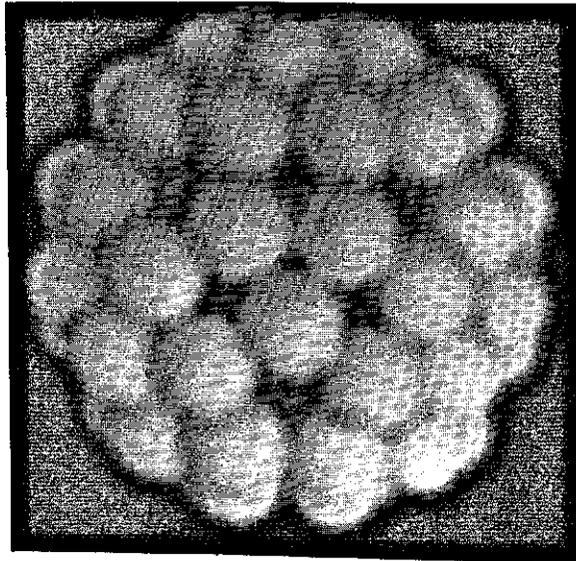
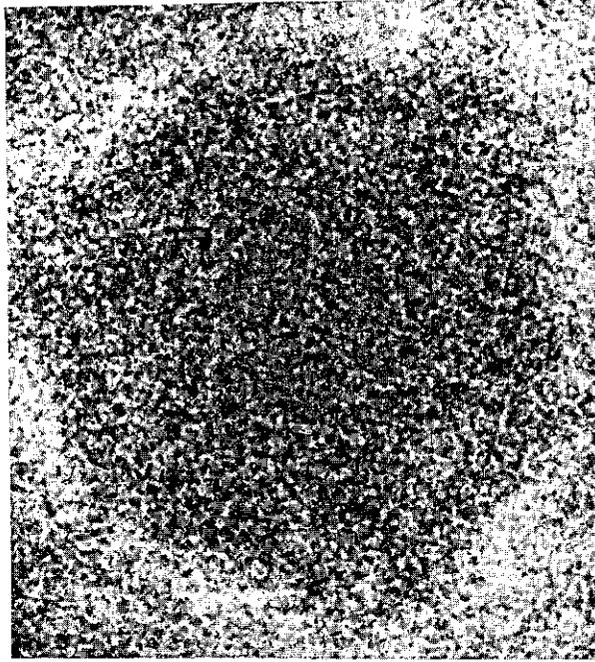


PHOTO 16. A particle of cowpea mosaic virus (Vu isolate) photographed by the rotation method. Rotated $n = 3$. The original print was made in reversed contrast. The photograph below, especially made out of focus for a better comparison, represents a 60 subunit model (5:3:2 symmetry) of table tennis balls in its threefold symmetry. The three subunits in the centre, and the hexagonal outline of the particle, can be seen in the micrograph above. Magnification: $\times 2,700,000$.

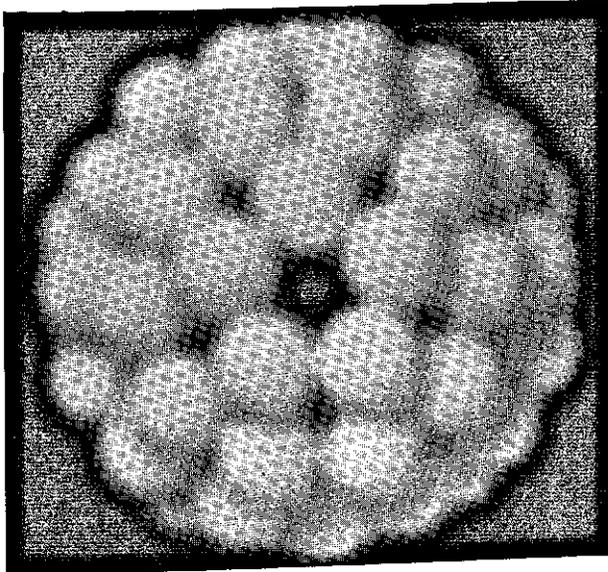
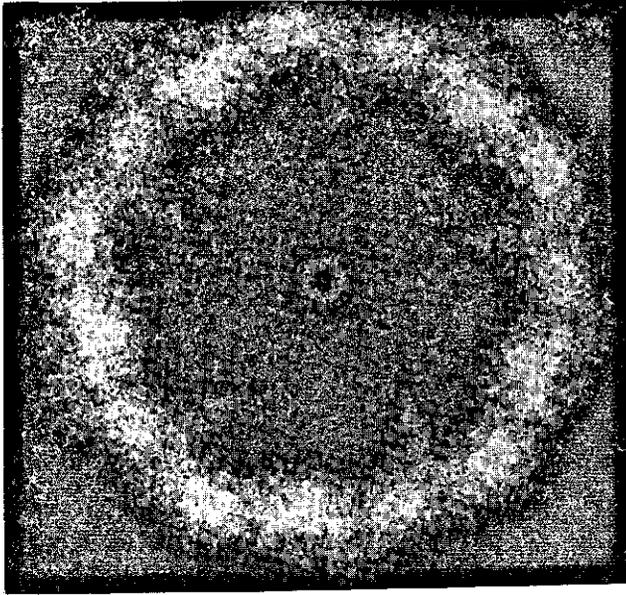


PHOTO 17A. An empty particle 'core' of cowpea mosaic virus (Vu isolate) photographed by the rotation method. Rotated $n = 5$. The photograph below especially made out of focus for a better comparison, represents the 60 subunit model with 5:3:2 symmetry, in its fivefold symmetry. The number of subunits (15) can be counted and the more or less circular profile of the particle, can be seen in the micrograph above. Magnification: $\times 2,700,000$.

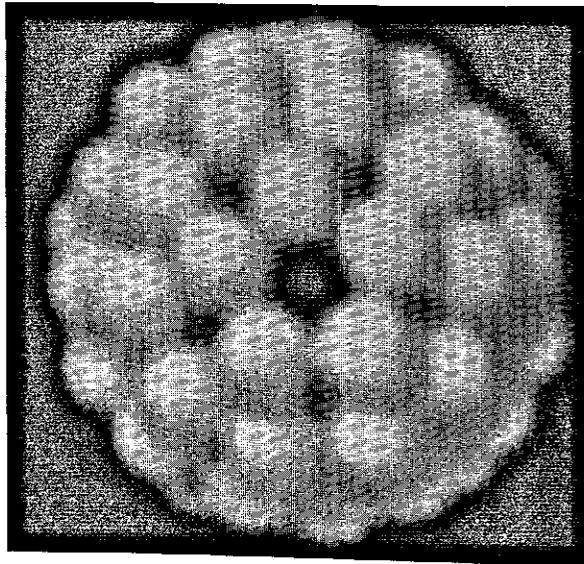
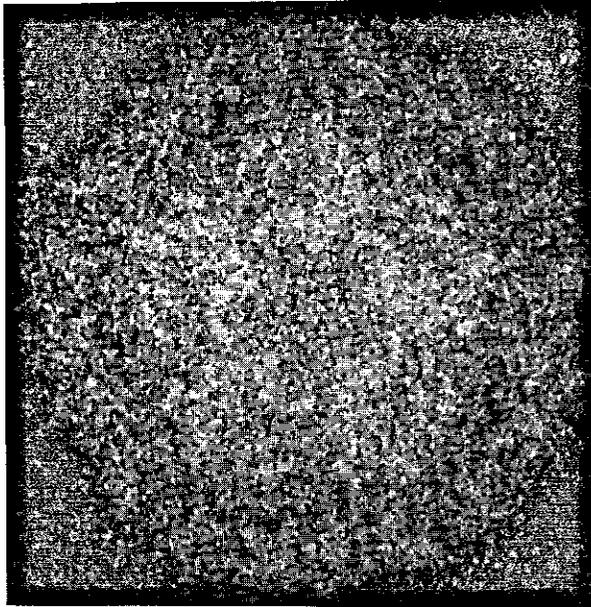


PHOTO 17B. A particle of cowpea mosaic virus (Vu isolate) photographed by the rotation method. Rotated $n = 5$. The photograph below especially made out of focus for a better comparison, represents the 60 subunit model with 5:3:2 symmetry, in its fivefold symmetry. The central ring of five subunits and more or less circular profile of the particle, can be seen in the micrograph above. Magnification: $\times 2,700,000$

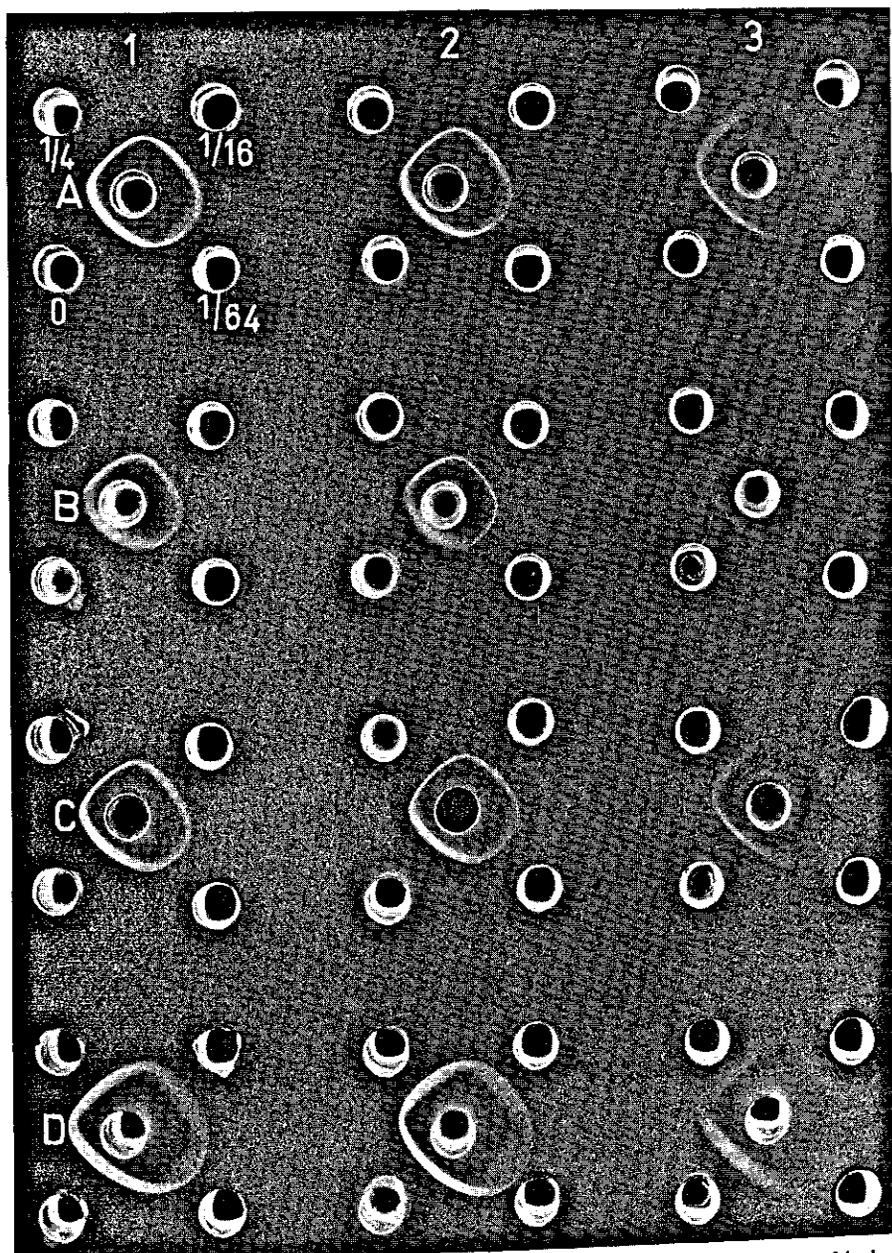


PHOTO 18. A photograph of the Ouchterlony double-diffusion test showing relationship between Vu, Vs, Trinidad, and Nigerian isolates of cowpea mosaic virus. The central wells contained the antigens; horizontal row A: Vu isolate crude sap, row B: Vs isolate crude sap, row C: Trinidad isolate crude sap, row D: Trinidad isolate purified virus. The surrounding wells contained dilutions of antisera against Vu isolate (Vertical row 1), Vs isolate (Vertical row 2), Nigerian isolate (Vertical row 3). The dilution pattern is indicated on the top left.

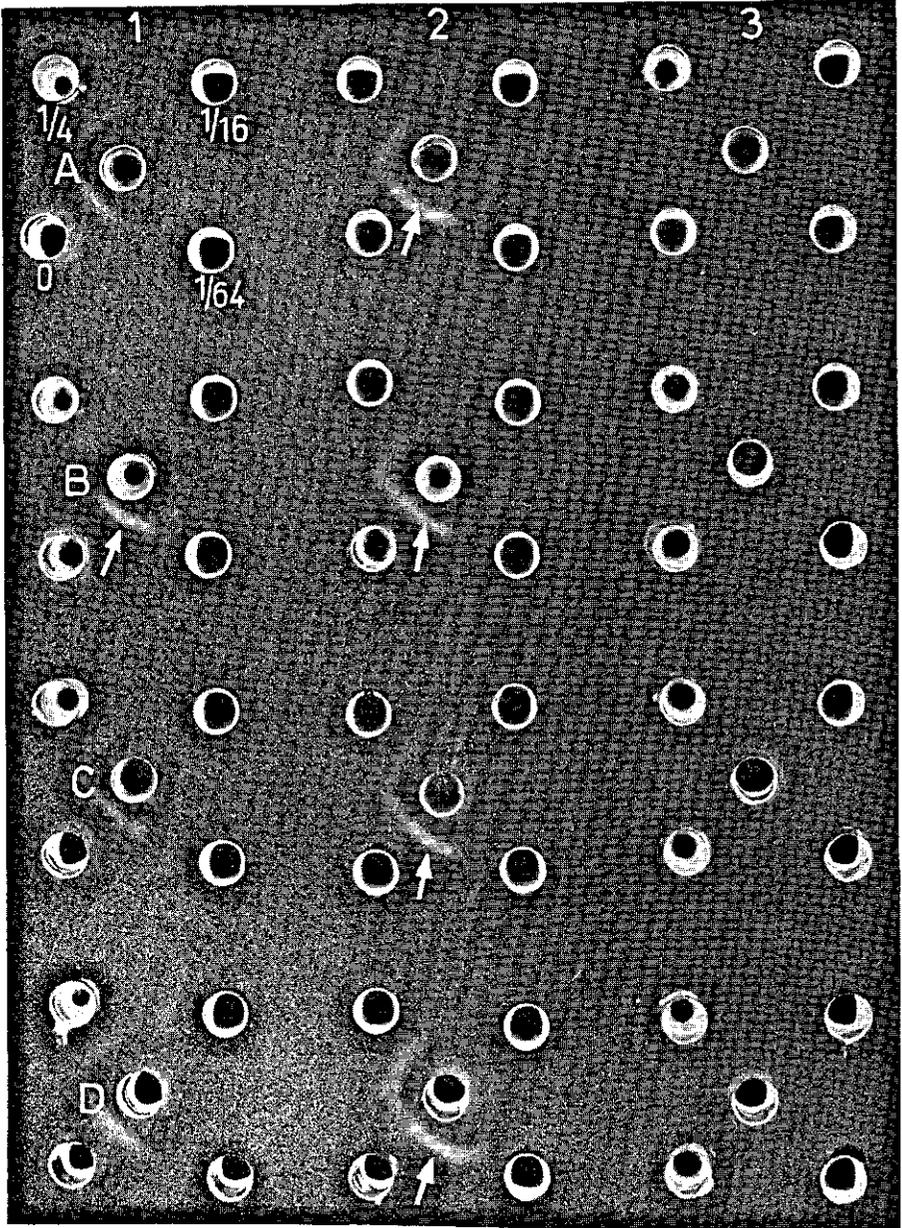


PHOTO 19. A photograph of the Ouchterlony double-diffusion test showing relationship among cowpea mosaic virus isolates, red clover mottle virus, and bean pod mottle virus. The central wells contained the antigens; horizontal row A: Vu isolate crude sap, row B: Vs isolate crude sap, row C: Trinidad isolate crude sap, row D: Trinidad isolate purified virus. The surrounding wells contained dilutions of antisera against red clover mottle virus, Dutch isolate (Vertical row 1), and bean pod mottle virus antiserum from Scott (Vertical row 2). Vertical row 3 contained dilutions of normal serum. The dilution pattern is indicated on the top left. The faint precipitin lines are pointed by arrow.

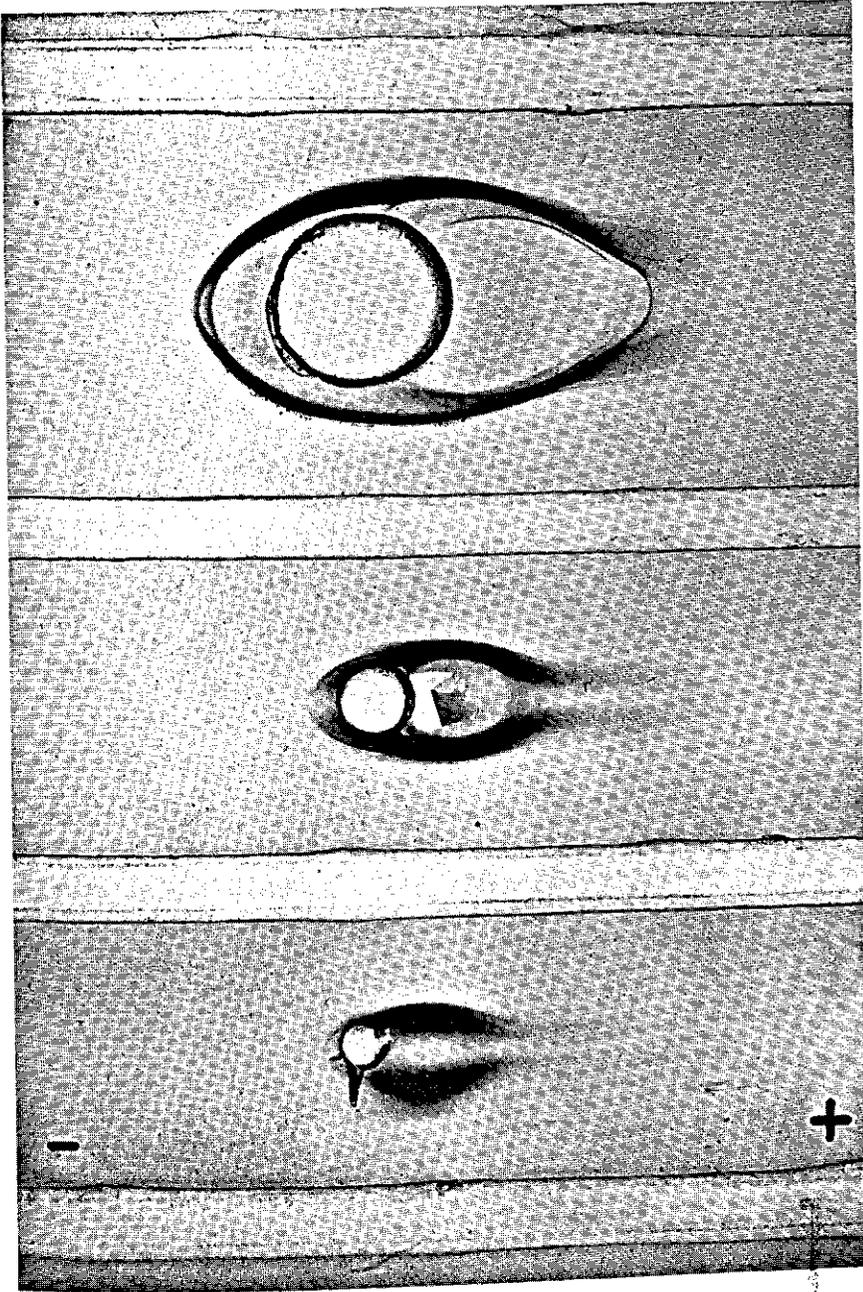


PHOTO 20. Immunoelectrophoresis in agar gel of cowpea mosaic virus (Vu isolate). Electrophoresis was carried out in 0.05 M veronal buffer pH 8.6 for 60 minutes with a current of ca. 10 ma and 60 volts (6 volt/cm). Precipitin bands were stained with amido black.

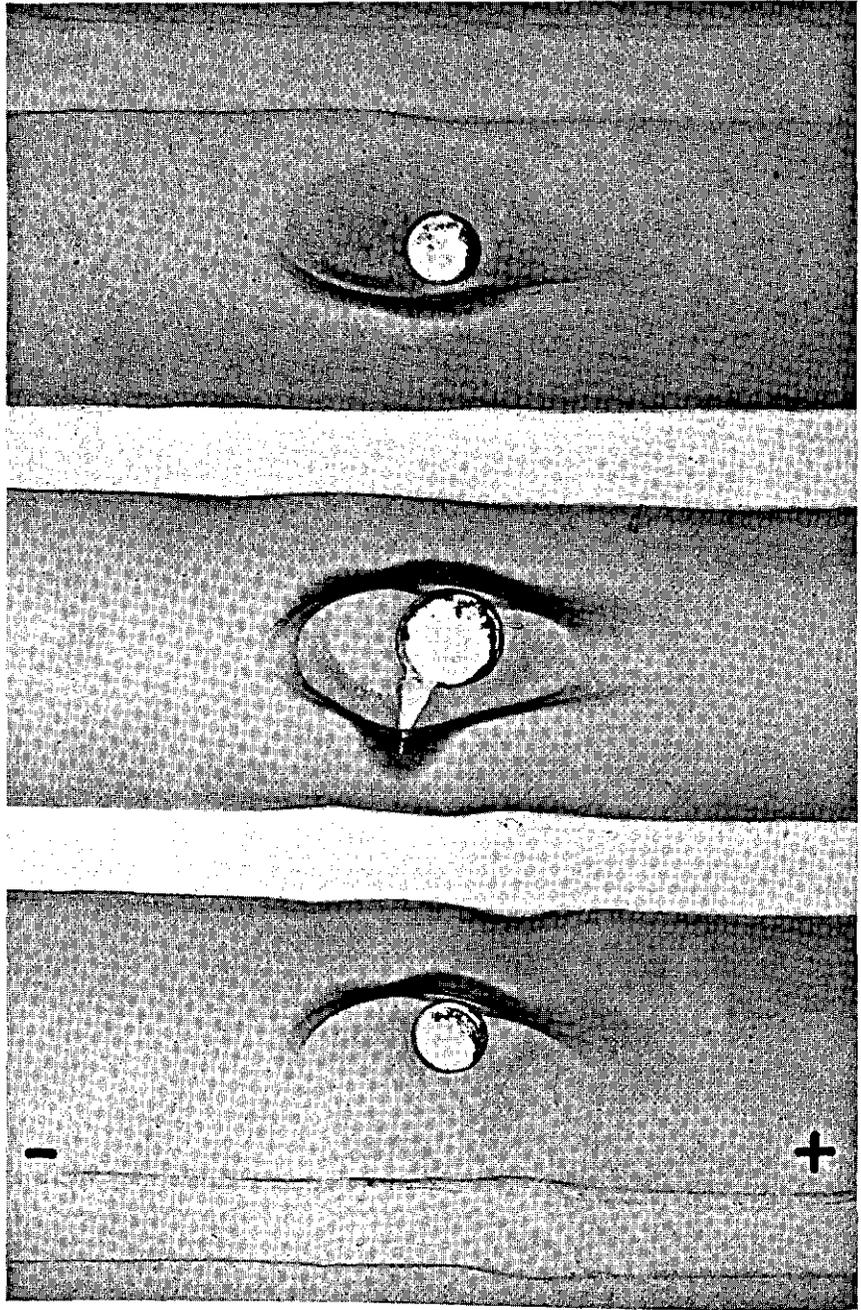


PHOTO 21. Immunoelectrophoresis in agar gel of cowpea mosaic virus (Vu isolate). Electrophoresis was carried out in 0.1 M phosphate buffer pH 5.2 for 60 minutes with a current of ca. 10 ma and 60 volts (6 volt/cm). Precipitin bands were stained with amido black.

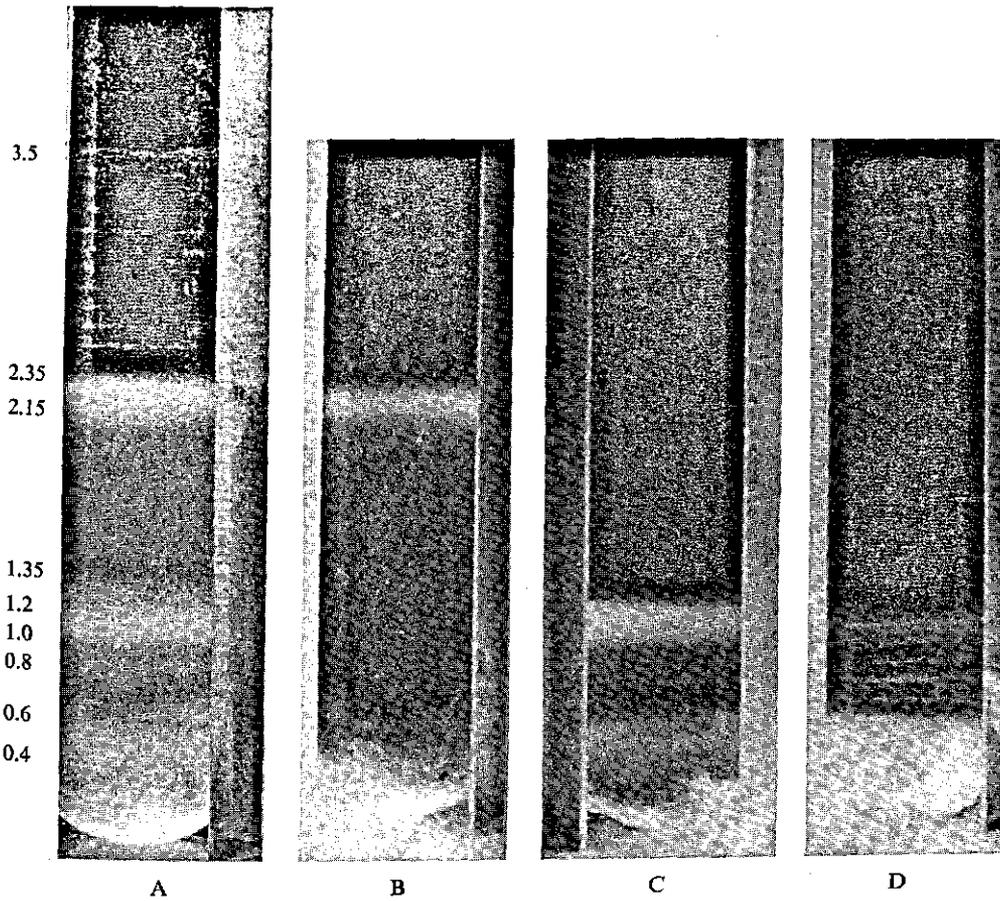


PHOTO 22. Fractionation of cowpea mosaic virus (Sb isolate) in a caesium chloride solution (density = 1.45). The tubes were photographed after 24 hr. centrifugation at 35,000 rev/min in a Spinco SW39 rotor. The zones could be slightly higher or lower depending on exactly where the virus is layered, but their relative positions remain the same. (A) unfractionated virus showing the top (2.15-2.35), middle (0.8-1.35), and bottom (0.4-0.6) components. Sometimes the middle component gave more than one zone as shown above. (B) Top zone after recentrifuging 3 times in CsCl. (C) Middle zone after recentrifuging one time in CsCl. Quite a lot of bottom component was still present as contaminant. Complete purification could be achieved by 2 more centrifugations. (D) Bottom zone after recentrifuging twice in CsCl. Slight contamination with the middle component can be seen. Further purification was accomplished by one more centrifugation.