

## The Purification and Properties of Chronic Bee-paralysis Virus

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### SUMMARY

Purified preparations of chronic bee-paralysis virus were obtained by clarifying water extracts of paralysed bees with ether and carbon tetrachloride; the virus particles were concentrated from the clarified extracts either by centrifugation or precipitation with ammonium sulphate. The preparations contained particles of three sizes, all approximately 220 Å wide and ellipsoidal in outline, but about 410, 540 or 640 Å long with sedimentation coefficients ( $S_{20, w}$ ) of 97, 110 and 125 respectively. The shortest particles contained least nucleic acid, and preparations containing mostly short particles were less infective than those containing mostly long ones. The particles contained ribose nucleic acid with a molar base ratio of G 20%—A 24%—C 28%—U 28%. When incubated in cold acid or alkali solutions (1 N), the virus particles formed empty rounded protein shells.

### INTRODUCTION

The disease of bees (*Apis mellifera* L.) called 'paralysis' was shown by Bailey, Gibbs & Woods (1963) to be caused by a virus, which they described and named chronic bee-paralysis virus. This virus has since been found to be widespread in many countries (Bailey 1965*a*, 1967).

Preparations of the virus made by Bailey *et al.* (1963) from artificially infected bees were too impure and dilute for detailed studies of their properties. We now describe an improved method of purifying chronic bee-paralysis virus and some of the properties of the purified preparations.

### METHODS

Naturally paralysed bees were obtained from two affected bee colonies; 2000 to 3000 sick bees were evicted each week from their hives by their healthy companions. They were collected by placing boxes under the hive entrances, and stored at  $-20^{\circ}$ .

Most of the techniques used have already been described (Bailey, 1965*b*; Bailey & Gibbs, 1964; Bailey *et al.* 1963; Gibbs, Nixon & Woods, 1963; Gibbs *et al.* 1966). For electron microscopy, 2% neutral sodium phosphotungstate, 1% uranyl acetate or 1% uranyl formate (Leberman, 1965) were used as negative stains. Some preparations were mounted on collodion-coated copper grids and shadowed with an alloy of 80% platinum + 20% iridium at an angle of 35 degrees. Purified preparations of the virus were fractionated by centrifugation in sucrose density gradients (Gibbs *et al.* 1966). Each gradient was displaced from its tube by pumping dense sucrose solution

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(500 g./l.) into the bottom of the tube. The displaced liquid was passed through an LKB Uvicord flow absorptiometer (connected to a chart-recorder to produce an absorption diagram of the gradient in 254 m $\mu$  wavelength light), and collected in approximately 0.5 ml. samples, which, by comparison with the absorption diagram, could be pooled appropriately.

Infectivities of preparations were determined by injecting each of a series of dilutions of each preparation into groups of bees (1  $\mu$ l./bee), which were then kept in small cages at 35°, and deaths recorded.

## RESULTS

### *Virus purification*

Paralysed bees were triturated in a mixture of tapwater (1 ml./bee) and either carbon tetrachloride (0.25 ml./bee), or ethyl ether (0.25 ml./bee) followed by carbon tetrachloride (0.25 ml./bee) (Wetter, 1960), or a mixture of equal volumes of chloroform and *n*-butanol (1 ml. mixture/bee) (Steere, 1956). The resulting emulsions were centrifuged at 8000 g for 10 min. and the aqueous phase of each removed and tested. Extracts clarified with chloroform + butanol were much less infective than those made with other chemicals (LD 50 dilution 10<sup>-5</sup> to 10<sup>-6</sup>).

Separate but comparable extracts clarified with carbon tetrachloride, with or without ether pretreatment, were purified and concentrated by one cycle of differential centrifugation (1.35  $\times$  10<sup>7</sup> g min. and 8  $\times$  10<sup>4</sup> g min.). The preparation made with carbon tetrachloride alone contained few particles, and sedimenting the virus from the clarified extract into a pellet of either Celite (Johns Manville & Co., London) or 600-mesh carborundum powder did not affect the final yield. (Plain water extract, which was not readily clarified, gave the same result.) The purest and most concentrated preparation was obtained from the extract treated with ether and carbon tetrachloride; a preparation containing the equivalent of 50 bees/ml. contained about 10<sup>12</sup> particles/ml. Extracts made this way from heads of paralysed bees contained ten times as many particles as similar extracts of the same weight of whole bees.

Chronic bee-paralysis virus was precipitated when saturated ammonium sulphate solution was added to ether-carbon tetrachloride extracts of paralysed bees. The virus was fully and reversibly precipitated when the extracts were half saturated with ammonium sulphate, whereas no particles were precipitated from one-third saturated extracts, and the precipitate from two-thirds saturated extracts yielded few particles and much amorphous material. Virus preparations were made by half saturating ether-carbon tetrachloride extracts with ammonium sulphate, collecting the precipitate by centrifugation, and dialysing the resuspended precipitate to remove ammonium sulphate. These preparations were as pure as those prepared by differential centrifugation. They contained most of the particles and 10% or more of the infectivity of the extracts from which they had been prepared. Virus particles could also be precipitated from ether-carbon tetrachloride extracts by adding 10 to 20% of acetone at 18° but the precipitate yielded only a very small proportion of the original virus when it was resuspended.

### *Properties of the virus*

*Electron microscopy.* Purified preparations of chronic bee-paralysis virus contained many irregularly shaped particles (Pl. 1, fig. 1, 2) most of which were approximately ellipsoidal in outline whether shadowcast or mounted in uranyl formate, uranyl

acetate, or sodium phosphotungstate negative stains. None of the particles was penetrated by these negative stains, or showed substructure or an obvious pattern on its surface, though many had a small irregular protuberance at one end (Pl. 1, fig. 3). Particles mounted in sodium phosphotungstate had a modal width of 220 Å and most were about 410 Å, 540 Å, or 640 Å long. The shortest particles were the most numerous in all preparations; about 55% of the particles in one preparation were 350 to 500 Å long, 25%, 500 to 600 Å long and 20%, 600 to 750 Å long. In shadow cast preparations some particles cast long shadows, whereas others did not and seemed to be flattened to various extents (Pl. 1, fig. 4). This is perhaps the reason for some, at least, of the variation in shape and size of the particles.

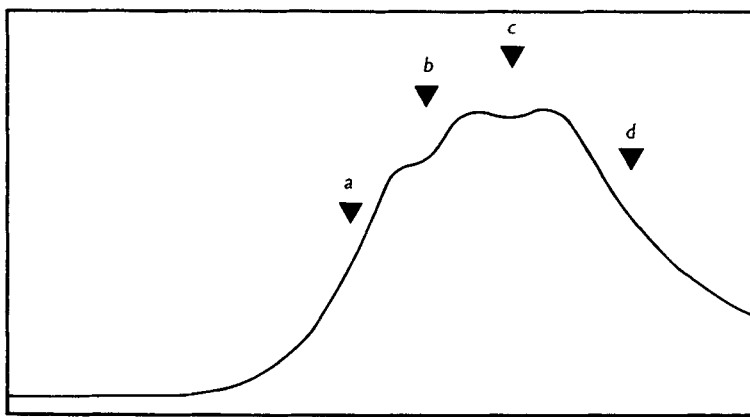


Fig. 1. Components of chronic bee-paralysis virus separated by centrifuging in sucrose density gradient; optical density diagram of gradient was obtained using 254 m $\mu$  wavelength light. Vertical axis, optical density; virus sedimenting from left to right. Pooled fractions between *a* and *b*, top component; between *b* and *c*, middle component; and between *c* and *d*, bottom component.

*Centrifugation.* Centrifuging in the analytical centrifuge (Pl. 1, fig. 5), or in sucrose density gradients (Fig. 1) resolved the chronic bee-paralysis virus preparations into three components, which, in order of increasing sedimentation rate, we call the top, middle and bottom components. Samples of the three components taken from sucrose density gradients corresponded to the three sizes of particles seen in electron micrographs (Fig. 2; Pl. 1, fig. 1, 2; Table 1). Attempts to fractionate the samples from density gradients by a second cycle of centrifugation in density gradients usually failed, because the fractions obtained contained only small amounts of virus with very little infectivity (whether they were tested separately or recombined). We examined preparations made from bees from two separate colonies, from artificially infected bees, from whole bees, from bees' heads, and from bees injected with terminal dilutions of the bottom component only. Regardless of their source and whether made by differential centrifugation or by precipitation with ammonium sulphate, all preparations separated into these components which always occurred in the same relative proportions.

*Chemical analysis.* No precipitate formed in chronic bee-paralysis virus preparations when they were brought to N-KOH or N-HCl. However, electron microscopy of

preparations so treated showed that the particles were penetrated by the negative stain, and seemed to be empty 'shells' (Pl. I, fig. 6, 7). 'Shells', prepared from virus particles kept in  $N$ -KOH for 18 hr at  $18^\circ$  and then dialysed exhaustively against 0.01 M-borate buffer (pH 8.0), precipitated at pH values of 6.0 and less. They remained separate in neutral phosphotungstate but clumped in uranyl formate which is acid

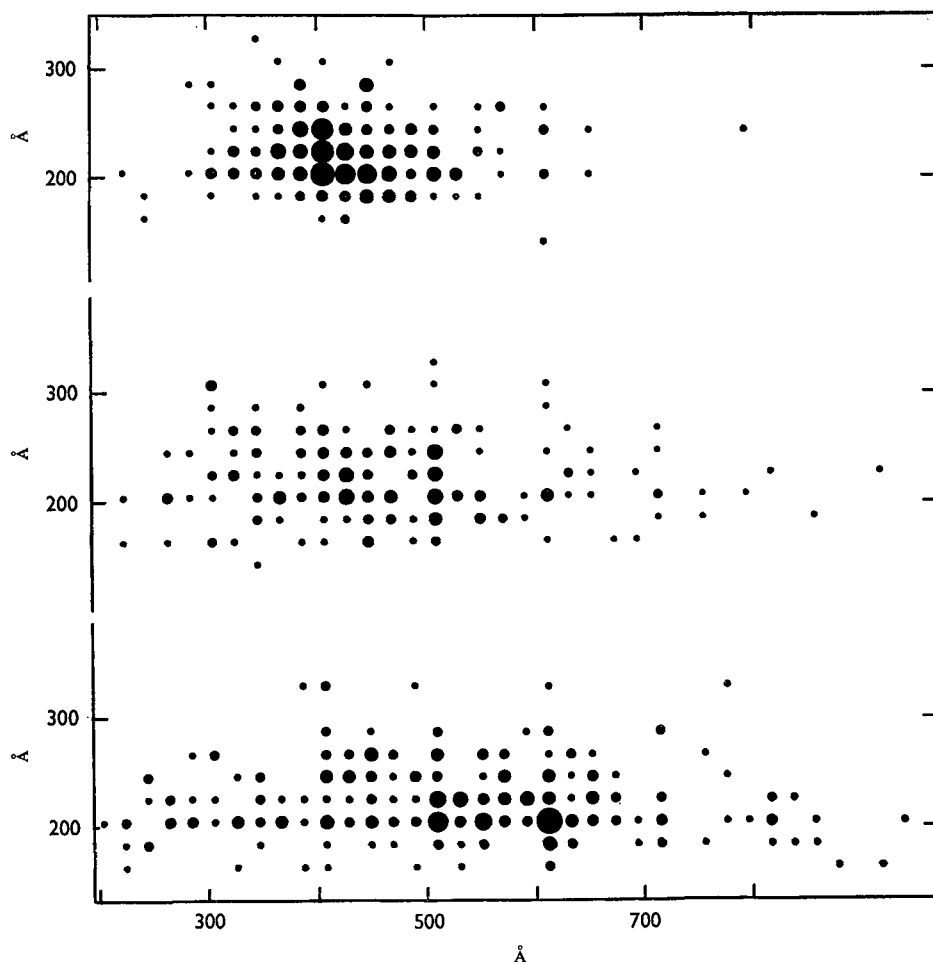


Fig. 2. Numbers of particles of different sizes in top component, middle component and bottom component (pooled fractions); see Fig. 1. The greatest linear dimension of each particle is called its length, the width is measured at right angles to this. The area of each spot is proportional to the number of particles of that size.

(Pl. I, fig. 6, 7). They were more rounded than particles in untreated preparations, and were 200 to 300 Å  $\times$  200 to 500 Å along axes at right angles (Fig. 3), with the shell wall 30 to 40 Å thick in preparations mounted in phosphotungstate and 40 to 50 Å thick in those mounted in uranyl formate. Some were obviously broken, but none showed any substructure when mounted in either of the three negative stains used, whether fixed with formaldehyde (1% for 30 min.) or not. The 'shells' were resolved into three components in the analytical centrifuge with sedimentation coefficients of

Table 1. *Properties and amounts of the components of chronic bee-paralysis virus preparations*

	Component*		
	Top	Middle	Bottom
Sedimentation coefficient ( $S_{20, w}$ )	97	110	125
Modal length of particles (Å)	410	540	640
Amount of component estimated from			
(a) Dimensions and numbers of particles† (%)	46	28	26
(b) Areas in schlieren diagram (Pl. 1, fig. 5) (%)	44	31	25
(c) Areas in u.v. absorption diagrams (Fig. 1) (%)	27	31	42
Ratio $a/b$ ‡	0.6	1.0	1.7
Infectivity§: Experiment 1 (%)	7	70	23
Experiment 2 (%)	8	31	61

\* Band or boundary formed when centrifuged in sucrose density gradient or analytical centrifuge respectively.

† Numbers of particles in different size categories (components) were estimated in electron micrographs; volumes of particles were calculated assuming them to be prolate spheroids.

‡ This ratio is related to the nucleic acid content of the component.

§ Calculated from the LD 50 concentrations.

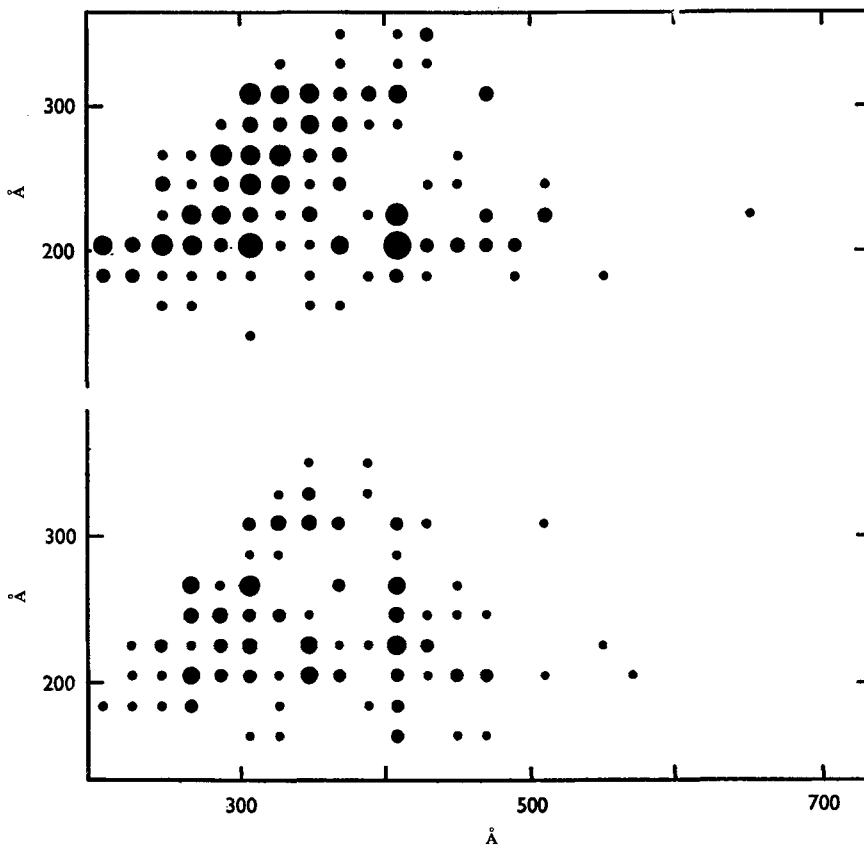


Fig. 3. Numbers of 'shells' of different sizes in alkali-treated preparations mounted in phosphotungstate (above) or uranyl formate (below) negative stains.

62, 65 and 71 *S* (Pl. 1, fig. 5); the middle component had the greatest area in the schlieren diagram, and the fastest moving component the smallest. The 'shells' prepared with alkali had the absorption spectrum of protein rather than nucleo-protein (Fig. 4), and had an optical density in 260  $m\mu$  wavelength light about one quarter that of the preparation from which they had been made.

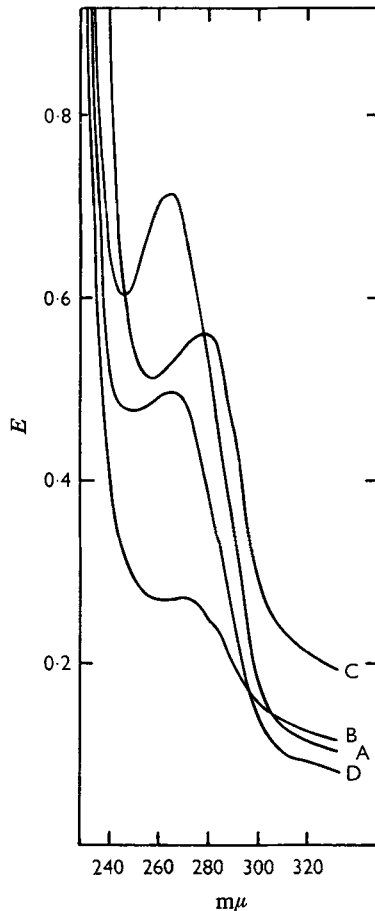


Fig. 4. Ultraviolet absorption spectra: A, purified chronic bee-paralysis virus preparation; B, 'bee protein' (< 30 *S*); D, the virus components obtained by fractionating A in sucrose density gradients (both B and D diluted 1/3); C, 'shells' prepared from A using alkali (C undiluted). Spectra were measured in an Optica recording spectrophotometer.

Perchloric acid, added to neutralize and precipitate the alkali in a virus preparation kept in *N*-KOH for 18 hr at 18°, also precipitated the virus protein. The supernatant fluid and washings of the precipitate were combined, concentrated by drying, and analysed chromatographically using an isopropanol + water (7:3, v/v) solvent in an ammonia atmosphere. All the material extracted from the virus that absorbed ultraviolet light migrated on the chromatogram, and resolved into two spots ( $R_f$  values about 0.2 and 0.4), indicating that the virus contained only ribose nucleic acid; deoxyribose nucleic acid would not have been hydrolysed by the alkali treatment and

would not have migrated in the chromatogram. The molar ratio of the bases in the nucleic acid of chronic bee-paralysis virus was estimated by a chromatographic method similar to that described by Markham (1955), using a tertiary butanol + HCl solvent. A total of seven estimates, using two separately made preparations purified by centrifugation in sucrose density gradients, gave the results:

$$G, 20.2 \pm 0.9; \quad A, 24.1 \pm 0.3; \quad C, 28.2 \pm 0.9; \quad U, 27.5 \pm 0.7$$

Analyses of the nucleic acid content of the virus were inconclusive. Chemical estimates of the nitrogen content of two preparations, and spectrophotometric estimates of their nucleic acid after hydrolysis, suggested they contained 29 and 34% nucleic acid. However, samples of the components of chronic bee-paralysis virus preparations from sucrose density gradients had ratios of optical densities at wavelengths 260 and 280  $m\mu$  ranging from about 1.45 to 1.48 (top component) and 1.53 to 1.56 (bottom component), which are equivalent (Paul, 1959), respectively, to a 10 to 20% nucleic acid content.

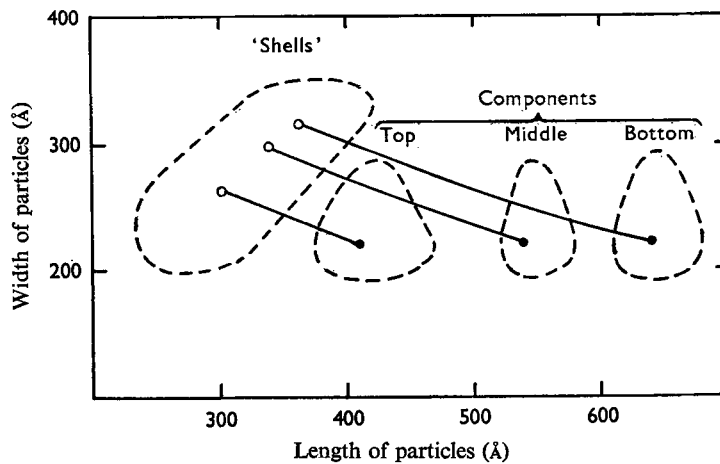


Fig. 5. Diagram to illustrate that the intact virus particles (black discs indicate modal dimensions) could become the empty 'shell' particles (open circles indicate possible dimensions) by a change of shape when treated with alkali. Each unbroken line gives the dimensions of prolate spheroids of the same surface area. Broken lines indicate the range of sizes of particles found; variation may be caused by flattening.

*Serological tests.* In micro-Ouchterlony gel-diffusion tests (Mansi, 1958) chronic bee-paralysis virus preparations made by centrifugation gave two bands of precipitate when tested with homologous antiserum, whereas the separated component particles from such preparations each produced a single band of precipitate; in suitably designed tests, the bands produced by these components were completely confluent. The second band of precipitate given by unfractionated virus preparations was apparently produced by a small molecular weight antigen from the bees, because it was confluent with the single band of precipitate produced by extracts of apparently healthy bees; was confluent with the single band produced by slow sedimenting material ( $< 30S$ ) from crude virus preparations; formed nearer the antiserum well than the band of precipitate produced by the separated virus component; was not produced by preparations that had been precipitated with ammonium sulphate; and was not produced after the

antiserum has been 'absorbed' by diluting it with 7 volumes of an ether-carbon tetrachloride extract of whole apparently healthy bees (10 bees/ml. of 0.85% NaCl solution). Extracts of the heads of paralysed bees contained at least eight times as much antigen as extracts made from an equal weight of the thoraces and abdomens of the same bees. A clear and diagnostic band of precipitate was obtained using absorbed antiserum and an extract made from the head of a single paralysed bee (one head ground in 0.05 ml. saline and 0.02 ml. ether). In tests with unabsorbed antisera, extracts of heads gave a much fainter 'bee antigen' band of precipitate than extracts of whole bees. However, the 'bee antigen' was immunogenic even when very dilute, for an antiserum prepared by injecting rabbits with chronic bee-paralysis virus extracted from heads and purified by ammonium sulphate precipitation still reacted strongly with 'bee antigen'.

In precipitation tests in tubes, unabsorbed chronic bee-paralysis virus antiserum had a titre of 1/32 against either extracts of healthy bees or the slow sedimenting material from unfractionated virus preparations, whereas the titre against virus fractions, regardless of whether these contained mainly top or bottom component, was 1/512. The titre of the antiserum was slightly decreased when it was absorbed with an extract of healthy bees, whereas its titre in gel-diffusion tests (1/64) was unaffected.

The 'shells' produced by treating chronic bee-paralysis virus preparations with alkali were tested with the antiserum prepared against intact virus. In gel-diffusion tests, the 'shells' produced a diffuse band of precipitate (irrespective of concentration), which crossed over and was only partially confluent with the band produced by intact virus, and in tube precipitation tests the antiserum titre was only one quarter (1/128) its homologous titre.

The band of precipitate, which formed in gel diffusion plates using chronic bee-paralysis virus and its homologous antiserum, was narrowest and densest when the antigen and antiserum concentrations were such that it formed 31% of the distance from the edge of the antigen well to the edge of the antiserum well. This suggests (Polson, 1958) that the antigen had a diffusion coefficient of  $0.97 \times 10^{-7} \text{ cm.}^2 \text{ sec.}^{-1}$  at 18°, corresponding with a sphere of about 400 Å diameter or spheroid with a similar volume, and, therefore, that it was mainly composed of whole virus particles, not subunits.

#### DISCUSSION

The particles of chronic bee-paralysis virus with their elliptical shape and variable size seem unlike those of other known viruses, except perhaps those of lactic dehydrogenase virus (De Thé & Notkins, 1965). The invariable presence of three serologically indistinguishable components in the same relative proportions in all the virus preparations we examined, suggests that these components are not contaminants or artifacts. The similarity of the proportions of the components obtained in estimates from electron microscopy and from analytical centrifugation (Table 1) suggests that there was a negligible Johnston-Ogston (1946) effect in the analytical centrifuge. Further calculations from the different properties of the three components (Table 1) suggest that the proportion of nucleic acid in the particles in different components differs more than would be expected from their relative sizes, if the 'walls' of all particles are the same thickness (i.e. more of the central cavity of the long particles is filled with nucleic acid than in the short particles). If this is so, the relationship between the different



sizes of chronic bee-paralysis virus particles and their nucleic acid contents differs from that found with, for example, tobacco rattle virus (Harrison & Nixon, 1959), bean pod mottle virus (Bancroft, 1962) or alfalfa mosaic virus (Bancroft & Kaesberg, 1960; Kelley & Kaesberg, 1962).

Estimates of the nucleic acid content of the virus differed. Chemical analysis of two unfractionated preparations gave an average content of about 30%, whereas measurements of the optical density of the separated virus components in ultraviolet light suggested a nucleic acid content between 10% (top) and 20% (bottom). The relative sedimentation coefficients of the intact virus and 'shell' components suggest (Reichmann, 1965) nucleic acid contents of 22% (top), 26% (middle) and 28% (bottom). However, it is unlikely that intact virus particles were converted to 'shells' simply by loss of nucleic acid and change of shape (Fig. 5), because the relative amounts of the three components also changed (Pl. I, fig. 5).

The sedimentation coefficients of the intact chronic bee-paralysis virus particles are small compared with those of other virus particles of the same size, and this suggests they are more than usually hydrated.

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#### EXPLANATION OF PLATE

- Fig. 1. Electron micrograph of chronic bee-paralysis virus top component in phosphotungstate.
- Fig. 2. Electron micrograph of chronic bee-paralysis virus bottom component in phosphotungstate.
- Fig. 3. Some chronic bee-paralysis virus particles with a protuberance at one end.
- Fig. 4. Electron micrograph of shadowcast chronic bee-paralysis virus preparation.
- Fig. 5. Schlieren diagram of centrifugal analysis of a chronic bee-paralysis virus preparation (bottom) and the alkali-treated preparation (top) made from it.
- Fig. 6. Alkali-treated chronic bee-paralysis virus preparation mounted in phosphotungstate.
- Fig. 7. Alkali-treated chronic bee-paralysis virus preparation mounted in uranyl formate.

