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The Multiplication Regions and Interaction of Acute and Chronic Bee-paralysis Viruses in Adult Honey Bees

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SUMMARY

Serological and infectivity tests showed that acute bee-paralysis virus accumulated in the heads of acutely paralysed bees, especially in the hypopharyngeal glands, and that much virus also occurred in the brain, where particles resembling acute bee-paralysis virus were made visible by electron microscopy. Similar tests showed that chronic bee-paralysis virus was concentrated in the brains of chronically paralysed bees. Electron microscopy of the brains showed particles resembling chronic bee-paralysis virus but these may have been synaptic vesicles or sectioned microtubules, since similar particles were also seen in the brains of apparently healthy bees. These particles also resembled particles that were seen in sections of pellets of purified chronic bee-paralysis virus, and that were electron-transparent in the centre.

Many bees injected with acute bee-paralysis virus and kept at 35° remained apparently healthy though they contained at least as much virus as bees injected with acute bee-paralysis virus and kept at 30°, all of which died of acute paralysis. Conversely, chronic bee-paralysis virus multiplied more at 30° than at 35°, though it killed bees more slowly at the lower temperature. When acute bee-paralysis virus and chronic bee-paralysis virus were injected together into single bees, acute bee-paralysis virus multiplication was depressed at 35° and chronic bee-paralysis virus multiplication was depressed at 30°.

INTRODUCTION

By infectivity assay, Bailey & Gibbs (1964) found little difference in the concentration of acute bee-paralysis virus in different parts of the bodies of paralysed bees previously fed with the virus. The serological titre of acute bee-paralysis virus was also much the same in various parts of bees that had been injected with the virus. The corpora pedunculata ('mushroom bodies') of the brains of acutely paralysed bees appeared abnormal by light microscopy and this seemed possibly associated with brain infection and the death of bees very soon after they show symptoms. Furgala & Lee (1966), however, using electron microscopy, examined sections of moribund bees infected with acute bee-paralysis virus and saw acute bee-paralysis virus-like particles only in the fat-body, not in the midgut, thoracic muscles, thoracic and abdominal ganglia and brain.

Infectivity assays suggested that the heads of bees naturally infected with chronic bee-paralysis virus contained about 100 times more virus per unit fresh weight than did thoraces, eviscerated abdomens or midguts (Bailey, 1965*a*) and serological assays suggested about ten times more virus per unit fresh weight of the head than of whole

bees (Bailey, Gibbs & Woods, 1968). Therefore, this virus may accumulate largely in the brain, which forms much of the head tissue, though this appeared normal by light microscopy. Electron microscopy of sections also indicated that chronic bee-paralysis virus may be neurotropic because Lee & Furgala (1965) found that thoracic and abdominal ganglia of chronically paralysed bees contained chronic bee-paralysis virus-like particles whereas the fat-body and thoracic muscles did not. However, the identification and distribution of the particles described by Lee & Furgala (1965) in ganglia of chronically paralysed bees is in some doubt for two reasons: (*a*) sections of synaptic vesicles, which occur in normal nervous tissue, somewhat resemble these particles, and (*b*) their centres are transparent to electrons, which is unusual for a virus particle positively stained with uranyl acetate.

For these reasons, we studied the multiplication of acute bee-paralysis virus and chronic bee-paralysis virus in more detail and their interaction in individual bees.

METHODS

Virus stocks. All our colonies of bees seem to contain acute bee-paralysis virus without showing symptoms and apparently healthy bees are the only known natural source of the virus. Bees become acutely paralysed when injected with concentrated extracts of other bees (Bailey, Gibbs & Woods, 1963) and in this way a laboratory source was established. Stocks of bees killed by acute bee-paralysis virus were preserved at -20° and fresh material was prepared by injecting apparently healthy bees each with the equivalent of 10^{-8} of a killed bee and then incubating the injected bees at 30°. Similar attempts to maintain laboratory stocks of chronic bee-paralysis virus by serial injection often led to the bees developing acute paralysis. Subsequent tests by infectivity, serology and electron microscopy showed that acute bee-paralysis virus invariably occurred in chronic bee-paralysis virus preparations from laboratory maintained stocks, but was undetectable in naturally paralysed bees. Fresh chronically paralysed bees were obtained, therefore, by injecting apparently healthy bees each with the equivalent of 10^{-7} of a naturally paralysed bee from some collected from one source in the field and preserved at -20°.

Isolation of tissue. Brains and hypopharyngeal glands were dissected from heads of bees held under a solution of 0·85% NaCl in water. The glands were removed easily, free from other tissues. All tissues adhering to the brain, except the finest tracheae, were removed with fine forceps. The brains and glands were washed in several changes of saline.

Tissue infectivity. Weighed amounts of tissue were triturated in known volumes of a 4:1 mixture of water and carbon tetrachloride. The extracts were clarified by centrifugation at 8000 **g** for 10 min., suitably diluted, and 1 μl. injected into each bee as described by Bailey *et al.* (1963).

Serology. Tissues were extracted in 0·85% saline + ethyl ether (Bailey *et al.* 1968). Individual heads, brains, and hypopharyngeal glands were ground with a glass rod in 1 drop of ether in a small conical centrifuge tube, and 0·05 ml. saline added. Pooled samples of heads, thoraces and abdomens were extracted in 5 parts by weight of saline + $\frac{1}{4}$ vol. of ether followed by $\frac{1}{4}$ vol. of CCl_4 and cleared by centrifuging at 8000 **g** for 10 min. The emulsified tissue, or clarified extracts and dilutions of these in saline, were tested by micro-immunoprecipitation-in-gel (Mansi, 1958) with antiserum

prepared in rabbits against purified chronic bee-paralysis virus or acute bee-paralysis virus (Bailey, 1965*a*; Bailey & Gibbs, 1964) and absorbed with 7 vol. clarified saline-ether-carbon tetrachloride extracts of apparently healthy bees (10 bees/ml. saline) (Bailey *et al.* 1968).

Electron microscopy. Pieces of brain were fixed in cold cacodylate-buffered 5% glutaraldehyde at pH 7 for 2 hr, drained and transferred without washing to cold 1% osmium tetroxide in veronal buffer at pH 7 for a further 4 hr. Both fixatives contained 0·1 M-sucrose, 0·01 M-$CaCl_2$ and 0·01 M-$MgCl_2$. After a wash in 50% acetone at room temperature, tissues were transferred to 70% acetone saturated with uranyl acetate, in which they remained overnight. Dehydration in 100% acetone was followed by embedding in Epon (Luft, 1961) and sectioning on a Reichert Om U2 ultramicrotome with glass knives. Sections were collected on unfilmed 400 mesh grids, stained in the Reynolds lead citrate, and examined in a Siemens Elmiskop I electron microscope at 60 kv. Thicker sections were taken from the same blocks, mounted in immersion oil and examined by phase contrast in the light microscope.

Pellets of purified chronic bee-paralysis virus (Bailey *et al.* 1968) were processed in the same way as the tissue.

RESULTS

Infectivity and serology

Extracts of brains from acutely or chronically paralysed bees were very infectious (Table 1). Precipitation-in-gel tests were made with acute bee-paralysis virus or chronic bee-paralysis virus antiserum on extracts of individual heads, brains and pairs of

Table 1. *Infectivity of extracts of brain tissue from bees with acute and chronic paralysis*

Source	Tissue	Infectivity*
	Acute paralysis	
Stock 1	6 whole brains	7
Stock 2	10 mid-brains	8
	10 optic lobes	8
	10 sub-oesophageal ganglia	8
Stock 3	6 whole brains	7
	5 mid-brains less mushroom bodies	5
	20 mushroom bodies	5
Water-injected bees	6 whole brains	<2
	Chronic paralysis	
Stock 1	6 whole brains	8
Stock 2	6 whole brains	8
Stock 3	5 mid-brains	7
	5 suboesophageal ganglia	7
	5 optic lobes	7

* Logarithm of the number of LD50 doses by injection per mg. wet weight of tissue.

hypopharyngeal glands of bees injected with acute bee-paralysis virus or chronic bee-paralysis virus (Table 2). Reactions were specific for the homologous antisera (Pl. 1 *a*). It should be noted that a positive result indicates the presence of much antigen,

but a negative result does not necessarily imply absence of all antigen, because more than 10^{10} particles per ml. are needed to give a visible reaction.

Quantitative serological tests were made on parts of bees that were killed by injection with either acute bee-paralysis virus or chronic bee-paralysis virus, or both (Table 3, 4).

Table 2. *Serological tests for acute bee-paralysis virus and chronic bee-paralysis virus in head tissues of bees with acute and chronic paralysis respectively*

Part of bee	Dilution* (w/w)	Proportion of positive results: Acute paralysis	Chronic paralysis
Individual whole heads	1/6	8/9	6/6
Individual brains	1/20	0/10	8/8
Pairs of hypopharyngeal glands	1/48	5/16	0/10

* Each part extracted in 0·05 ml. saline and tested against 1/8 antiserum by immuno-diffusion.

Table 3. *Serological titre* of acute bee-paralysis virus and chronic bee-paralysis virus in parts of bees with acute and chronic paralysis respectively*

Part of bee	Acute paralysis	Chronic paralysis
Heads	24	48
Thoraces	<6	12
Abdomens	<6	6

* Reciprocal of the dilution end-point (w/w) of antigen in a pooled sample of 10 parts tested as in Table 1.

Table 4. *Serological titre* of the virus concentration in heads of bees killed by acute bee-paralysis virus or chronic bee-paralysis virus or a mixed inoculum at* 30° *and* 35°

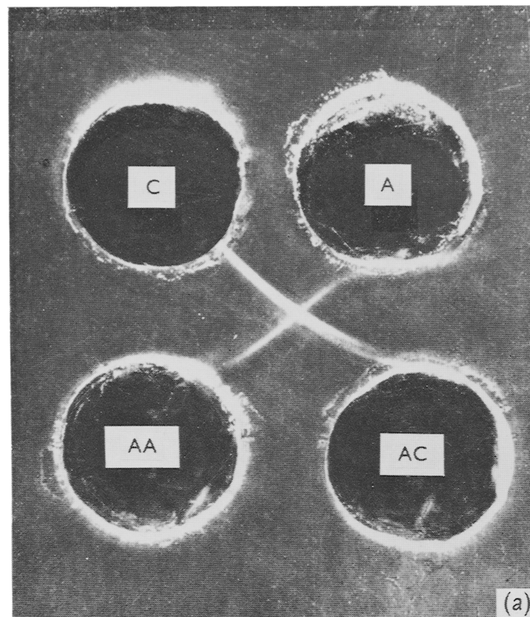
Inoculum†	Antiserum	30°	35°
ABPV	ABPV	31·9 (6; 5·0)‡	33·0 (4; 9·0)
ABPV+CBPV	ABPV	33·6 (5; 5·8)	8·4 (5; 2·4)
CBPV	CBPV	96·0 (4; 0)	42·0 (4; 6·0)
CBPV+ABPV	CBPV	36·0 (4; 6·9)	42·0 (4; 6·0)

* As in Table 3.

† 10^{-8} or 10^{-7} of the equivalent of one bee killed by acute bee-paralysis virus or chronic bee-paralysis virus respectively injected into each bee.

‡ Figures in parentheses indicate the number of observations and the standard error of the mean.

Half the injected bees died 6 or 7 days later except those injected with acute bee-paralysis virus at 35°, half of which died after 11 days. As previously observed (Bailey, 1965*b*), chronically paralysed bees died about a day sooner at 35° than at 30°; but chronic bee-paralysis virus alone multiplied more at 30° than at 35° ($P < 0{\cdot}001$) (Table 4). The amount of acute bee-paralysis virus in dead bees injected with it alone was the same at 30° and 35°, but further tests showed that the mean serological titre of acute bee-paralysis virus in samples of 10 heads of bees kept at 35° for 7 to 21 days after they were injected, but still seemingly normal was 48·0 (8 observations; standard error 7·8). This was at least as much as in bees killed by acute bee-paralysis virus at 30° or 35° (Table 4); moreover, the titre in the live bees increased steadily to 96, the greatest



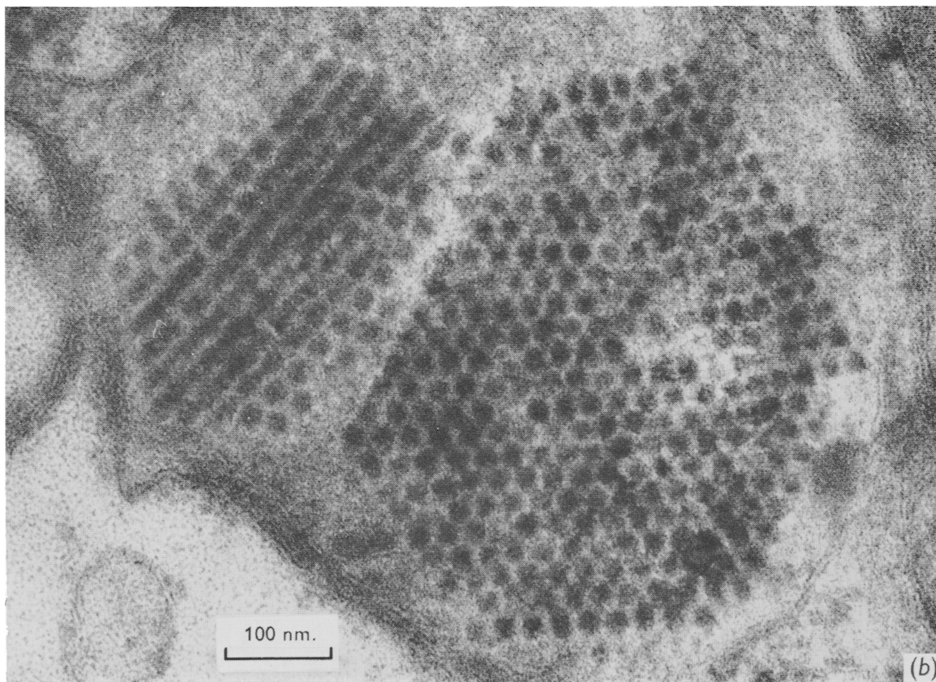


Plate 1. *a* Precipitin lines from extracts of whole heads of bees with acute paralysis (A) and chronic paralysis (C) against their homologous antisera (AA and AC) absorbed with an extract of healthy bees.

b Crystalline aggregates of particles in the midbrain of an acutely paralysed bee.

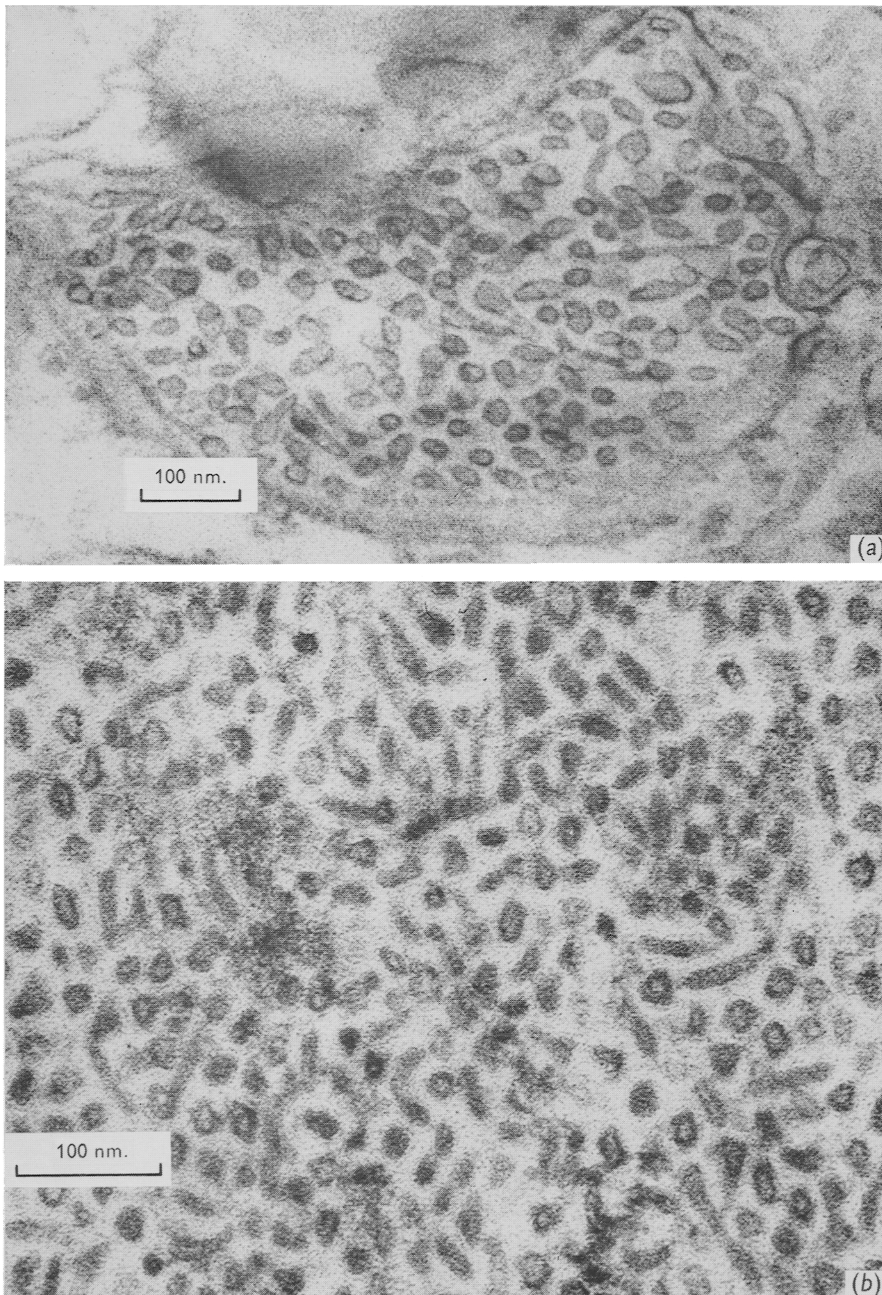


Plate 2. *a* Particles resembling chronic bee-paralysis virus in the midbrain of a chronically paralysed bee. The appearance of synaptic vesicles, found in healthy brains, is similar.

b Pellet of purified chronic bee-paralysis virus, fixed, sectioned and stained with uranyl acetate and lead citrate. Some particles have a central electron-transparent region which may be artifact or may represent a hollow or a protein core. The outer protein capsid is not stained.

obtained, after 21 days. Thus each virus multiplied most at the temperature at which the injected bees lived longest.

When both viruses were injected into each bee, chronic bee-paralysis virus did not interfere with the multiplication of acute bee-paralysis virus at 30°, but it multiplied less than when alone ($P = 0{\cdot}001$); by contrast, at 35° chronic bee-paralysis virus was unaffected by the presence of acute bee-paralysis virus but depressed the multiplication of acute bee-paralysis virus ($P = 0{\cdot}02$) (Table 4).

Electron microscopy

Electron microscopy of sections of the midbrain of acutely paralysed bees showed occasional crystalline arrays of dense particles 25 nm. in diameter (Pl. 1 *b*). Mushroom bodies of acutely paralysed bees contained more crystals of similar particles, though the crystals were smaller and less regular, and also many similar, dispersed particles. Neither crystalline groups nor individual particles like these were seen in midbrains or mushroom bodies of normal or chronically paralysed bees.

Sections of midbrains of chronically paralysed bees contained ovoid particles (Pl. 2*a*) resembling those in purified negatively stained preparations of chronic bee-paralysis virus. However, most particles were electron-transparent in the centre and similar particles were found in midbrains of both acutely paralysed and apparently healthy bees. Sections of sedimented purified chronic bee-paralysis virus had similar ovoid particles, many with hollow centres (Pl. 2 *b*). Some images showed a faint layer, probably representing the protein coat of the virus particles, surrounding the dense material.

DISCUSSION

Our infectivity tests showed that much acute bee-paralysis virus occurs in the brains of bees with acute paralysis and that the concentration of virus in brains is about the same as the average concentration throughout the body (Bailey *et al.* 1963). Different parts of the brain are similarly infective, but particles of acute bee-paralysis virus are very unevenly distributed within at least some of the parts, and are difficult to find by electron microscopy of tissue sections. The dense, isometric particles, 25 nm. across, seen as individuals or crystalline arrays in midbrains and mushroom bodies of acutely paralysed bees, are probably particles of acute bee-paralysis virus. Furgala & Lee (1966) did not find such particles in brains of bees injected with acute bee-paralysis virus but they may have examined parts of the brain where the virus was very sparse.

Our infectivity tests also showed that much chronic bee-paralysis virus occurs in the brains of chronically paralysed bees, but the results of electron microscopy were difficult to interpret. Sections of sedimented purified virus had some individual particles that were uniformly dense but others that were dense rings, electron transparent in the middle. After treatment with uranyl acetate and lead citrate, the nucleic acid of the particle should stain densely, so it seems that, at least after fixation and embedding, many of the particles of chronic bee-paralysis virus have their nucleic acid arrayed peripherally. These particles resemble objects occurring in normal nerve tissues, which have been called synaptic vesicles or microvesicles, or are cross-sectioned microtubules in axons. It is difficult, therefore, to decide whether the similar hollow ovoid particles in sections of brains of paralysed bees are chronic bee-paralysis virus particles. Many probably are not, because brain extracts of apparently healthy bees, which contained

similar particles, were not infective. The particles illustrated by Lee & Furgala (1965) in nerve ganglia of chronically paralysed bees may have been those of the virus, because very many were observed in closely packed groups. We saw no large aggregates of particles so are left in doubt about the distribution of chronic bee-paralysis virus in brain tissue. Although extracts of this tissue were very infective, virus in it may have been sparse because the LD50 of chronic bee-paralysis virus, like that of acute bee-paralysis virus, is only about 100 particles (Bailey *et al.* 1963).

Acute bee-paralysis virus accumulates more in the hypopharyngeal glands, and possibly other tissues of the head, than in the brain, whereas chronic bee-paralysis virus may multiply mostly in the brain. Thus, the similar titres of chronic bee-paralysis virus and acute bee-paralysis virus in the presence or absence of the other virus at 35° and 30° respectively and their lack of serological relationship suggest that they may have different multiplication sites. If so, the depressed multiplication of one virus at the appropriate temperature is not necessarily due to specific interference by the other. The unaffected virus probably kills the host before the other virus has multiplied completely.

The large amount of acute bee-paralysis virus in apparently healthy bees that had been injected with the virus and kept at 35°, compared with the amount in killed bees, shows that bees tolerate infection of most tissues and suggests that they are killed only when some small but vital region is invaded. An analogous process seems to occur at 30° with chronic bee-paralysis virus, which kills injected bees more slowly, even though more virus accumulates, than at 35°. Bailey & Gibbs (1964) found little difference between the amount of acute bee-paralysis virus in the heads, thoraces and abdomens of bees that had been injected with large doses of virus and had begun to show signs of paralysis 2 days later. After 3 days, however, there was some indication that the relative amount of virus in the heads was increasing. Acutely paralysed bees died much later after injection in our experiments than in those of Bailey & Gibbs (1964) because they were injected with less virus. This enabled much virus to accumulate in the glands and possibly other tissues of the head before vital centres were affected, especially of the bees kept at 35° that survived longest.

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