Evidence Suggesting Nonassociation of Mycoplasma With Pea Disease

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ABSTRACT

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Sixty mycoplasma isolates, with uniform colony type, were derived from five of 31 experiments using diseased pea tissue extracts as inoculum. Five of these isolates were identified serologically as *Mycoplasma gallisepticum*. The DNA of isolate Y12-5P consisted of 34.8% guanine-cytosine base composition, was 92% homologous with that of *M. gallisepticum* reference isolate S6, and constituted a genome size of 4.3-4.6 $\times$ 10^8 daltons. Isolate Y12-5P was found to be highly pathogenic to turkeys, but not to plants. Two isolates produced growth responses at three temperatures comparable to those of *M. gallisepticum* reference strain, S6. Mycoplasmalike structures visualized in diseased pea cytoplasm by ultrathin section electron microscopy were probably vesicles of host-cell membranes enclosing cytoplasm, ribosomes, and DNA-like strands. These lines of evidence suggest that the etiology of the previously reported pea disease may have been viral, that *M. gallisepticum* isolates derived in these studies originated from animal rather than plant sources, and that these isolates were introduced repeatably by an unknown means in attempts to cultivate microorganisms from diseased plant tissues.

Additional key words: isolation, pathogenicity, temperature optima, DNA homology, genome size, ultrastructural cytology.

Following the reports by Japanese workers (6, 15) that mycoplasmalike organisms were associated with four plant diseases, Oregon State University scientists discovered membrane-bound bodies in the cytoplasm of pea tissues infected by alfalfa mosaic virus, and were able to cultivate mycoplasmalike organisms when extracts of diseased pea tissue were introduced into mycoplasma-selective media (12). These membrane-bound bodies occurred profusely in cells of plants inoculated mechanically or by aphids with the supposed pathogenic complex, but were absent in healthy tissues. Believing that preliminary evidence suggested the presence of mycoplasma in infected pea tissue, plant science and veterinary research personnel at Oregon State University conducted a series of experiments attempting to isolate and identify the causal agent(s).

MATERIALS AND METHODS

The first 43 experiments (Table 1) were conducted by veterinary researchers, and the remaining 31 were conducted in two laboratories by the senior author. Although many mycoplasmalike colonies had been observed in the previously published study (12), none had been successfully established as cloned isolates. In the second and third series of experiments during 1970-74, however, 60 single-colony isolates were derived in five of 31 experiments, using RYE (rabbit-meat infusion, yeast-extract) medium (1).

The uniformity among isolates from these experiments suggested a rather specific origin of the isolates. We therefore tested the hypothesis that, irrespective of their precise role in plant pathogenesis, these isolates represented mycoplasma strains unique to plants. For this purpose, we evaluated their comparative pathogenicity in pea plants and turkeys, their temperature optima for growth, their DNA characteristics, and their relationship to membrane-bound bodies within pea parenchyma cytoplasm.

Mycoplasma isolation.—Initial isolation studies (12) (Table 1, Experiments 1-43) utilized nine types of mycoplasma-selective media commonly used among veterinary mycoplasma researchers. Success with cloning mycoplasma colonies, however, was coincident with our use of RYE medium (1).

Cultivars of several legume species *(Pisum sativum, Vicia faba, Phaseolus vulgaris,* and *Trifolium hybridum)* infected with a supposed infectious complex including alfalfa mosaic virus (618Y) (12) were sources of inoculum for mycoplasma isolation studies. *Pisum sativum* cultivar Perfected Wales was used most frequently. Leaf and stem tissues from infected plants were surface-sterilized with 1% sodium hypochlorite, rinsed four times in sterile

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phosphate-buffered saline solution (PBS), and aseptically triturated to produce an aqueous extract. Extracts from healthy plants served as inoculum controls. Two 0.1-ml aliquots of these extracts were placed into replicated 5-ml quantities of broth medium, and incubated at 25, 32, and 37 C. Tubes of noninoculated broth served as medium controls. Tubes of broth in which microbial growth was indicated by red color induction (reduction of 2, 3, 5-triphenyl tetrazolium chloride), were sub-transferred to tubes of fresh broth at 1:25 volume ratios, at 2- to 4-day intervals. Cultural series which contained either no microflora or primarily bacterial microflora, as determined by assay on mycoplasma-selective and bacteriological agar media, were discarded. Isolated mycoplasmalike colonies were aseptically withdrawn from agar plates by means of a micromanipulated capillary tube, placed into 0.2 ml portions of broth media (9), and incubated at 37 C. Such "microcultures" were sequentially transferred from broth to agar for the purpose of selecting single-colony clones of mycoplasma isolates.

A cloned, purified isolate from each of five cultural series (Table 1, Series Y7, Y12, YS3, YS4, and YS6) was selected for definitive studies. Each isolate was identified by growth inhibition serology (3) as *Mycoplasma gallisepticum*. This identity was confirmed in two other laboratories (*personal communications* from L. Hayflick and J. Tully, respectively). The characteristics of these isolates therefore were compared to those of *M. gallisepticum* reference isolates S6 and PG31.

Our attempts and those of an independent laboratory to isolate mycoplasmas from the serum component used in our RYE medium were negative; *M. gallisepticum*, however, recently has been detected in swine tissues (M. F. Barile et al., *personal communication* of *unpublished results*).

Inoculation of pea plants and turkeys.—Mycoplasma inoculum, to be used for inoculating pea plants and turkeys, was prepared by seeding active broth cultures onto mycoplasma-selective agar medium; 10 days later which the resultant colony masses were aseptically resuspended in fresh RYE broth medium. This inoculum was immediately applied to the leaves of young pea plants by mechanical abrasion or introduced aseptically into the suborbital sinuses of 12-week-old turkey poults. Groups of 25 pea seedlings, grown in isolation chambers, were abraded, respectively, with colony suspension of mycoplasma isolate Y12-5P, reference isolate S6, fresh RYE broth medium, or sterile phosphate-buffered saline. Plant inoculation experiments at Corvallis were repeated at intervals when isolate Y12-5P had been transferred in broth culture 12, 16, 21, 26, and 31 times following single-colony cloning. The volumetric dilution of Y12-5P broth cultures during 12 passages had been 5.97×10^{16}. Additional plant inoculation experiments at Corvallis (ROH) and Beltsville (RFW) were conducted when isolate Y12-5P had been broth-transferred 32-60 times.

Four turkey poults, kept in isolation cages during the 6-week test period, received sinus injections of aseptically prepared Y12-5P colony suspensions (12th-broth-passage stage), and four isolated control poults received sinus injections of sterile phosphate-buffered saline.

Determinations of temperature optima for growth.—Two of our mycoplasma isolates, Y12-5P and CP12, were tested with *M. gallisepticum* reference strain S6 for their ability to grow at three temperatures. Seventy-two-hour growth curves at 37, 32, and 25 C were determined by assaying cultures for colony-forming units. Data plotted graphically represented the average number of colony-forming units derived from three cultural assays.

Extraction and purification of DNA.—Mycoplasma cells were harvested from the culture medium, resuspended in saline-EDTA (0.15 M NaCl, 0.1 M ethylenediaminetetraacetate, pH 8.0), and lysed with the addition of 2% sodium dodecyl sulfate. Cells of *Escherichia coli* were washed with saline-EDTA and frozen prior to DNA extraction.

Deoxyribonucleic acid was purified by a modification of the technique by Marmur (18), utilizing neutralized, saline-EDTA-equilibrated phenol for deproteinization before and after ribonuclease treatment. Purity of the final DNA preparations was judged by the absorbance ratios from readings taken at 230 nm, 260 nm, and 280 nm (1-cm light path).

Deoxyribonucleic acid base composition.—Purified

TABLE 1. Use of selective media to isolate mycoplasma from tissues of legume species infected by a supposed pathogenic complex

Experiment number	Culture series	Dates	Inoculum[a]	
1 - 43	600 - 670	19 Nov 68 to 17 Sept 70	618Y in several legume species	Evaluation of nine types of selective media; microflora primarily bacteria; various pseudocolonies; occasional mycoplasmalike colonies (MLC); unsuccessful single-colony cloning or isolation.
44 - 64	Y1 - Y12	1 Nov 70 to 15 Dec 71	618Y A5 in *Pisum sativum*	MLC successfully established as mycoplasma isolates, with the use of RYE[b] medium; numerous single-colony isolations in Series Y7 and Y12.
65 - 74	YS1 - YS10	13 Oct 73 to 15 Mar 74	618Y A5 in *Pisum sativum*	Successful single-colony isolations in series YS3, YS4, and YS6[c].

[a]Inoculum consisted of sap extracts from plants mechanically inoculated with the pathogenic complexes (cultures) designated as 618Y and 618Y A5, respectively.
[b]Rabbit-meat infusion, yeast-extract medium (Al-Aubaidi, 1970).
[c]Purified isolates from each of five cultural series (Y7, Y12, YS3, YS4, and YS6) were identified by growth inhibition serology as *Mycoplasma gallisepticum*. Identification confirmed in independent laboratories.

DNA in 0.1 concentration SSC (0.15 M NaCl plus 0.15 M sodium citrate, pH 7.0) was placed in quartz cuvettes at approximately 25 μg/ml concentration and heat-denatured in an automatic recording spectrophotometer (Gilfort Instruments, Model 2000). Results were plotted as relative absorbance versus temperature, corrected for thermal expansion. The percentage G+C base composition was calculated by the methods of Mandel et al. (17).

Genome size determination.—Deoxyribonucleic acid samples being prepared for mycoplasma genome size determinations were placed in 3× (3-fold concentrated) SSC and sheared by double passage through a French press at approximately 1,020 atmospheres pressure (15,000 psi). Sheared DNA was dialyzed to equilibrium against 100-150 volumes of 3× SSC plus 20% dimethylsulfoxide (DMSO), filtered, and prepared for renaturation. The optical renaturation technique (21) for genome size determination consisted of placing sheared, dialyzed DNA samples in 3× SSC plus 20% DMSO into cuvettes (40 to 60 μg/ml concentration), subjecting them to heat denaturation, and monitoring renaturation spectrophotometrically.

Electron microscopy.—Plant tissues and mycoplasma

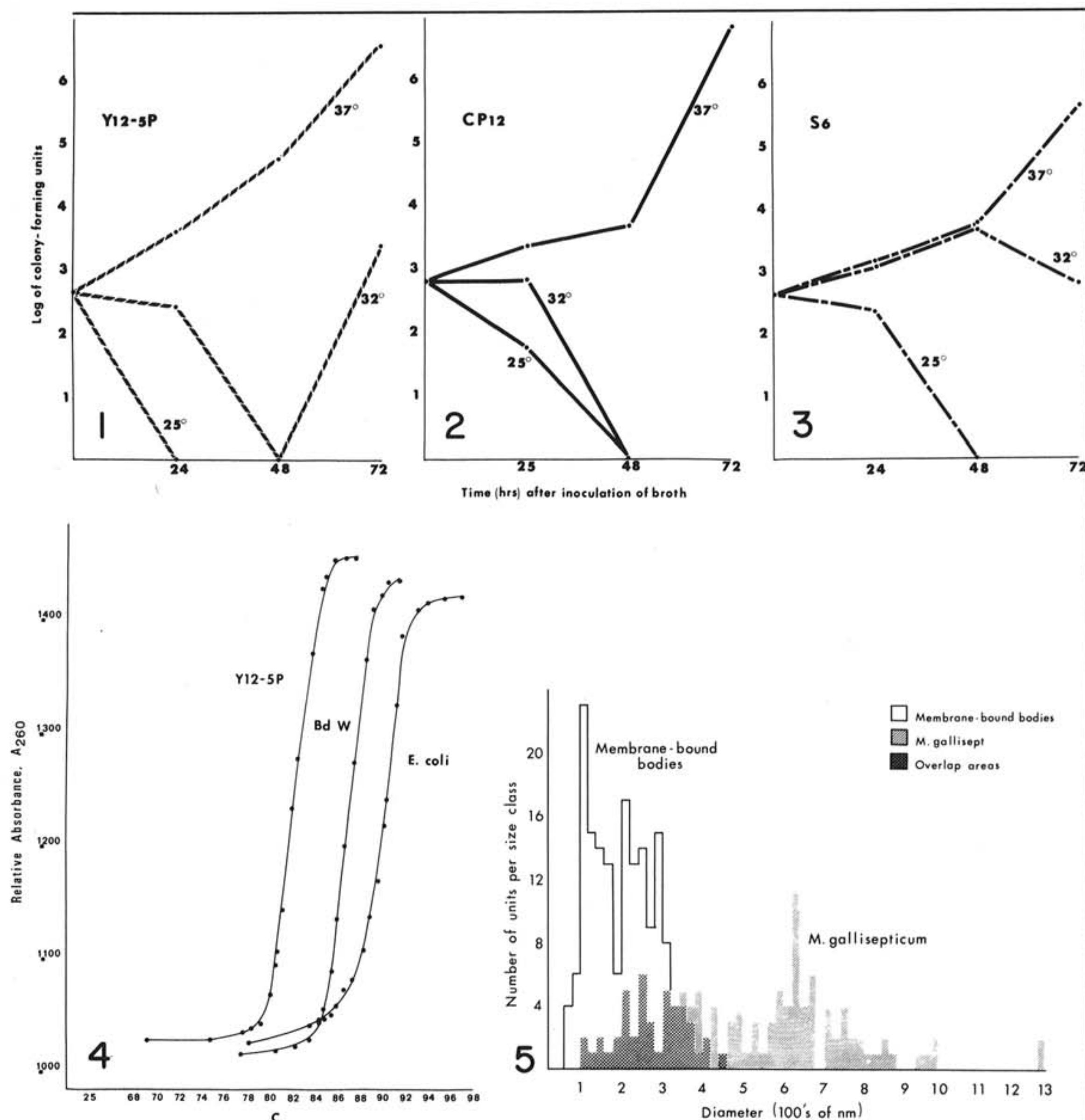

Fig. 1-5. Growth responses of *Mycoplasma gallisepticum* isolates **1)** Y12-5P, **2)** CP12, and **3)** reference strain S6. **4)** Melting curves of purified DNA from Y12-5P isolate of *M. gallisepticum,* compared to those of purified DNA from host-independent *Bdellovibrio* sp. strain W (Bd W) and from *Escherichia coli.* **5)** Histogram of size distributions of membrane-bound bodies from diseased pea *(Pisum sativum* L.) tissue and of *M. gallisepticum* cells (isolate Y12-5P).

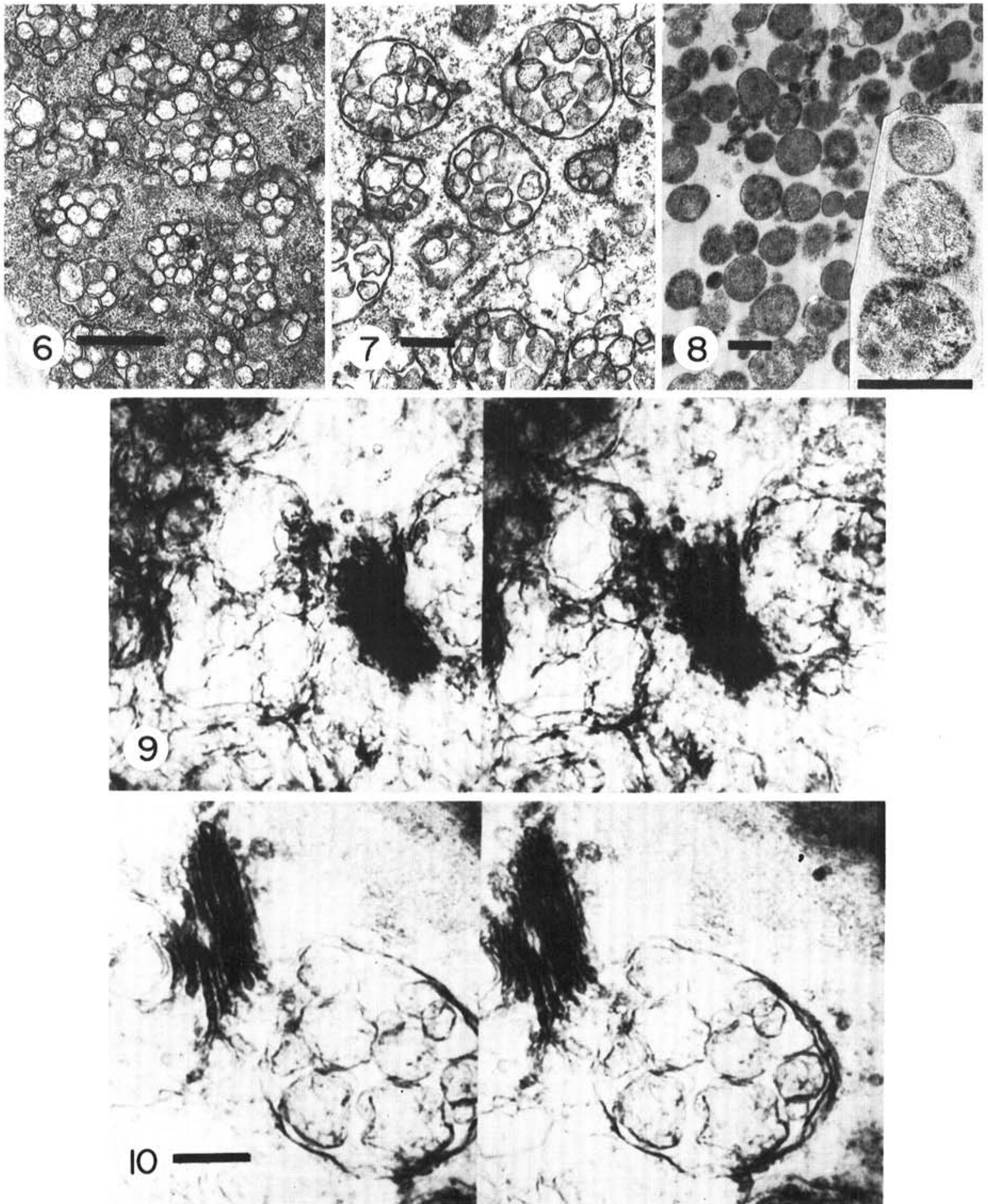

Fig. 6-10. 6-7) Membrane-bound bodies in diseased pea mesophyll cytoplasm, occurring in clusters within membrane enclosures. **8)** *Mycoplasma gallisepticum* cells within colony thin-section; inset shows exterior membrane, cytoplasm, ribosomes, and either nucleoids or DNA strands. **9-10)** Stereo-pairs of stacked serial-section-micrograph transparencies showing three-dimensional views of diseased pea mesophyll cytoplasm; note close association of membrane-bound bodies with the Golgi apparatus (densely stained), suggesting that these bodies are specialized vesicles elaborated by the Golgi apparatus; to be viewed through stereo optics. Reference bars: (6-8), 500 nm; (9-10), 250 nm.

colonies were prepared for thin-section electron microscopy by previously described procedures (11). Sections were examined with a Philips EM-300 electron microscope, operating at 60 kV. Membrane-bound bodies found within infected pea parenchyma cells were studied definitively by three-dimensional, serial section electron microscopy. For this purpose, electron photomicrographs of parenchyma serial sections were converted to positive transparencies (8, 10), stacked in serial order with plate glass spacers approximating the placement of thin sections within the tissue, and viewed by projecting a light through the three-dimensional assembly of transparencies.

RESULTS

Pathogenicity of isolate Y12-5P.—Some of the plants inoculated with colony suspensions of mycoplasma isolate Y12-5P developed symptoms in seven of 16 experiments, whereas control plants abraded with colony suspensions of reference isolate S6 or RYE broth or PBS remained symptomless. These symptoms were inconsistent, however, resembling virus-induced mosaic in some cases and growth regulator disturbances in others. More significantly, we were unable to reisolate mycoplasma from these plants, and no mycoplasmalike structures were observable in the cells of affected plants by thin-section electron microscopy.

In contrast, all turkeys injected with isolate Y12-5P had developed acute sinusitis 6 days after injection, and exhibited *M. gallisepticum*-specific agglutinating antibodies in their serum 2 weeks after injection. Control turkeys remained healthy during the 6-week test period.

Temperature optima for growth.—Isolate Y12-5P (Fig. 1) did not grow at 25 C, produced limited growth at 32 C, and grew well at 37 C. Isolate CP12 (Fig. 2) failed to grow at 32 C within the 72-hour test period. The limited growth at 32 C by *M. gallisepticum* reference strain S6 (Fig. 3) was similar to that of isolate Y12-5P. Growth optima at 37 C and limited or no growth at 32 C suggest that isolates Y12-5P and CP12 were not adapted to temperatures within greenhouse-grown plants.

Deoxyribonucleic acid characteristics.—The DNA of isolate Y12-5P was compared to that of *M. gallisepticum* reference isolates S6 and PG31. The DNA base composition (%G+C) of isolate Y12-5P, determined by the methods of Mandel et al. (17), was 34.8, closely comparable with the previously reported (16) base composition for S6 (35.2) and PG31 (35.7). The thermal melting point curves obtained for Y12-5P and two standards, are illustrated in Fig. 4.

Genome sizes of isolates Y12-5P, S6, and PG31 were determined from optical renaturation data (22), using *E. coli* as a standard. The hypochromicity curves for denatured, sheared DNA of isolates Y12-5P and PG31 were exactly superimposed, and these curves closely approximated that for DNA from isolate S6. The genome size of isolate Y12-5P was $4.3 - 4.6 \times 10^8$ daltons, very closely approximating our estimates for isolates S6 and PG31 and previously reported values (2) for the genome size of isolate PG31 ($4.9 \pm 5 \times 10^8$ daltons).

Deoxyribonucleic acid homology between isolate Y12-5P and reference isolates S6 and PG31 was estimated by renaturation kinetics, as the percent relative reassociation. Deoxyribonucleic acid from isolate Y12-5P reassociated with S6 DNA at 92% the rate of self-reassociation, and with PG31 DNA at 90% the rate of self-reassociation.

The DNA characteristics of isolate Y12-5P thus were essentially indistinguishable from those of reference isolates S6 and PG31.

Size comparison of Y12-5P cells and membrane-bound bodies.—Frequency distributions of diameter measurements of membrane-bound bodies from diseased pea tissues and cells of isolate Y12-5P (thin sections of mycoplasma colonies) were compared (Fig. 5). Membrane-bound bodies ranged from 60 to 440 nm. Eighty percent of these bodies were between 100 and 300 nm in diameter. The cell diameter of isolate Y12-5P ranged from 100 to 1,300 nm. The two structure-size populations, despite some size commonality, were statistically distinguishable.

Ultrastructural cytology of diseased pea tissues.—Parenchyma cells of diseased pea plants exhibited malformed or degraded organelles and profuse clusters of membrane-bound bodies within the cytoplasm (Fig. 6). Membrane-bound bodies consisted of an outer membrane enclosing cytoplasm, ribosomelike structures and DNA-like strands, and were in turn contained within a membrane (Fig. 7). Recent ultrastructural comparisons indicate that cells of isolate Y12-5P (Fig. 8) are more smoothly ovoid in shape and contain much denser cytoplasm than membrane-bound bodies.

Examination of membrane-bound bodies by three-dimensional electron microscopy (Fig. 9, 10) suggested a structural relationship of membrane-bound bodies to the Golgi apparatus.

Viruslike particles were not discernible in our pea tissue thin sections, although alfalfa mosaic virus was derived from infected tissue by chloroform extraction and differential centrifugation (12).

DISCUSSION

In recent years, cytoplasmic and organelle vesiculation has become a widely observed phenomenon (4, 8, 13, 14, 20). Several workers have associated vesiculation with plant virus infections (5, 7, 13, 19, 21). In the latter reports, cytoplasmic or organelle vesicles usually did not resemble procaryotic organisms and in addition were accompanied by particles representative of the viral agent. However, vesicles in pea enation mosaic virus-infected tissues observed by De Zoeten et al. (5), appeared to contain cytoplasm, ribosomelike structures and DNA-like strands, and occurred in membrane-enclosed clusters similar to those shown in Fig. 5-8.

Investigating the possible involvement of a mycoplasmalike organism, De Zoeten et al. (5) inoculated pea plants with purified viral ribonucleic acid (RNA) and found that tissues infected by pea enation mosaic virus RNA also contained these same structures. Furthermore, they found that these structures persisted in cells after infected plants had received oxytetracycline and heat treatments and concluded that they therefore were virus-induced vesicles. Strands of DNA-like material within the structures were removable from ultrathin tissue sections

by treatment with 1% DNase, but not by 0.1% DNase, 1% RNase, or water.

Our work now suggests, in agreement with the conclusions of De Zoeten et al. (5) that the membrane-bound bodies previously reported by our laboratory (12) are probably vesicles of host-cell membranes. We suggest, without definitive proof, that these membrane-bound bodies arose as vesicular elaborations from the Golgi apparatus.

These several lines of circumstantial evidence, therefore, suggest that *M. gallisepticum* was derived during our studies from animal, rather than plant sources, that this organism was introduced repeatably by unknown means during attempts to cultivate microorganisms from diseased plant tissues, and that the previously reported (12) disease of pea may have been induced solely by alfalfa mosaic virus.

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