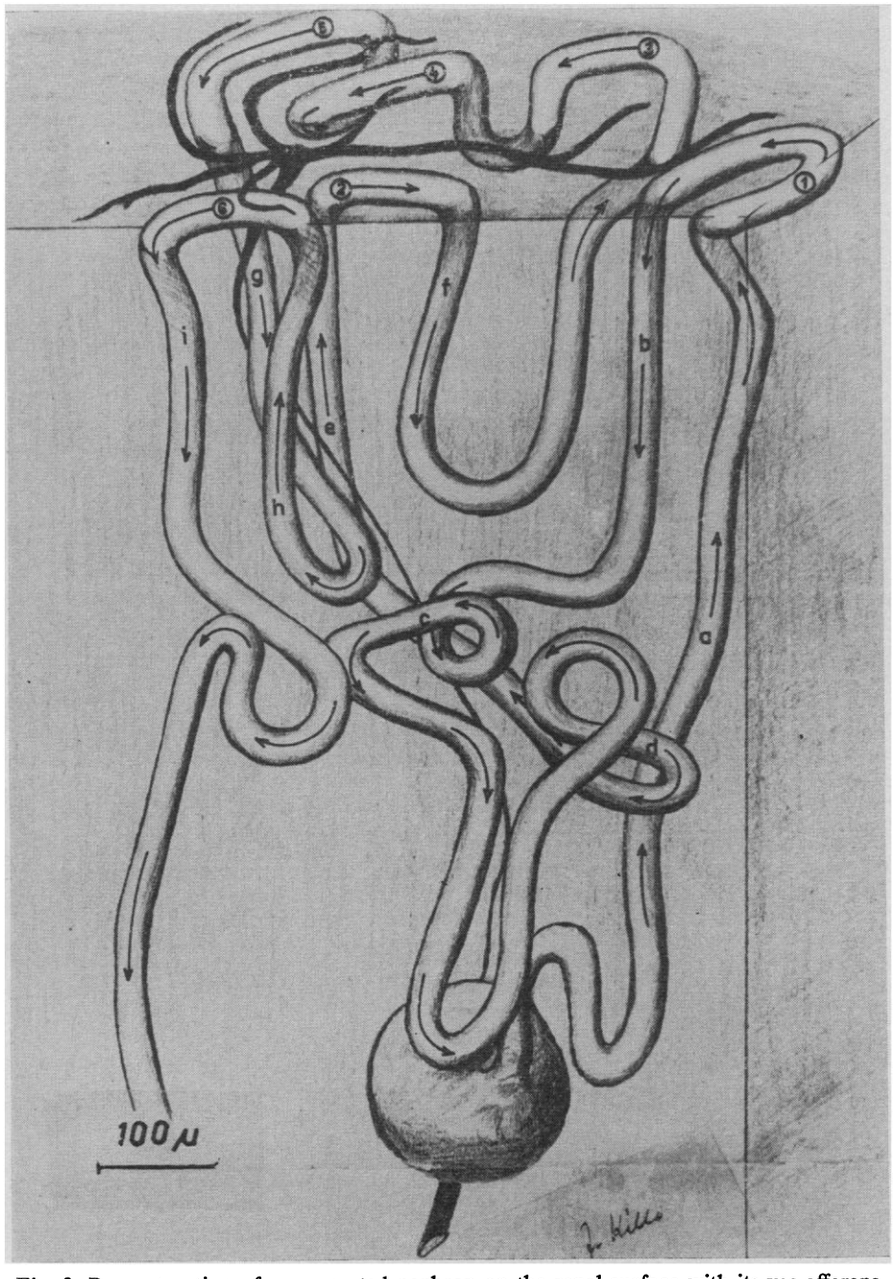

Fig. 3. Reconstruction of a macerated nephron on the renal surface with its vas afferens, glomerulus, and vas efferens after filling the arterial system and the proximal convolution with Latex. (Numbers) Number sequence of proximal loops; (letters) sequence of proximal tubular loops of the whole nephron; and (arrows) flow-direction of tubular fluid flow.

model might be useful in explaining the mechanism of the glomerular tubular balance, or, in a more detailed view, the interaction of glomerular filtration rate and the intracapillary reabsorptive capacity for the reabsorbate actively transported by proximal tubules.

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Plant-Pathogenic Mycoplasma-Like Organism: Maintenance in vitro and Transmission to Zea mays L.

Abstract. *Mycoplasma-like bodies, isolated from corn plants that were infected with Rio Grande strain of corn stunt, were maintained in an artificial cell-free medium. The agent apparently multiplied. Leafhopper vectors that were injected with the pure cultures transmitted the agent to healthy corn plants and induced the corn stunt disease.*

Japanese workers (*1*) recently proposed that yellow diseases of plants may be caused by mycoplasma-like organisms. Their evidence was based on (i) the presence of mycoplasma-like bodies in the phloem elements of infected plants and their absence in healthy plants, (ii) the therapeutic effect of tetracycline antibiotics on infected plants, and (iii) the disappearance of the mycoplasma-like bodies from the phloem of plants treated with tetracyclines. In the past 2 years, mycoplasma-like organisms have been reported as the probable etiological agents of at least 24 plant diseases (*2*, *3*).

Corn stunt, a yellows-type disease affecting corn *Zea mays* L. and teosinte *Euchlaena mexicana* L., is transmitted by several species of leafhoppers (*4*). Before 1968 the causal agent of corn stunt was considered to be a virus (*5*), but mycoplasma-like bodies have been found in both diseased corn plants and in inoculative insect vectors (*2*, *6*). Further evidence supporting the mycoplasma hypothesis of etiology came from results of chemotherapy of diseased corn plants and from blockage of vector transmission by the application of tetracycline antibiotics (*7*).

Maintenance of the agent in pure culture must be achieved before the hypothesis can be fully tested by Koch's postulates. Hampton *et al.* have reported the purification and growth in artificial medium of a mycoplasma isolated from infected pea plants *Pisum sativum*, but their results did not clearly state how Koch's postulates were fulfilled (*8*). Evidently, the tests of infectivity were conducted with a purified preparation from infected plants and not with the pure mycoplasma cultures.

We now report maintenance in vitro and evidence of growth in cell-free medium of a plant pathogenic mycoplasma-like organism, the corn stunt agent. Its original isolation, cultivation

in cell-free medium, injection into leafhopper vectors, transmission to healthy plants in which disease was induced, and its reisolation are described.

The Rio Grande strain of corn stunt (*9*) and the vector *Dalbulus elimatus* (Ball) (*4*) were used throughout the experiments. The procedure for rearing insects and maintaining infected corn plants has been described (*9*).

Initially we determined the stability of the corn stunt agent in buffer during varying lengths of time. Stem tissue from diseased plants was triturated in a protective buffer (*10*) containing 0.1*M* glycine, 0.03*M* $MgCl_2$, and 0.01*M* Na_2SO_3 at *p*H 7.3. The extract was squeezed through two layers of cheesecloth and centrifuged for 12 minutes at 5000*g*. All work was performed in a cold room or refrigerated centrifuge at 4°C. Samples of the low-speed supernatants were tested for infectivity after storage for 0, 4, 24, and 72 hours at 4° and 25°C. To assay the infectivity of each sample, 50 to 75 uninfected adult *Dalbulus elimatus* were injected. The insects were then caged in their respective groups on healthy corn plants in a controlled environment chamber (25°C, 16-hour day). Approximately 21 days after injection the surviving insects were caged singly on young corn seedlings, left to feed 7 days, and then discarded.

Insects injected with samples main-

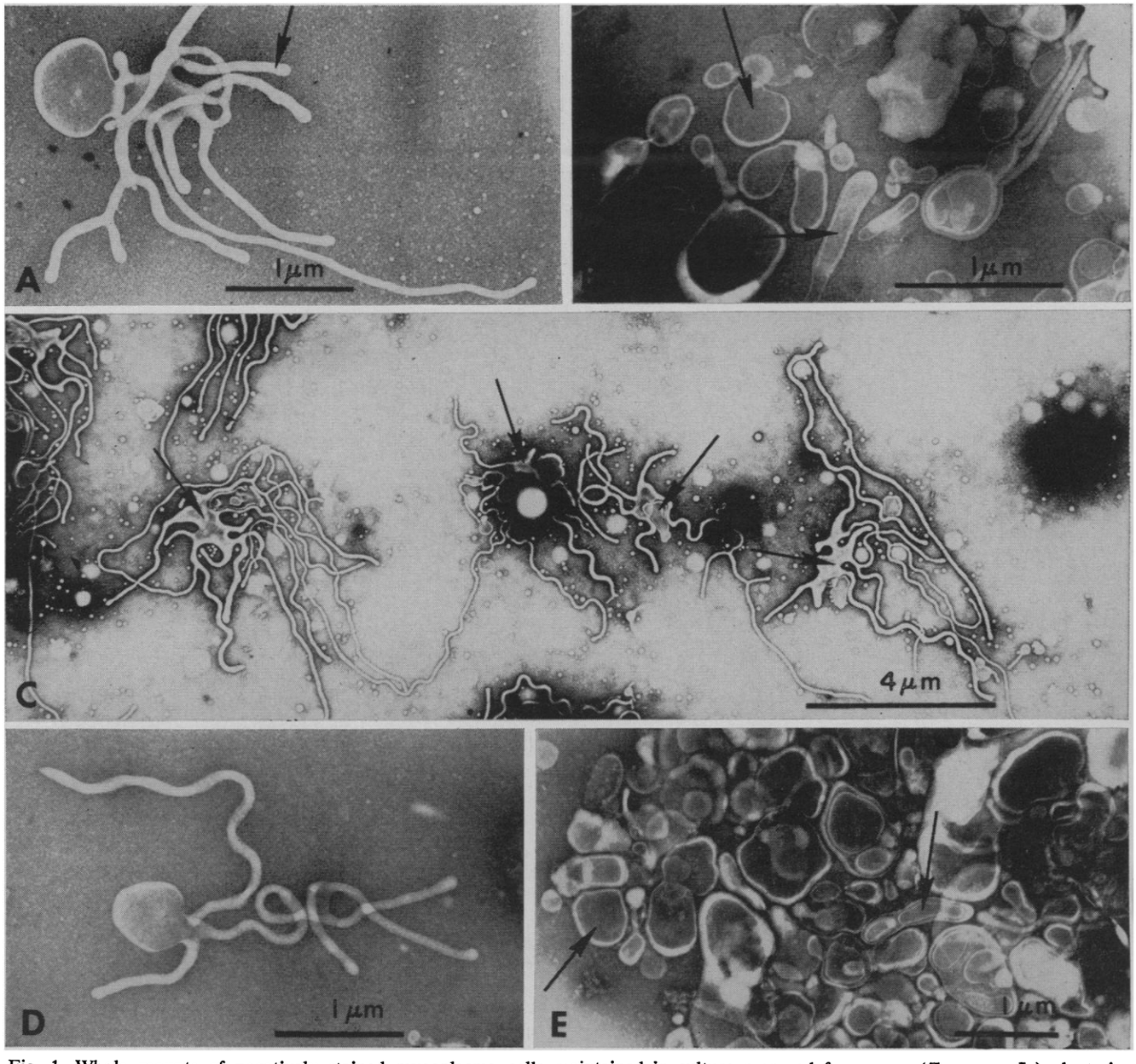

Fig. 1. Whole mounts of negatively stained mycoplasma cells maintained in culture prepared from corn (*Zea mays* L.) plants infected with Rio Grande strain of corn stunt. (A) Filamentous form of mycoplasma cell commonly found in primary cultures. Note the filaments (arrows) which emerge from the main body. (B) Round (arrow) and elongated (arrow) forms of numerous mycoplasma cells found in primary cultures. (C) Filamentous mycoplasma cells (arrows) observed in subcultures 43 days old. (D) Filamentous form of a mycoplasma cell observed in primary cultures prepared from diseased corn plants which were inoculated with mycoplasma cells from primary corn stunt cultures. These cultures (E) also contained round (arrow) and elongated (arrow) forms of mycoplasma cells.

tained at 4°C for 0, 4, and 24 hours transmitted corn stunt to 5 of 50, 2 of 20, and 1 of 16 plants tested, respectively. Fifteen insects injected with the sample maintained for 72 hours were not infective. Insects injected with the samples maintained at 25°C for 4 hours transmitted corn stunt to 2 of 48 plants tested. None of the insects injected with samples maintained for more than 4 hours at 25°C transmitted corn stunt.

Several kinds of artificial media were used in attempts to cultivate the mycoplasma-like agent of corn stunt, but only one is described here. The medium was prepared in two stock solutions (*11*).

Solution 1 was sterilized by filtration through a 0.22-μm Millipore filter, solution 2 by autoclaving for 20 minutes at 121°C. The complete medium (with a final *p*H of 6.8) consisted of 10 ml of solution 1, all of solution 2, 20 ml of horse serum, and 1 ml of penicillin G (10,000 units).

The cultures were set up as follows. A 15- to 20-cm portion of stem, 5 cm above ground, was removed from a diseased corn plant. The stem pieces were placed for 1 to 2 minutes in a large dish that contained 70 percent ethanol. The pieces were held briefly in a flame and put immediately into a dish that contained either 0.1 percent $HgCl_2$ or 10 percent Clorox (5.25 percent sodium hypochlorite) for 15 minutes. After being washed four or five times in sterile distilled water, the outer sheath leaves were removed from the stem and the stem pieces were placed in a dish of fresh culture medium. Cubes of atactosteles (0.125 cm^3) were taken from the center of the stem and placed individually in test tubes that contained 3 ml of fresh medium. After 24 hours the tissues were removed from the test tubes and were discarded.

An alternative culture method was to put a cube (0.125 cm^3) of atactostele tissue into approximately 0.1 ml of culture medium in a sterile petri dish. The tissue was crushed with forceps, and the plant juice was mixed with the medium. Approximately 0.1 ml of the medium plus plant juice was transferred to a test tube that contained 3 ml of medium. Control cultures were prepared, in the same manner, from healthy plants of approximately the same age. The cultures were maintained in incubators at 25°C.

To assay for infectivity in the cultures after varying periods of incubation, two culture tubes were combined and centrifuged for 15 minutes at 17,000*g*. The pellet was resuspended in 0.2 ml of fresh medium and used as the inoculum for injecting 50 to 100 healthy, adult *D. elimatus* leafhoppers. Samples from the inoculum were also examined by electron microscopy after they were stained with 2 percent sodium phosphotungstate acid (*p*H 7.0).

In three of five experiments infectivity was obtained from cultures which were 5, 8, and 14 days old (Table 1). All the infective cultures contained organisms that morphologically resemble mycoplasma (Fig. 1, A and B). These organisms generally had an irregular-shaped main body approximately 1 to 2 μm in width. Some organisms (Fig. 1A) had several filaments, 50 to 150 nm wide and up to 12 μm in length, emerging from their surfaces. Other organisms (Fig. 1B) had round to elongated forms. Some cultures were centrifuged for 15 minutes at 17,000*g*, and the pellets were fixed and dehydrated in Epon 812. Ultrathin sections of the cultivated organisms revealed the presence of a single-unit membrane surrounding each mycoplasma cell (Fig. 2, A and B). Generally, ribosome-like particles and the centrally located strands of network, presumably nucleic acids, were also observed within the cells. Thus, the organisms in culture were morphologically indistinguishable from those bodies observed in the sections of diseased plants and insects and in infective extracts from plants with corn stunt disease (*2*), and they were also similar to those seen in thin sections and negatively stained prepara-

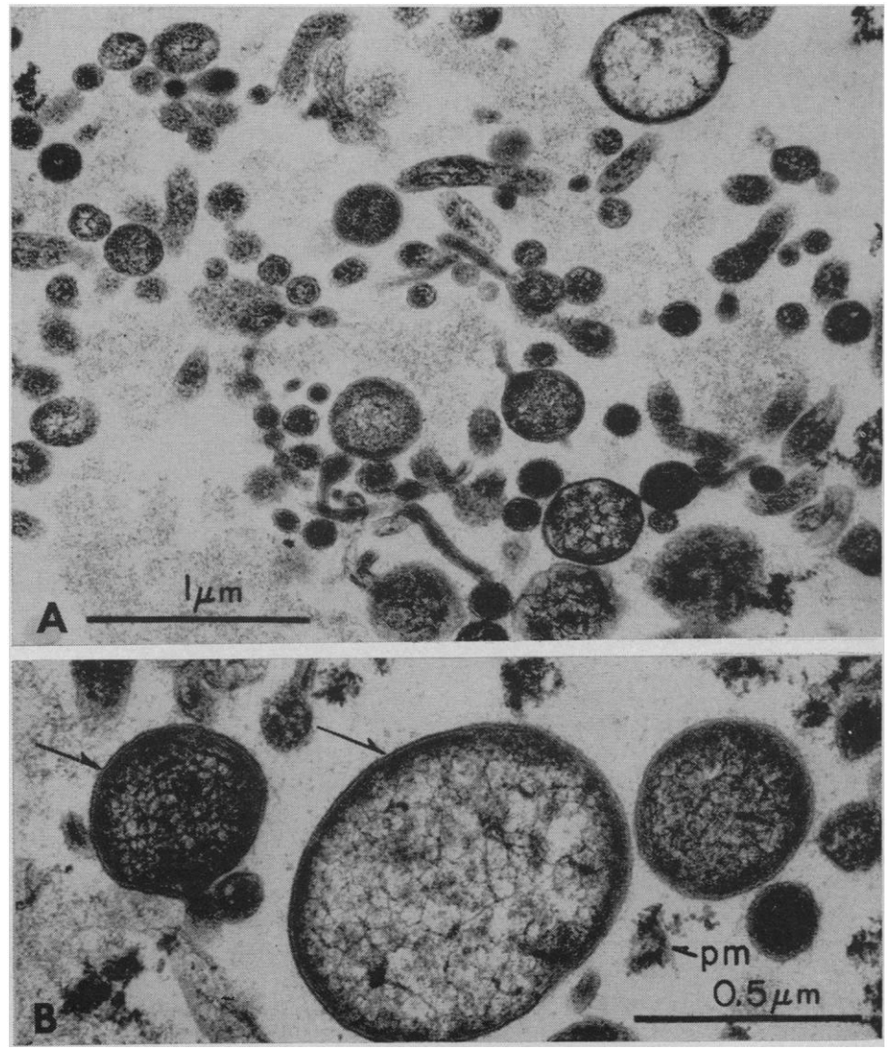

Fig. 2. (A) Ultrathin section of mycoplasma cells prepared from 43-day-old subcultures (see Fig. 1C). (B) High magnification of mycoplasma cells prepared from subcultures. Note the unit membrane (arrows) surrounding each cell and the netlike strands within the larger bodies; *pm*, precipitated medium.

Table 1. Transmission of corn stunt agent by *Dalbulus elimatus* injected with mycoplasma cultures isolated from corn plants infected with the Rio Grande strain. Twenty noninjected insects from stock colonies were tested in each experiment and all were noninfective. All the infective colonies transmitted to two or more test plants.

Exp. No.	Age of culture (days)	No. insect colonies infective/ tested	Mycoplasma-like cells present in inoculum*
1	5	1/5	Yes
2	8	18/21	Yes
3	8	0/12	No
4	14	0/5	No
5	14	11/14	Yes

* Samples of the inoculum were placed on Formvar-coated grids, stained with 2 percent phosphotungstate acid, pH 7.0, and examined by electron microscopy.

tions of various mycoplasma species that infect man and other higher animals (*12*). The primary cultures containing mycoplasma-like cells occasionally contained some plant debris, but this material usually disintegrated and disappeared within 1 to 2 weeks and only the mycoplasma cells remained in culture. Organisms resembling fungi, bacteria, or viruses were never observed in the pure cultures. In experiments 3 and 4, injected insects did not transmit corn stunt, and mycoplasma-like cells were not observed in the inocula by electron microscopy. Control cultures prepared from healthy plants never contained organisms that resembled mycoplasma.

The corn stunt agent is not stable in simple buffer at room temperature for more than 4 hours, but it can be maintained for several weeks in a complex cell-free artificial medium. Electron microscopy of negatively stained samples from 4- to 6-day-old cultures and from 14-day-old cultures generally revealed a higher number of organisms in the older cultures, but precise quantitative experiments were not conducted. Turbidity in the culture tubes usually increased slightly during the experiment; however, it is well known that viable counts of mycoplasma do not necessarily parallel turbidity (*13*). Several attempts were made to subculture the organism. In some, 0.2 to 0.5 ml of the primary culture that contained the mycoplasma organisms was added to 3 ml of fresh medium. In one experiment, electron microscopy of samples from 12 tubes containing subcultures that were 43 days old revealed the highest concentration of mycoplasma-like cells (Figs. 1C and 2, A and B). Samples of these subcultures were injected into 50 healthy adult leafhoppers. Three out of 30 surviving insects transmitted corn stunt to corn seedlings. Forty-five noninjected control insects were noninfective. The data suggest that multiplication has occurred under these culturing conditions.

Diseased plants from experiments 2 and 5 (Table 1) were used for preparing primary corn stunt cultures. The cultures prepared from these plants contained organisms that resembled mycoplasma (Fig. 1, D and E). The reisolation of an organism that was indistinguishable from the one injected into the leafhopper vectors from primary cultures (Fig. 1, A and B) satisfies the last criterion of Koch's postulates: (i) the mycoplasma was consistently associated with corn stunt disease and was consistently isolated from plants infected with corn stunt disease; (ii) the organism was isolated in pure culture; (iii) the disease, with characteristic symptoms, was reproduced in plants (healthy leafhoppers artificially injected with pure mycoplasma cultures transmitted the agent to healthy corn plants); and (iv) the mycoplasma was reisolated from the inoculated plants and identified with the one originally isolated.

We believe that the hypothesis of organismal etiology has been proved for the corn stunt disease and that this report demonstrates the pathogenicity of a mycoplasma to plants. We must now recognize a new class of organisms causing diseases in plants. The agent of corn stunt disease has been shown to multiply in its insect vector, *Dalbulus maidis* (DeLong & Wolc.) (*14*), and it has been demonstrated that the Rio Grande strain of corn stunt agent is pathogenic to the vector *D. elimatus* (*15*). Accordingly, this report also establishes a new class of organisms that infect invertebrates.

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11. Solution 1 contained: T.C. 199 medium (IX), 100 ml; Schneider's drosophila medium, 50 ml; Difco CMRL-1066, 50 ml; Yeastolate, 2 g. Solution 2 contained: Difco PPLO broth base, 1.5 g; sucrose, 8.5 g; double-distilled water, 69.0 ml. The three liquid mediums in solution 1 and the horse serum were purchased from Grand Island Biological Company.
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16. Supported in part by an experimental research grant from Fairleigh Dickinson University, Teaneck, New Jersey. We thank I. Greenberg for technical assistance.

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Differentiation of Populations

Ehrlich and Raven's provocative paper (*1*) never makes clear whether large-scale, intermittent gene flow is being relegated to the same never-never land as the steady, "trickle" variety. Extreme, unpredictable, and largely unexplained fluctuations in numbers occur widely in natural populations, even in *Euphydryas aurinia* (*2*), a European butterfly which shares many biological characteristics with Ehrlich's *E. editha*. The lack of gene flow during the period of observation of a given situation implies nothing about the past or the future. The Hesperiid butterfly, *Euphyes bimacula*, is a well-known colonial species (*3*) in which gene exchange among demes cannot as a rule be observed. Still, it had a "population explosion" all over the northeastern United States in 1968 (*4*) which produced extensive mixing of populations, at least in upstate New York. Had the Stanford group been studying *E. bimacula* for 10 years through 1967, they could have stated