PROTOPLASMIC INCOMPATIBILITY IN *PODOSPORA ANSERINA*: A POSSIBLE ROLE FOR ITS ASSOCIATED PROTEOLYTIC ACTIVITY

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ABSTRACT

A mutation (*modD*) was selected in a gene involved in the control of protoplasmic incompatibility. Previous results (LABARERE and BERNET 1979) showed that *modD* decreased the density of protoperithecia and caused a defect in ascospore germination. In addition, *modD* has a third defect: when *modD* stationary cells were isolated in order to obtain further development, renewal of growth rarely ensued. Instead, the *modD* cells lysed or produced microthalli from which normal growth never occurred. These defects were suppressed by β-phenyl pyruvic acid, a protease inhibitor, and by the presence of a mutation (*modC*) that suppresses the proteases associated with protoplasmic incompatibility. The stationary wild-type cells' regeneration was inhibited by β-phenyl pyruvic acid at levels that maintained *modD* cells' regeneration. These results suggest a biological role for the proteases associated with protoplasmic incompatibility.

IN *Podospora anserina*, protoplasmic incompatibility is due to the pathological effects of specific lytic proteases (BEGUERET 1973; BEGUERET and BERNET 1973). Indirect arguments (BOUCHERIE, BEGUERET and BERNET 1976) suggested that these proteases, which are never found in wild-type cultures—neither in cell extracts nor in the culture filtrates (LABARERE 1978)—had a role in protoperithecium formation.

A mutant strain (*modD*) defective in protoperithecium formation and ascospore germination was previously described (LABARERE and BERNET 1979). In this same strain, we now show a deficiency in the renewal of growth from stationary cells. A function for the proteases associated with protoplasmic incompatibility, wider than that originately postulated, is suggested by the association of these three defects.

MATERIALS AND METHODS

Podospora anserina is a secondary homothallic ascomycete. Occasional uninucleate ascospores give rise to clones that behave as heterothallic strains and whose life cycle resembles closely that of *Neurospora crassa*, with the exception that *Podospora* microconidia do not germinate. (For details see ESSER 1974).

Barrage and protoplasmic incompatibility: The term "barrage" designates an unpigmented area formed on the confrontation line between strains of unlike genotype. In the barrage area, mixed cells formed by the fusion of hyphae from the different strains are destroyed by a lytic process (RIZET and SCHECROUN 1959; BERNET, 1963) called protoplasmic incompatibility.

Incompatibility gene nomenclature: In the *R/V* nonallelic incompatibility system, incompatibility genes are designated by capital letters (*R* and *V*) because only one incompatibility allele is known at both the *r* and *v* loci. At the *c*, *d* and *e* loci, which give rise to the *C/D* and *C/E* systems, studies of 16 geographical races, called (A), (B), (C), etc., revealed allelic series (BERNET 1965, 1967); *c(s)*, *d(A)*, etc., designate genes *c* and *d* from the geographical races (s) and (A). To simplify nomenclature, capital letters *C*, *D* and *E* are also used. Allelic incompatibility systems involve the *v* locus, as well as the *b*, *q*, *s* and *z* loci (RIZET and ESSER 1953; BERNET 1967). At these five loci, only two wild-type alleles have been identified; for example, the two incompatibility alleles of the *z* locus are designated *Z1* and *Z2*.

Self-lysing (SL) strains: SL strains are lethal strains in which growth stops in their early development, and the cells lyse as the consequence of the presence of nonallelic incompatibility genes (strains *CD*, *RV*, etc.). Crosses between strains whose barrage is the result of a nonallelic interaction, which does not always function in the mating process, are the source of the self-lysing strains; for instance, *RV* and *CD* strains derive from the fertile crosses female *rV* × male *RV1* and female *Cd* × male *cD*.

The modC *mutation*: *modC* was selected from the SL strain *RV*, mutagenized by UV, on the basis of the cell lysis suppression and growth restoration (LABARERE and BERNET 1977). The *modC* mutation suppresses the proteases associated with protoplasmic incompatibility (LABARERE and BERNET 1978).

The modD *mutation*: *modD* was derived from a SL strain (mutagenized by nitrosoguanidine) in which self-lysis was the result of the interaction between the nonallelic incompatibility genes *C* and *E*. At 26°, the *modD* strain produces protoperithecia in low density; most of the ascospores are killed in the course of incubation. At 32°, the *modD* strain exhibits a wild-type phenotype (LABARERE and BERNET 1979).

Procedure to measure regeneration from stationary cells: Mycelia were grown on cellophane discs (3 cm in diameter) and placed on solid medium in a Petri dish. After three days of culture, apical hyphae passed over the edge of the cellophane disc and invaded the growth medium. Three or four days later, when growth had stopped at the limit of the Petri dish, the cellophane discs were expected to have only stationary cells on their surface; at this time the medium below the cellophane was still free of mycelium. The cellophane disc was then removed, placed on a fresh medium for 12 hr and removed once more. Mycelial colonies appeared on the surface of the second medium. Those located on the edge of the print of the disc arose from the renewed growth of the hyphae cut when the cellophane disc was removed from the first culture. Numerous colonies also appeared in the center of the print of the cellophane disc, but they had an undetermined origin.

Drugs: β-phenyl pyruvic acid, a competitive inhibitor for certain proteolytic enzymes (GERATZ 1965; BARRETT 1967), suppressed cell destruction in SL strains and inhibited the proteolytic enzymes active in the crude extracts of normal strains, as well as the lytic proteases associated with protoplasmic incompatibility (DELETTRE, BOUCHERIE and BERNET 1978). With the doses used in this work to investigate cell regeneration (up to 0.08 M), the drug has practically no effect on the growth of a normal strain. β-phenyl pyruvic acid, cycloheximide and 5-fluorouracil were obtained from Sigma Chemical Co.

RESULTS

Effect of the modD *mutation on barrage, protoplasmic incompatibility and cell regeneration*

The effect of *modD* on barrage formation was first investigated in the nonallelic incompatibility system, *C/E* : *c(s)/e(A)* and *c(A)/e(F)* interactions. When *Ce* and *cE* strains were confronted on solid medium, no barrage occurred only when both strains carried *modD*. The nonformation of barrage was also observed in the other two nonallelic systems investigated (*C/D* and *R/V*), as well as in all five

allelic systems (*B1/B2*, *Q1/Q2*, etc.). At standard conditions (26°), *modD* completely suppressed barrage formation.

As previously reported, the defects in protoperithecium formation and ascospore germination in *modD* were completely suppressed at 32° (LABARERE and BERNET 1979). We also observed that barrage suppression is temperature sensitive. At 32°, barrage was observed in all confrontations involving strains carrying *modD*. The results suggested that barrage suppression is a third defect associated with *modD*, not the consequence of an additional mutation.

In *Podospora anserina*, most of the nonallelic gene interactions and one allelic interaction (*Z1/Z2*) result in the sterility of specific crosses. Previous investigations showed that unsuccessful hybridization was the consequence of the destruction of the trichogyne as the result of its fusion with a microconidium of unlike genotype (ESSER 1959; BERNET 1965). In the nonallelic *C/E* system [*c(s)/e(A)* interaction] and in the allelic *Z1/Z2* system, fertility rates (protoperithecium per microconidium) in the hybridizations of female *E* × male *C* and female *Z1* × male *Z2* were 5×10^{-4} and 3×10^{-3}, respectively. When the parental strains carried the *modD* mutation, fertility rates for the crosses of female *E* × male *C* and female *Z1* × male *Z2* were 8×10^{-4} and 5×10^{-3}, respectively. Thus, it appears that *modD* has practically no effect on protoplasmic incompatibility.

To demonstrate a possible lack of effect of the *modD* mutation on protoplasmic incompatibility, anastomoses between *C* and *E* or *Z1* and *Z2* hyphae were carried out in an oil chamber. Normally, cell disintegration occurs after a delay of four to five hours following hyphal fusion (BERNET 1965). When the strains carried *modD*, complete cell destruction also occurred in less than five hours. This lack of effect of *modD* on protoplasmic incompatibility was also observed in the case of SL strains. Strains *CE* and *RV* carrying the *modD* mutation contained as many disintegrated cells as the standard (*modD*$^+$) self-lysing strains.

From these results, we deduced that *modD* suppresses only the secondary consequence of protoplasmic incompatibility, *i.e.*, barrage formation. According to BEISSON-SCHECROUN (1962), barrage is the consequence of intensive cell regeneration, which is responsible for the formation of the unpigmented area. Consequently, it might be suspected that *modD* affects cell regeneration. Since *modD* is temperature sensitive, we investigated cell regeneration in a *modD* strain at 26° and 32° in comparison with a wild-type reference strain. The results in Table 1 show that the regeneration ability of the *modD* strain at 26° was only 10% of that

TABLE 1

Number of colonies generated at 26° and 32° from stationary cells of a modD *strain and a reference wild-type strain*

Genotypes	26°	32°
modD	11:25*	172:129
wild type	158:342	187:114

* The first number designates colonies that appeared on the edge of the cellophane disc and the second, those arising in the center.

of the wild-type strain. At 32°, as expected, the *modD* strain exhibits a nearly wild-type capacity. Results, not reported, indicated that the nonpermissive temperature (26°) affected cell regeneration only when it was applied during cell regeneration.

A proteolytic activity is associated with the onset of modD *cell regeneration*

The *modD* strain was grown in liquid medium in an oil chamber. Hyphae were fragmented with a De Fonbrune micromanipulator in order to isolate hyphal fragments containing three stationary cells; these cells were between 24 and 48 hours old. About 50 hyphal fragments were observed.

In nine cases, regeneration occurred in the same way as for wild-type cells: growth resumed simultaneously at both ends in the first two hours of their incubation. In more than half of the remaining cases (33 of 50) a phenomenon that we call "aborted" regeneration was observed: at both ends of the hyphal fragment, protoplasm extended but linear growth never exceeded 10μ per day (instead of more than 100μ per hour in normal conditions). This pathological growth may continue for many days, but finally stops. This feature did not revert after subsequent exposure to permissive conditions (32°). In the remaining eight cases, cell disintegration occurred in the two cells located at the ends of the hyphal fragments: large vacuoles appeared, accompanied by streaming of cytoplasmic granules, and about ten hours later cell lysis was terminated by protoplasmic extrusion. These symptoms strongly resembled those described for protoplasmic incompatibility in Neurospora (GARNJOBST and WILSON 1956) and Podospora (RIZET and SCHECROUN 1959; BERNET 1965).

The *modC* mutation was selected on the basis of a suppression of protoplasmic incompatibility (LABARERE and BERNET 1977); further results (LABARERE and BERNET 1978) showed that it abolished the lytic proteases associated with protoplasmic incompatibility. In order to determine if cell destruction and aborted outgrowth specific to regenerating *modD* cells might be related to protoplasmic incompatibility, we investigated cell regeneration in the double-mutant strain, *modD modC*. Results from the experiments reported in Table 2 show that the *modC* mutation completely abolishes this *modD*-associated defect and agree with the idea that *modD* cell death (or aborted regeneration) and protoplasmic incompatibility are related phenomena.

TABLE 2

Effect of the modC *mutation on* modD *cell generation*

Genotypes	Number of colonies*
modD	11:25
modD modC	142:364
wild type	158:342

* The first number designates colonies that appeared on the edge of the cellophane disc and the second, those arising in the center.

A protease inhibitor, β-phenyl pyruvic acid, protected Podospora cells from protoplasmic incompatibility and self-lysis (BOUCHERIE and BERNET 1977; DELETTRE, BOUCHERIE and BERNET, 1978). Cell regeneration of *modD*, *modC* and a wild-type strain was compared on media supplmented with β-phenyl pyruvic acid. As shown in Figure 1, lower doses of the inhibitor (up to 0.03 M) restore the wild-type regeneration capacity to the *modD* strain. With higher doses (0.045 M to 0.06 M), cell regeneration in wild-type strains was progressively inhibited, whereas cell regeneration in the *modD* strain was maintained. Regeneration in the *modD* strain also dropped sharply at concentrations greater than 0.06 M. Low doses of β-phenyl pyruvic acid (0.025 M) inhibited the renewal of normal growth from stationary cells of the *modC* strain. Stationary *modC* cells, in the presence of the drug, produce an abnormal mycelium with reduced growth (Figure 2). This reduced growth was never observed from apical *modC* cells. To test whether β-phenyl pyruvic acid also affects growth, mycelia were grown on cellophane discs for only 36 hours. The mycelia had not reached the edge of the cellophane disc and contained hyphal tips. They were placed on medium supplemented with β-phenyl pyruvic acid. Growth of all three strains was stopped at 0.15 M (Figure 3). Thus, regeneration is especially sensitive to β-phenyl pyruvic acid, and the differences in sensitivity among the three strains during cell regeneration were not due to differences in permeability to the drug.

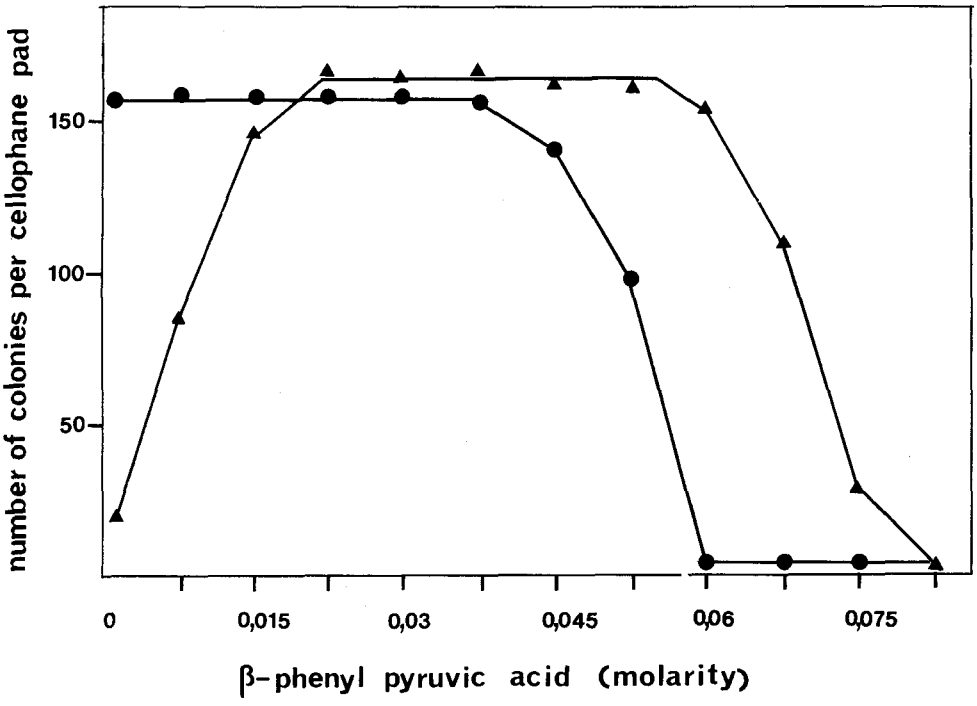

FIGURE 1.—Effect of β-phenyl pyruvic acid on cell regeneration in the *modD* mutant strain (▲——▲) and a reference wild-type strain (●——●); only colonies formed at the limits of the cellophane disc were counted.

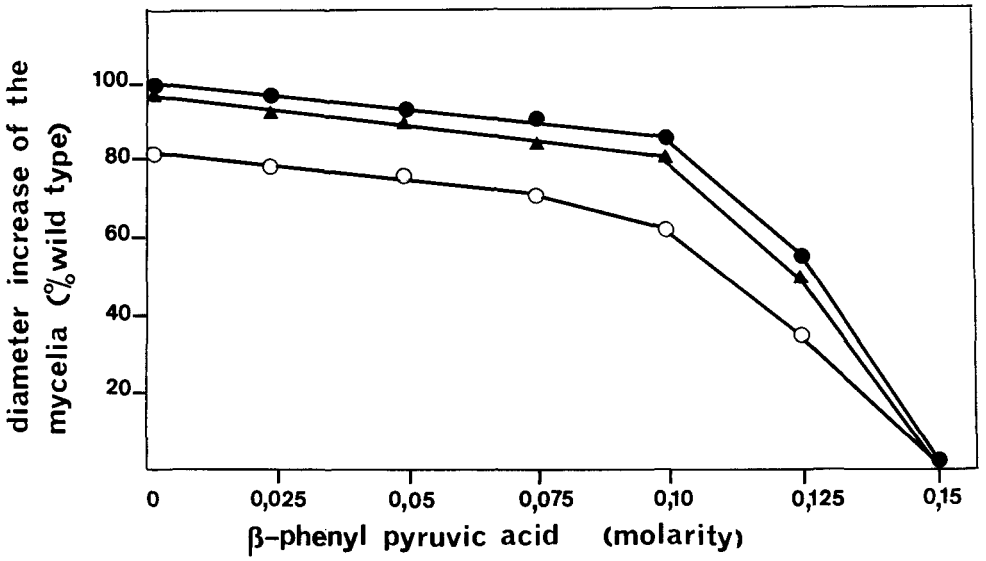

FIGURE 2.—Effect of β-phenyl pyruvic acid on *modC* (○——○), *modD* (▲——▲) and wild-type (●——●) cultures. The initial cultures had grown for 36 hrs on cellophane discs laid down on standard medium prior to their transfer to medium containing β-phenyl pyruvic acid.

These results emphasize that proteolytic activity is necessary for regeneration from stationary cells. The extreme sensitivity of *modC* strains to β-phenyl pyruvic acid supports the idea that the proteolytic activity postulated for cell regeneration may be related to the proteolytic activity specific to protoplasmic incompatibility. The strong resistance shown by the *modD* strain gives additional

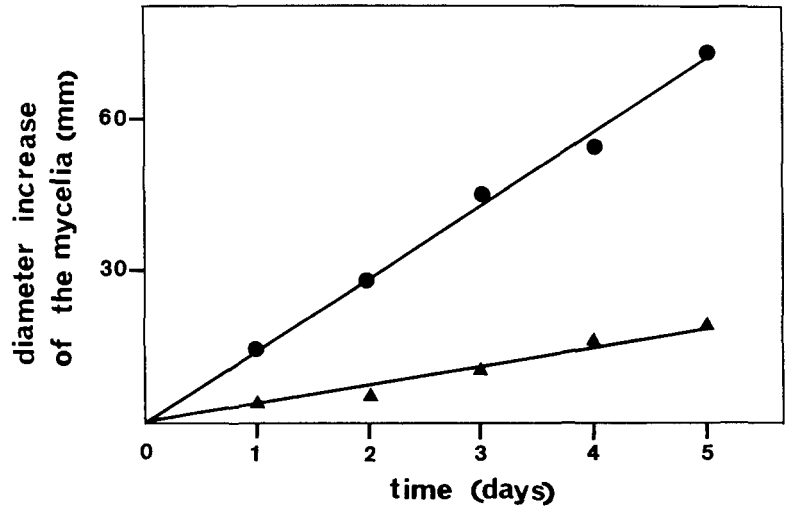

FIGURE 3.—Effect of β-phenyl pyruvic acid (0.025 M) on the growth of *modC* cultures obtained as renewed growth of stationary cultures (▲——▲) and from growing cultures (●——●).

TABLE 3

Effects of salts on regeneration of modD *and wild-type stationary cells*

Genotypes	Ammonium* acetate	Ammonium* sulfate	Sodium* acetate	Sodium* sulfate
modD	149†	110	18	21
wild type	172	148	138	154

* Ammonium and sodium ions are 0.5M.
† Numbers of colonies that arose on the edge of the cellophane disc.

support to this interpretation and can be explained only if the proteolytic activity responsible for the lysis of *modD* cells is the proteolytic activity needed for cell regeneration.

Effect of some inhibitors and ions on modD *cell regeneration*

The results obtained with temperature-sensitive, self-lysing strains (BEGUERET and BERNET 1973; BOUCHERIE and BERNET, 1977; BOUCHERIE, 1979) were consistent with the idea that the formation of the proteases associated with protoplasmic incompatibility required protein synthesis involving pre-existing and stable messenger RNAs whose translation was especially sensitive to the NH_4^+ ion.

Experiments with cycloheximide and 5-fluorouracil were carried out on cultures grown in oil chambers. When hyphal fragments of *modD* identical to those described above were incubated in the presence of 5-fluorouracil (150 μg per ml), cell destruction was observed in 18 of the 52 cases examined. With cycloheximide (only 2 μg per ml because of the cytotoxic effect of the drug), cell destruction occurred in only three of the 52 fragments investigated. It can be deduced that the frequency of *modD* cell destruction is increased by 5-fluorouracil (×2) and decreased by cycloheximide (×0.3). Thus, the initiation of the proteolytic activity associated with *modD* cell regeneration involves protein synthesis dependent on preformed mRNAs. We also investigated the effect of the NH_4^+ ion on *modD* cell regeneration. The results in Table 3 show that *modD* cell regeneration approached the wild-type level on a medium supplemented with ammonium acetate and ammonium sulfate. All of these results emphasize the physiological relation between biogenesis of the proteolytic activity of protoplasmic incompatibility and that involved in the death or the abnormal development of stationary *modD* cells.

The *modD* strain thus represents a special case of a self-lysing strain. In SL strains (*CD*, *RV*, etc.) cell destruction occurs during development of the mycelium: 18 to 36 hours (according to the loci) after spore germination. In the *modD* strain, lysis occurs when resting cells (the dormant ascospore, LABARERE and BERNET 1979) or stationary cells have to undergo further development.

DISCUSSION

Before discussing these results, we will consider protoplasmic incompatibility in fungi and its possible relation to similar phenomena in other groups.

Protoplasmic incompatibility in fungi: In *Neurospora crassa*, protoplasmic incompatibility results only from the interaction of allelic genes (WILSON and GARNJOBST 1966); cytological symptoms accompanying the fusion of unlike genotypes resemble those described in Podospora. In Neurospora, about ten incompatibility loci are known (PERKINS 1975; MYLYK 1975; PERKINS and BARRY 1977).

In *Podospora anserina*, five loci (*b,q,s,v,z*) are involved in allelic interactions, and five loci (including the *v* locus responsible for the allelic *V/V1* interaction) determine nonallelic interactions (RIZET and ESSER 1953; ESSER 1954; BERNET 1965). These nine different loci were found in a sample of 16 geographic isolates from different regions (BERNET 1967). In *Podospora anserina*, two additional nonallelic systems, *F/G* and *K/L*, were created after the selection of induced mutations in a normal reference strain (DELETTRE and BERNET 1976).

Protoplasmic incompatibility was also shown in the myxomycetes *Didymium iridis* (COLLINS and CLARK 1968; LING and LING 1974) and *Physarum polycephalum* (CARLILE and DEE 1967), and in the ascomycete *Endothia parasitica* (ANAGNOSTAKIS 1977). In the genus Aspergillus, the failure of heterokaryon formation was found in many species (GRINDLE 1963; CATEN 1971; CROFT and JINKS 1977); it has not been determined whether the phenomenon is associated with the destruction of the fused cells.

Three main hypotheses have been proposed to explain the common occurrence of protoplasmic incompatibility in fungi. ESSER (1971) considers that protoplasmic incompatibility is a breeding system since it occasionally also occurs between sexual cells (the trichogyne of the protoperithecium and the microconidium in ascomycetes). ESSER and BLAICH (1973) proposed that homogenic incompatibility (heterothallism) prevents inbreeding, whereas heterogenic incompatibility (as they call protoplasmic incompatibility) has the opposite role of restricting outbreeding. CATEN (1972) proposed that protoplasmic incompatibility might serve as a defense mechanism protecting the organism against cytoplasmic invasion (viral infection or suppressive cytoplasmic factors). HARTL, DEMPSTER and BROWN (1975) proposed that vegetative incompatibility prevents a kind of exploitation of heterokaryons (heterosis) by nuclei that would be nonadaptive in homokaryons. These three hypotheses postulate that the phenomenon has a biological advantage, and that the incompatibility systems are maintained by natural selection.

Protoplasmic incompatibility in higher plants and animals: Graft incompatibility in plants, especially in fruit trees (HERRERO 1951; MOSSE and SCARAMUZZI 1956; BRIAN and DURON 1971), is very similar to the barrage process. The development of a necrotic line at the union of stock and scion results sooner or later in the final dissociation of the participants. Two additional phenomena may be related to protoplasmic incompatibility. The death of hybrids early in their development is a common phenomenon in interspecies hybridization of plants (for review see STEBBINS 1958). In intraspecies crosses, the formation of necrotic lethal hybrids has also been observed: in all cases, it was shown that hybrid lethality resulted from the interaction of complementary lethal genes (WIEBE 1934; CALD-

WELL and COMPTON 1943; HEYNE, WIEBE and PAINTER 1943; OKA 1957; SAUNDERS 1960; SAWANT 1956; HERMSEN 1963). The necrotic hybrids strongly resemble the self-lysing strains (*CD*, *CE*, *RV*, etc.) of *Podospora anserina*, in which the self-lysing trait is also dominant (BERNET, BEGUERET and LABARERE 1973; LABARERE, BEGUERET and BERNET 1974).

These features are also observed in the phenomenon of hypersensitivity when cell necrosis is induced in plants in response to invasion by pathogenic viruses, bacteria or fungi. The biochemical similarities between hypersensitivity and protoplasmic incompatibility were outlined by BOUCHERIE and BERNET (1978). Like protoplasmic incompatibility and hybrid necrosis, hypersensitivity is the result of a specific interlocus relationship, namely the interaction of a dominant gene of the pathogen (the avirulent allele *Av*) and a dominant gene of the host (the resistance gene *R*) (FLOR 1956; for reviews, see DAY 1976 and ELLINGBOE 1976). Furthermore, cytological symptoms accompanying cell destruction in the cases of hybrid necrosis (PHILIPS and REID 1975) closely resemble those described in hypersensitivity (COLENO 1973).

Lethal hybrids have also been described in animals (for review, see DOBZHANSKY 1951); cell destruction was also observed in the development of interspecific hybrids (BALTZER 1940). DOBZHANSKY (1951, p. 203) proposed that hybrid in-

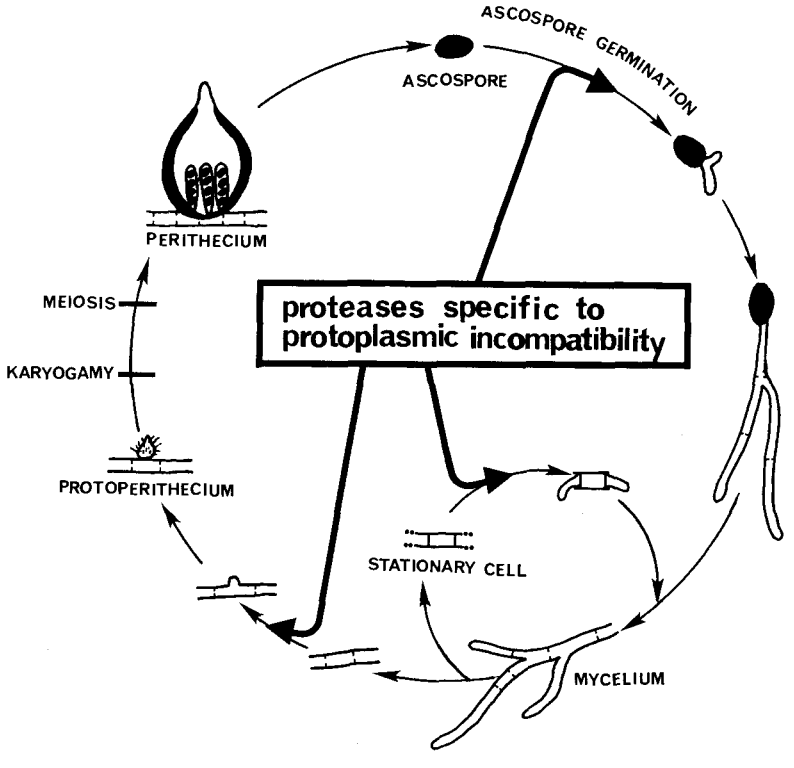

FIGURE 4.—Hypothetical model for the involvement of proteases specific to protoplasmic incompatibility in the developmental cycle of *Podospora anserina*.

viability was the consequence of nonallelic interactions, a phenomenon resulting from divergent mutations in specific loci.

Protoplasmic incompatibility and necrotic phenomena are clearly widespread. Our previous results in *Podospora anserina* showed that protoplasmic incompatibility was the consequence of the abnormal presence of specific proteases (BEGUERET and BERNET 1973; BOUCHERIE, BEGUERET and BERNET 1976). Despite systematic investigations, these proteases have never been found in extracts of normal strains or in their culture filtrates (LABARERE 1978). Studies carried out on *mod* mutations (*modA*, *modB* and *modC* genes), selected on the basis of the suppression of protoplasmic incompatibility and on the inhibition of its associated proteases, showed that the strains carrying these mutations had defects in the formation of protoperithecia (BOUCHERIE, BEGUERET and BERNET 1976; LABARERE and BERNET 1977). It was postulated (BOUCHERIE and BERNET 1974; LABARERE and BERNET 1978) that the proteases specific to protoplasmic incompatibility had a role in the formation of protoperithecia.

The results of our examination of the *modD* strain lead us to propose that the proteolytic enzymes associated with protoplasmic incompatibility are also involved in ascospore germination and in the renewed growth of stationary cells. This hypothesis is shown schematically in Figure 4. The proteases have a role whenever a resting protoplasm is induced to undergo further development. It is noteworthy to observe that the proteases might be involved in a destabilization of the low and reversible state of differentiation specific to stationary cells; this break can result either in dedifferentiation for renewal of growth or initiation of the formation of protoperithecia.

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