CHEMICAL INDUCTION OF HAPLOID FRUITING BODIES IN SCHIZOPHYLLUM COMMUNE*

BY THOMAS J. LEONARD† AND STANLEY DICK

INDIANA UNIVERSITY, BLOOMINGTON

Communicated by Ralph E. Cleland, January 2, 1968

The formation of fruiting bodies in the higher Basidiomycetes is the most dramatic expression of differentiation and morphogenesis that can be found among the fungi. Although fruiting is normally a property of dikaryotic mycelia,[1, 2] which in heterothallic species are formed from the interaction of compatible homokaryotic strains,[3] it is by no means restricted to the dikaryophase. The extensive literature on the physiology, development, and genetics of Basidiomycetes contains numerous references to the sporadic formation of normal or subnormal fruiting structures on the haploid mycelia of a number of species.[1, 4–9] Among the principal conditions that have been reported to trigger fruiting body development in haploid mycelia are mycelial aging,[1] nutrient exhaustion,[8] and mechanical injury.[1] Occasional reports have also indicated that chemical substances, e.g., the metabolic products of unrelated species of microorganisms, can initiate the process of fruiting.[10, 11] Moreover, the specific occurrence of haploid fruiting in individual strains of any one species and its absence in other such strains have implicated an important genetic component in the control of the process.[1, 7, 9] In all these cases, to be sure, full expression of the haploid fruiting response has required appropriate environmental conditions of growth, e.g., low CO_2 tension, adequate illumination, suitable limits of pH and temperature, and an adequate supply of essential nutrients.

The present study originated from the chance observation of an intermycelial reaction between a haploid strain of the Basidiomycete *Schizophyllum commune* and an imperfect fungus, *Hormodendrum cladosporioides*, which resulted in the induction of haploid fruiting bodies in the former. The present communication reports (*a*) the demonstration of a fruiting-inducing substance (FIS) in cell-free extracts of the isolate of *Hormodendrum* and the presence of materials with similar activity in certain stages of *S. commune* itself; (*b*) the physiological factors which affect the expression of FIS-induced fruiting; and (*c*) the preliminary attempts to characterize the inducing substance.

Materials and Methods.—The mycelia of *S. commune* used for the major portion of this study were three homokaryons, isogenized, with the exception of their mating-type factors, by ten generations of backcrossing with strain 699, and heterokaryons derived from their intermating. In addition to this isogenic series, haploid strains of diverse origin and mating type were used for a number of tests. Some of these strains were obtained from Professor John R. Raper of Harvard University, whereas other strains were newly isolated from fruiting bodies collected in the vicinity of Bloomington, Indiana.

The isolate of *H. cladosporioides* employed throughout this study was obtained as a laboratory contaminant and maintained on Difco's Cantino-PYG medium; this medium permitted little conidiation of the fungus and allowed maximal production of active FIS.

All fruiting assays, test matings, and spore isolations were carried out on a medium (MM) that contained 20 gm glucose, 1.5 gm asparagine, 1.0 gm K_2HPO_4, 0.46 gm KH_2PO_4, 0.5 gm $MgSO_4 \cdot 7H_2O$, 120 μg thiamine hydrochloride, and 17 gm agar per liter of dis-

tilled water. Glucose was always sterilized separately from the remaining components in order to minimize caramelization. The pH of the medium after autoclaving was 6.8.

Cell-free extracts of *Hormodendrum* were prepared as follows: 10-day-old surface mats were established from conidia sown on 500 ml of PYG broth in 3-liter Fernbach flasks and incubated at room temperature (23–28°C) without forced aeration or agitation. The mycelial pads were washed several times in distilled water, pressed dry between filter papers, and then macerated for 1 min with an equal volume of distilled water in a Waring Blendor. The resulting suspension of conidia and hyphal fragments was passed through a French pressure cell (American Instrument Corp.) at 16,000 psi. The brei was centrifuged at 17,000 $\times$ g, and the supernatant was filter-sterilized by passage through Millipore filters of 0.35-μ pore size. Although all operations subsequent to breakage of the cells were originally carried out at 2–4°C, it later became evident that such precautions were not necessary for the preparation of active extracts.

Cell-free extracts of homokaryotic or dikaryotic mycelia of *S. commune* were prepared from cultures grown in 10-liter carboys of liquid MM with forced aeration. Mycelia were harvested after 3 or 4 days, and then treated as described above for *Hormodendrum*. Haploid and dikaryotic fruiting bodies of *S. commune* were extracted by grinding with an equal weight of sand and approximately 10 times their weight of distilled water. After centrifugation to remove debris, the extracts were filter-sterilized.

Assays for FIS-activity were carried out as follows: rectangular wells, 4 mm $\times$ 24 mm, were cut from the agar 5 mm in advance of the mycelial front of 4-day-old colonies of *S. commune* homokaryons. The wells of one set of plates, which usually consisted of five replicates, were filled with the extract to be assayed, additional extract being added as needed to keep the wells filled for a period of 8 hr. Other plates received control materials: distilled water, uninoculated PYG broth, and uninoculated MM. The assay plates were then incubated overnight at room temperature in continuous light of 100 ft-c. Subsequent incubation took place under the same conditions, but with the plates inverted over watch glasses filled with 6% KOH.

The progression of morphological events leading to the formation of fruiting structures in *S. commune* has been divided into five stages:

Stage I. Masses of definitely aggregated cells; masses have no defined regular shape.

Stage II. Aggregated masses of cells take on regular, subspherical to cylindrical appearance. Apical pits not visible.

Stage III. Structure as in stage II, but with a macroscopically visible apical pit. Gills not visible.

Stage IV. Structures as in stage III, but gills macroscopically visible.

Stage V. Expansion of the gilled surface.

The onset of sporulation may be detected as early as stage III, but the presence or absence of sporulation is not essential to any of the stages.

Basidiospores from fruiting bodies of *S. commune* were isolated according to the procedures of Raper and Miles,[12] and mating types of monosporous cultures were determined by scoring the phenotypes of the heterokaryons formed with testers of known mating type.

Experimental Results.—The initial and unexpected observation that *Hormodendrum* colonies could stimulate haploid mycelia of *S. commune* to initiate the production of fruiting bodies revealed a number of interesting features of the fruiting response and led to an extensive series of experiments dealing with reactions between intact mycelia of a variety of fungal species and colonies of *S. commune*. Although the details of these studies will be reported elsewhere, a brief summary of the results is relevant to a consideration of the experiments with cell-free extracts reported below:

(1) In no case did contact with *Hormodendrum* prevent the further extension of the *Schizophyllum* colony. On the contrary, mycelia of *S. commune* generally

continued to grow over the imperfect fungus, the continued overgrowth being more dense on the surface of small *Hormodendrum* colonies than on large ones.

(2) *Hormodendrum* colonies were stimulatory to the production of stage III structures. Colonies 4 mm in diameter were as effective as colonies 60 mm in diameter, and smaller colonies were occasionally even more effective than larger ones. This last observation suggested that optimal concentrations of an inducing substance might be required and that supraoptimal concentrations might be slightly inhibitory.

(3) Fruiting of *Schizophyllum* mycelia did not appear to be inducible over all size ranges. A minimum colony size of 10 mm had to have been reached at the time of intermycelial contact for fruiting structures of stage III to form.

(4) The fruiting response was restricted to a limited region immediately adjacent to the zone of contact with the inducing mycelium. No clear indication of a telemorphotic response could be seen in any of the pairings and, whereever a fruiting response was elicited, it was seen only several days after actual contact had been made between two mycelia.

(5) In all cases of induction, fruiting structures (stages III and IV) were produced between three and five days after contact between the mycelia had been made. Considerable variation existed within these limits even in replicates of the same pairing.

(6) In the vast majority of these experiments the fruiting response did not progress beyond stage III; the small number of stage IV structures that did appear were never seen to sporulate.

In an attempt to study fruiting induction under more precisely controlled conditions, without the added uncertainties provided by the presence of a living inducing mycelium, work with cell-free extracts of *Hormodendrum* was initiated.

Preliminary treatments, under standard assay conditions, of the isogenic strains with filtrates of nine-day-old liquid cultures of *Hormodendrum* elicited no response. When nine-day-old mycelial pads of *Hormodendrum* were extracted, however, and the extracts applied to the above homokaryons, fruiting responses up through stage V were observed in most cases (Fig. 1). Control mycelia, exposed to uninoculated PYG or distilled water, continued to grow vegetatively and gave no fruiting response (Fig. 2). These results were repeated hundreds of times and strengthen the hypothesis that *Hormodendrum* mycelia contain and release metabolites during growth that induce vegetative mycelia of *S. commune* to form reproductive structures. The failure of culture-filtrates to induce the response is not entirely clear, but it is probably due to an insufficient concentration of these metabolites in the growth medium. On the basis of these results, a provisional hypothesis has been established that induced fruiting is a response to a single substance, *FIS;* although the postulated substance will henceforth, for convenience and simplicity, be referred to in the singular, it is understood that, in the absence of purified material, a complex of substances might equally well account for the results.

The developmental course of FIS-induced haploid fruiting was as follows: The growth-rate of *S. commune* mycelium was not affected by the treatment, and the hyphal margin continued its expansion at the rate of 0.4–0.5 mm/hour.

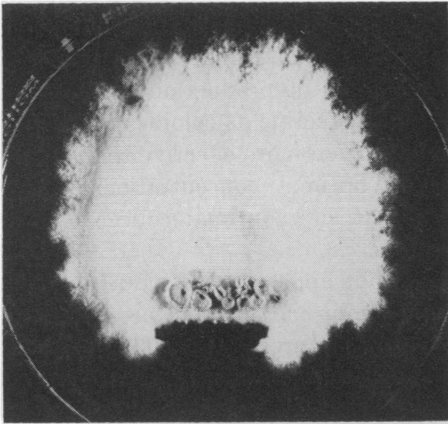

FIG. 1.—Homokaryotic culture 6 days after induction with FIS. Extract was added to the rectangular well adjacent to the fruiting bodies.

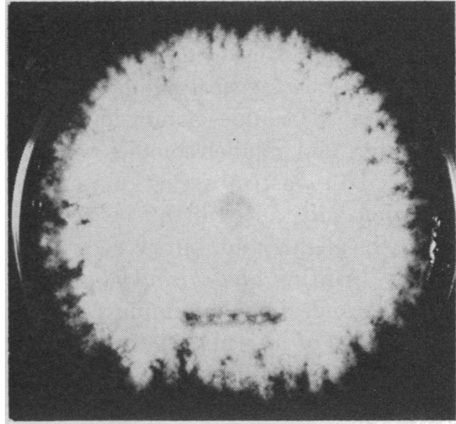

FIG. 2.—Control culture which received uninoculated PYG medium.

The first indication of an effect was the deposition of a brown pigment in a restricted region corresponding to the position of the mycelial front at the time of application of the extract. Within 48 hours after treatment, fruiting structures of stage II had appeared above the region of pigment-deposition; the fruiting reaction was always restricted to this region. By the following day, the fruiting structures had progressed to stages III, IV, or V. The number of fruiting bodies varied considerably, even in replicates of the same experiment. When a large number of structures were induced in a culture, only a few developed to stage V; the majority remained in stages III and IV. When only a few fruiting structures were induced, however, the majority usually developed through stage V. Although the sequence of morphogenetic events was the same in all instances of induction, the total time required for the formation of stage V fruiting bodies varied, even under seemingly identical conditions, from 72 to 144 hours.

FIS-induced haploid fruiting bodies were found to pass through the same stages of development as those normally produced by dikaryotic mycelia. Microscopic examination of the hyphae of induced haploid fruits, however, revealed hyphae of the type found in homokaryotic vegetative mycelia. Although haploid fruits produced decidedly fewer spores than normal, dikaryotic fruits, sporulation was abundant and the germination of the spores exceeded 95 per cent. Single-spore isolates from haploid fruiting bodies were invariably of the same mating type as the homokaryotic parent strain.

In an effort to determine the range of homokaryotic strains susceptible to FIS-induction, a series of 40 mycelia of disparate geographic origin and mating type were grown under standard conditions and exposed to FIS-containing extracts of proved activity; three replicates of each strain were so tested. Included in this study were strains which had been in laboratory cultivation for many years, as well as new strains which had been very recently isolated from nature. Of the older strains tested, only three strains, in addition to strains

from the isogenic series, were induced to fruit; the induced structures developed, however, only to stage III. Although fruiting was seen among the new isolates, the response was erratic. The range of responses in single-spore isolates from the same fruiting body varied from negative to the production of stage II and stage III structures; moreover, replicates of a single haploid isolate gave variable responses. In none of the cases, except for the strains of the isogenic series, however, did development proceed to stages IV and V. Although these results do not negate the striking cases of positive FIS-activity, they imply an important genetic component to the competence to respond to FIS.

Quantitative evaluations of the FIS-induced response have to date proved difficult, and only the subjective categories of "high," "moderate," "low," and "none" have been used as preliminary measures of the intensity of the induced fruiting response.

An investigation of the effect of concentration of FIS-containing extracts of initially "high" activity revealed the following relationships: (1) The range of dilution that would still show demonstrable activity was narrow. After dilution to 10^{-1} or 10^{-2}, most crude extracts showed no FIS-activity. (2) Within this range, the intensity of the response bore no continuous relationship to the concentration of FIS. (3) The removal of water from extracts under reduced pressure occasionally resulted in a loss of activity; this activity could be regained by diluting the concentrate to its original volume. These observations suggest that a threshold quantity of FIS is necessary for fruiting to occur and possibly that an optimal concentration is also essential. In the absence of more purified material, however, these conclusions can only remain tentative. The above results, nevertheless, indicate that the best available measure of the FIS-titer of a given active extract is the highest dilution factor that will just eliminate the fruiting response.

Individual batches of FIS-containing extract differed in their inducing potency; the reason for this variation is obscure, and it may reflect either uncontrolled fluctuations in culture conditions or inadvertent losses during the isolation procedure. The activity of any given extract, however, has remained stable and reproducible for up to one year at temperatures of $-20°$ or 4°C.

The stability of sterile preparations of FIS is a fortunate property that has facilitated progress in the attempts to characterize the material. Although work on the chemistry of FIS is still in progress and will be reported elsewhere in greater detail, the following information is presently available:

(1) FIS is heat-stable and can be autoclaved at 121°C for 15 minutes without loss of activity; incineration at 425°C, however, destroys the activity.

(2) FIS can be boiled for one hour at pH values as low as 1.0 and as high as 12.0 without loss of activity.

(3) FIS is dialyzable, and calibration of the dialysis membranes has thus far set an upper limit of 12,000 on its molecular weight; it may, of course, be considerably smaller than this.

(4) FIS is soluble in water, insoluble in diethyl ether at low and high pH values, and only slightly soluble in 70 per cent ethanol, *n*-butanol, and chloroform.

(5) FIS-activity is not destroyed by treatment with trypsin, chymotrypsin, pronase, DNase, and RNase.

Although the above studies were carried out with FIS obtained from *Hormodendrum*, it has recently become evident that fruiting-inducing activity is not limited to this one source. A number of other species of fungi were capable of inducing a similar response. Most striking of all was the discovery that FIS-activity could be detected in mycelial extracts of *S. commune* itself. Observations, to be reported more fully elsewhere, that mechanical injury to haploid mycelia could induce stage III structures along the injured surface suggested the possibility that FIS-like "injury substances" were released from injured cells. Consequently, attempts were made to recover FIS from various homokaryons and heterokaryons. Sterile extracts were prepared and assayed. A low fruiting reaction was initiated, but development was not observed to progress beyond stage II. Similar results were obtained with extracts from four-day-old vegetative dikaryons. In all of the above cases, however, the concentration of FIS-like material was rather low.

That the developmental stage of the organism was a critical factor in the successful detection of FIS-activity in *S. commune* was shown by experiments with fruiting bodies. Extracts were prepared from fruiting bodies of a number of types: dikaryotic fruiting bodies, FIS-induced haploid fruiting bodies, and abnormal fruiting bodies that had appeared spontaneously on very old haploid mycelia. Even relatively mild disruption of the fruiting tissue was sufficient to release uniformly active extracts. In fact, fruiting bodies, both from *S. commune* and at least one other species, *Agaricus bisporus*, have yielded the highest titers of inducing activity yet found in any source. Although preliminary studies with fruiting body extracts indicate that the properties and molecular size of the active principle are identical with those of *Hormodendrum*-FIS, conclusive proof of the chemical equivalence of the substances is still lacking.

Apart from this minor uncertainty, one point seems reasonably clear from the results with extracts from *S. commune:* during the course of development of fruiting bodies from vegetative tissues, active substances which have the capacity to regulate the process appear in pronounced quantities; these substances are either synthesized *de novo* in the fruiting tissue or are concentrated there as a result of translocation from other regions of the mycelium.

Discussion.—The results offered here shed light on a number of features of the biology of fruiting in Basidiomycetes. First of all, the action of FIS on *S. commune* confirms earlier scattered reports that diffusible metabolic products can influence and regulate mushroom morphogenesis.[11, 13–14] The isolation and partial characterization of the properties and effects of FIS represents the most complete analysis hitherto performed on a substance of this sort. Secondly, the demonstration that homokaryons as well as dikaryons can be induced to fruit by chemical means corroborates previous findings[1, 4–9] that fruiting competence in *S. commune* is not necessarily linked to sexual reproduction and the binucleate condition. Thirdly, and perhaps most significantly, the discovery that active inducing substances can be isolated from mycelia and fruiting bodies of S. *commune* itself lends support to the hypothesis that FIS or related substances

may in some way be involved in the natural course of fruiting body induction and development. Under these circumstances, at least, FIS appears to have all the properties of a naturally produced growth substance or hormone.

That FIS might serve as a common chemical basis for dikaryotic fruiting and for all the reported cases of homokaryotic fruiting in *S. commune* remains an interesting and testable possibility. For example, if FIS synthesis is required for all forms of fruiting, then specific inhibitors of the synthesis should also prove to be specific inhibitors of fruiting. Moreover, fruiting capacity in homokaryons and dikaryons should show positive correlation with the ability to produce and/or to respond to FIS. Finally, mutations which affect fruiting body initiation might, at least in some cases, be expected to affect metabolic reactions associated with FIS biosynthesis or utilization. Further work on these problems is anticipated following the chemical identification of FIS from the various sources mentioned above.

Thus far, all attempts to duplicate the effect of FIS with known vitamins, plant growth hormones, amino acids, nucleic acid components, and other simple nutrients have been ineffective beyond an occasional enhancement of vegetative aerial growth. FIS seems to be more specific in its action than these substances. Little can be said, however, concerning the underlying cellular mechanisms by which FIS exerts its effect. The appearance of brown pigment in the region where fruiting bodies ultimately arise suggests that one of the earliest stages in morphogenesis is the deposition of melanin-like pigments. Consequently, attempts are presently under way to determine whether FIS acts to induce phenoloxidases or related enzymes as a prerequisite to fruiting.

Summary.—A diffusible substance, which is capable of inducing fruiting bodies to develop in certain haploid mycelia of the Basidiomycete, *Schizophyllum commune,* has been detected in a number of fungi. Cell-free extracts of the substance, called fruiting-inducing substance (FIS), are stable to heat, acid and alkaline hydrolysis, and a number of degradative enzymes; the active principle is water-soluble and dialyzable. FIS is particularly abundant in fruiting bodies of *S. commune,* where it may function as a regulatory hormone.

* This work has been supported by a USPHS microbiology training grant (5T1-GM-503-07).
† Present address: The Biological Laboratories, Harvard University, Cambridge, Massachusetts.

[1] Raper, J. R., and G. S. Krongelb, *Mycologia,* **50,** 707–740 (1958).
[2] Raper, J. R., *Quart. Rev. Biol.,* **28,** 233–59 (1953).
[3] Raper, J. R., *Genetics of Sexuality in Higher Fungi* (New York: Ronald Press, 1966).
[4] Buller, A. H. R., *Researches on Fungi* (New York: Longmans, Green, 1924), vol. 3.
[5] Smith, A. H., *Mycologia,* **26,** 305–331 (1934).
[6] Biggs, R., *Mycologia,* **30,** 64–78 (1938).
[7] Dick, S., thesis: "The origin of expressed mutations in common-A heterokaryons of *Schizophyllum commune,*" Harvard University (1960).
[8] Niederpruem, D. J., H. Hobbs, and L. Henry, *J. Bacteriol.,* **86,** 1300–1308 (1964).
[9] Mishkin, S. L., thesis: "Genetic and cytological studies of a linked suppressor of the B-incompatibility factor and a haploid fruiting phenomenon in *Schizophyllum commune,*" Indiana University (1967).
[10] Raper, J. R., *Botan. Rev.,* **18,** 447–545 (1952).
[11] Urayama, T., *Botan. Mag.* (*Tokyo*), **70,** 29–30 (1957).
[12] Raper, J. R., and P. G. Miles, *Genetics,* **43,** 530–546 (1958).
[13] Gruen, H. E., *Plant Physiol.,* **38,** 652–666 (1963).
[14] Eger, G., *Arch. Mikrobiol.,* **50,** 343–356 (1965).