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COMPLEX.

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EXPERIMENTAL TAXONOMY OF THE
ENTOPHLYCTIS-RHIZOPHLYCTIS
CHYTRID COMPLEX

by

MOZAFFAR WADOOD HASSAN

A dissertation submitted to the Graduate Faculty in Biology
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy, The City University of New York

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ABSTRACT

EXPERIMENTAL TAXONOMY OF THE ENTOPHLYCTIS-RHIZOPHLYCTIS CHYTRID COMPLEX

by

MOZAFFAR WADOOD HASSAN

Adviser: Professor Solomon Goldstein

Features used in distinguishing taxa of the Entophlyctis-Rhizophlyctis group of chytrids include endobiotic or epibiotic nature of the thallus, whether sporangial development arises from the germ tube or by direct expansion of the zoospore, and the presence or absence of an operculum for zoospore discharge. A review of the literature, however, reveals that these morphological criteria are not sharply defined and vary considerably within differnt isolates of the same species depending upon substrate and environmental conditions. Structural and developmental variations in isolates of Entophlyctis and Rhizophlyctis make these criteria of questionable taxonomic worth.

Physiological parameters have commonly been used to identify genera and species of yeasts and pathogenic fungi. Several investigators have emphasized that nutritional characteristics should also be considered in the taxonomy of water molds, especially when the morphological and developmental criteria are not well defined.

Although the morphology of the *Entophlyctis*-*Rhizophlyctis* group of chytrids received considerable study, investigations of their physiology remained limited in number and scope. The present investigation was begun in order to expand our knowledge of the physiology of members of *Entophlyctis* and *Rhizophlyctis* as well as to to ascertain the value of nutritional data in the taxonomy of these chytrids. It was determined that substantially greater nutritional differences exist between the two genera than was found within either genus. *E. aureus* differed from *R. rosea* in the utilization of D & L arginine, D-lysine HCl, xylose, D-galactose and cellobiose whereas *R. harderii* was distinguished from *E. aureus* in the utilization of D-leucine, D-lysine HCl, L-methionine, D-valine, D-fructose, cellobiose, lactose,and raffinose. *Entophlyctis* sp. differed from *R. rosea* with respect to D & L arginine, arabinose, D-fructose, D-galactose, and raffinose. *R. harderii* showed physiological differences with *Entophlyctis* sp. in the utilization of D-leucine, D-lysine HCl, L-methionine, D-valine, arabinose, xylose, D-fructose, raffinose, cellobiose,and lactose.

The results obtained from nutritional studies suggest that these methods are of value in assessing the degree of similarity between the taxa of the *Entophlyctis*-*Rhizophlyctis* complex and may aid in studies of their taxonomy. Additional geographic isolates of each genus need to be analyzed in order to verify the constancy of nutritional differences.

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Introduction

Chytrids are considered among the most primitive of the true fungi. They are characterized by the presence of posteriorly uniflagellated zoospores and by a simple coenocytic thallus that lacks a true hyphal system. The thalli of the simpler forms in the group are unicellular and holocarpic in that they are entirely converted into one or more reproductive structures. However, in most chytrids the thallus is eucarpic, that is, the reproductive unit is formed from only a portion of the vegetative body and one to many branched or unbranched rhizoids are produced. The majority of these fungi are saprophytes occurring predominantly in fresh water and soil, but some attack algae and plants, and members of genera *Synchytrium* and *Physoderma* may parasitize economic plants such as potato and corn.

The recognition of chytrids as a taxonomic entity resulted from the studies of Alexander Braun (1851, 1855, 1856). Braun described his observations of chytrid-like organisms associated with the fresh-water algae *Hydrodictyon* and *Stigeoclonium*, but he was not quite sure as to whether they were parasitic. In his studies on chytrid-like organisms (1851, 1855), he described *Chytridium olla* and reported that it was a parasite on the oogonia of the alga *Oedogonium* and not a polymorphic form or antherozoid of the host as previously believed by Thwaites (1846). Gross (1851) described and figured an organism identifiable as the chytrid *Polyphagus euglenae* from *Euglena* (Nowakowski, 1878; Dangeard, 1886a, 1901; Scherffel, 1925; Bartsch, 1945). Unfortunately, he misunderstood the parasitic nature of the organism and believed the fungus to be a metamorphic form of the host. Later Braun (1856) described another chytrid associated with the alga Gloeococcus mucosus and named it Rhizidium apiculatum. Even after Braun's classic papers (1855, 1856), Pringsheim (1860) interpreted the zoospores of an endobiotic chytrid, Pringsheimiella (Couch, 1939), parasitic on Achlya as the antherozoids of the host. In 1865, de Bary and Woronin recognized chytrids as constituting a family, the Chytridiaceae, made up of three genera Chytridium (Braun, 1855), Rhizidium (Braun, 1856), and Synchytrium (de Bary and Woronin, 1865). Fischer (1892) in his well known monograph established a group, the "Archimycetes" coequal with Zygomycetes and Oomycetes. Fischer's Archimycetes (1892) included all the primitive aquatic Phycomycetes of simple body plan and consisted of chytrids, members of the Hypochytridiales (a small group with anteriorly uniflagellated zoospores), as well as members of the Lagenidiales (endobiotic forms that are primarily parasitic). Sparrow (1942, 1958, 1960) formed the order Chytridiales (chytrids) to include all the fungi which have a simple body plan and produce posteriorly uniflagellate zoospores. The single flagellum is of the whiplash type (the base of which is enveloped by a tubular covering; capable of active lashing movement). More recently, Alexopolous (1964) established the class Chytridiomycetes and included the Chytridiales as well as the Blastocladiales and the Monoblepharidales as orders. He considered the three groups as belonging to the class Chytridiomycetes chiefly on the basis that they all produce planogametes having a posterior whiplash flagellum

Morphological and developmental studies of many chytrids are known from unifungal culture on natural and artificial substrates (Rhizidium Zopf, 1885: Entophlyctis Karling, 1928, 1931; Booth, 1971: Cladochytrium Sparrow, 1931; Rhizophlyctis Ward, 1939; Stanier, 1942: Chytridium Koch, 1951). Several workers have described methods for their isolation, laboratory cultivation and maintenance (Zopf, 1887; Sparrow, 1931, 1935: Karling, 1935: Couch, 1939; Ward, 1939; Emerson, 1950: Koch, 1951). Zopf (1887) employed fern spores for the isolation of Rhizidium and other chytrids. This enrichment method was successfully utilized by Couch (1939) and his associates in establishing unifungal cultures of Rhizophlyctis, Rhizidiomyces, and other water molds but they used the pollen of Liquidambar styraciflua in place of fern spores. Emerson (1950) has stated that the pollen of gymnosperms and other plants serve as excellent bait for various members of the Chytridiales since pollen can be sterilized easily with propylene oxide. In an ecological study, Goldstein (1960a) reported on the relative efficacy of a variety of pollens as bait for the isolation of chytridiaceous fungi.

In addition to pollen, other natural and artificial substrates including algae, other water molds, wood, roots, grasses, leaves of higher plants, boiled filter paper, hair, horn, spider webs have also been used (Sparrow, 1939; Couch, 1939: Karling, 1941). In culturing these fungi, Couch (1939) reported good growth of Rhizophlyctis rosea on boiled filter paper, Sparrow (1939) observed species of Rhizidiomyces growing on moist blotting paper, and Karling (1941) found boiled straw of corn a suitable bait for isolating Cladochytrium replicatum. Sparrow (1931) was the first to report success in growing C. nowakowskii on corn meal agar, and Haskins and Weston (1950) grew R. rosea on a defined medium consisting of a mineral base with ammonium sulfate as the nitrogen source and glucose as the source of carbon.

Couch (1939), Stanier (1942), and Koch (1957) have outlined methods for securing bacterium-free cultures of chytrids on artificial media. Stanier's (1942) work indicated that chytrids were capable of digesting cellulose and he used a selective enrichment culture consisting of a mineral agar containing shredded filter paper for the isolation of Rhizophlyctis rosea. The fungus was subsequently isolated from the contaminating cellulose-decomposing myxobacteria by repeated streaking on fresh agar plates containing the filter strips.

Description of Entophlyctis and Rhizophlyctis.

Chytrids of the genera Entophlyctis and Rhizophlyctis are monocentric (possess a single reproductive center) and eucarpic. Rhizophlyctis is ubiquitous in soil and is probably the most widespread of all chytrids (Sparrow, 1960). Entophlyctis includes chytrids which are primarily inhabitants of fresh water, and grow on algae or other aquatic plants, but a few have been reported from soil (Sparrow, 1939; Haskins, 1946). The thalli of Entophlyctis and Rhizophlyctis are simple and consist of spherical or oval sporangia ranging in size from 20 to 230 µ and possess branched or unbranched rhizoids. These vegetative structures are aseptate, coenocytic, and do not attain the structural complexity of a hyphal system. Posteriorly uniflagellated zoospores bearing a single whiplash type flagellum are produced within a sporangium and upon maturity escape from it, swim for a while, encyst, and eventually germinate to form a new thallus. No sexual reproduction has been reported in these chytrids.

The genus Entophlyctis was established Fischer (1892) to include all species of lower Phycomycetes whose sporangia, rhizoids, and spores developed within the host cell. At first the genus included the species of Chytridium (Braun, 1855: Zopf, 1885), Rhizidium (Braun, 1856), and Diplophlyctis (Schroeter, 1893). Later Schroeter (1897) limited the genus Entophlyctis to the endobiotic (endophytic: intracellular) types whose zoosporangia lacked an apophysis (a subsporangial, globose structure bearing rhizoids). He segregated all apophysate endophytic species in his original genus Diplophlyctis (Schroeter, 1893). Later (1897) he also delineated Rhizidium to include chytrids that had one tap root-like rhizoidal axis, as distinguished from Entophlyctis which has several rhizoidal axes. The first accounts of Entophlyctis-like organisms were those given by Braun (1855, 1856) in two reports using the names Chytridium olla and Rhizidium apiculatum. They were observed to attack the algae Oedogonium and Gloecoccus. Cienkowski (1857) observed an Entophlyctis-like chytrids in the cells of Cladophora sp. and C. glomerata. Following Braun's creation of Rhizidium in 1856, Cienkowski placed them in that genus designating them as R. cienkowskii and R. confervae-glomeratae. Zopf (1885) discovered a form identical with Rhizidium apiculatum (Braun, 1856) on Gloecoccus mucosus and named the chytrid Chytridium apiculatum. The descriptions and figures by Braun (1856) and Zopf (1885) of R. apiculatum and C. apiculatum are the same except that Braun (1856) failed to observe rhizoids. Zopf (1885) and Sorokin (1889) in their studies of Cienkowski's chytrids noted that the organisms growing in the two hosts were morphologically identical and, therefore, they considered them as one species under the name Rhizidium cienkowskiana.

Dangeard (1886b) described Chytridium helioformes from the cells of Chara and in 1888 reported on it again as occurring in cells of Nitella and Vaucheria. In his two accounts (1886b and 1888) Dangeard gave no figures, but described the sporangia as spherical, being 10-20 µ in diameter with six to seven rhizoids, and producing spherical, uniflagellate zoospores that escape from the host cell through a sporangial neck. These chytrids remained in the genera Rhizidium (Braun, 1856; Cienkowski, 1857; Sorokin, 1889) and Chytridium (Dangeard, 1886b, 1888) until incorporated into Entophlyctis by Fischer (1892) and Schroeter (1897).

Karling (1928) was the first to give an extensive account of a member of this genus and detailed the occurence, development, and life cycle of Entophlyctis helioformes (R. apiculatum of Braun, 1855; C. apiculatum of Zopf, 1885). He reported the chytrid to be commonly associated with members of the algal family Characeae. Its zoospores contain a large, round refractile droplet, are 3-4 µ in diameter and have a single posteriorly attached flagellum 20-30 µ long. Upon exiting from the sporangium, the zoospore swims for 30 minutes to 2 hours and then comes to rest on the wall of the host, withdraws the flagellum, and forms a germ tube which subsequently penetrates the host and enlarges to become a new sporangium. As the thallus develops in the host cell, the zoospore body with its germ tube shrinks and ultimately disintegrates. The number of rhizoids produced on the surface of a sporangium varies from three to twelve. The rhizoids consist of a main trunk from which are developed numerous, small, anastomozing branches within the host cell. The mature sporangium measures 20-40 µ, has a wall and a granular cytoplasm containing a large number of refractile bodies, and it may bear one to three small discharge tubes. Zoospores begin to form in the sporangium about the time the sporangial tube starts to penetrate the wall of the host. Following Schroeter's (1897) concept of the delimitation of the genus Entophlyctis as, "endophytic types whose zoosporangia lacked an apophysis", Karling (1931) recognized ten species, namely, E. apiculata (Braun, 1855; Fischer, 1892; Schroeter, 1897), E. confervae-glomeratae (Cienkowski, 1857), E. cienkowskiana (Cienkowski, 1857; Zopf, 1885: Sorokin, 1889), E. tetrasporum (Fischer, 1892), E. bulligera (Zopf, 1884), E. vaucheria (Zopf, 1885; Schenk, 1920), E. spirogyrae (Fisch, 1884), E. heliomorpha (Dangeard, 1886b, Karling, 1928), E. characearum (Wildeman, 1896), E. brassicae and E. salicorniae (Nemec, 1912). Sparrow (1960) added four more species to the list of ten recognized by Karling (1931). Three species E. texana (Karling, 1941), E. pygmae (Sparrow, 1943) and E. aureus (Haskins, 1946) were discovered after 1931. However, E. rhizina, described by Minden in 1915, was not included in Karling's list (1931) probably because Minden (1915) considered the chytrid synonymous with E. vaucheria (Zopf, 1884) since he found it on Vaucheria germuta. Sparrow (1960) argued that the presence of a colored globule in the zoospores of *E. rhizina* distinguishes it from *E. vaucheriae* whose zoospores are characteristically without any colored globule. Since then (Sparrow, 1960), four more species, *E. lobata* (Willoughby and Townley, 1961), *E. molesta* (Canter, 1965), *E. reticulospora* (Cook, 1966), and *E. crenata* (Karling, 1967) have been described.

The genus *Rhizophlyctis* was established by Fischer (1892) and, as originally described in his monograph on the Phycomycetes, the genus also included *Rhizidium* (Braun, 1856). The lack of figures in Braun's original account of *Rhizidium* and the vagueness of his description led Fischer (1892) to interpret *Rhizidium* as synonymous with *Rhizophlyctis* Rhizidium was separated from Rhizophlyctis by Schroeter (1893) and Minden (1915). They emphasized the presence of a single main rhizoidal axis in *Rhizidium*, whereas several axes developed in *Rhizophlyctis*. The genus *Rhizophlyctis*, as characterized by Minden (1915), included monocentric, eucarpic, epibiotic chytrids whose sporangia develop from the direct expansion of the zoospores and possess several rhizoidal outgrowths. These morphological criteria for the genus have been maintained by Sparrow (1960) and he recognized eleven species belonging to *Rhizophlyctis* (Sparrow, 1960). The morphology and development of several species of *Rhizophlyctis* are known namely, *R. petersenii* (Sparrow, 1937), *R. rosea* (Ward, 1939; Stanier, 1942; Johanson, 1944; Haskins and Weston, 1950), *R. spinosa* and *R. chitinophila* (Karling, 1947, 1949), *R. harderii* (Uebelmesser, 1956). *R. rosea* is the most cosmopolitan member of the genus (Sparrow, 1960) and has been reported from soils of various parts of the world including France (Couch, 1939), the United States (Ward, 1939: Stanier, 1942; Sparrow, 1948), the Galapagos Islands

(Sorgel, 1941), and Brazil (Karling, 1947).

de Bary and Woronin (1865) first described Rhizophlyctis from flower pot soil. Ward (1939), in a study mainly devoted to the morphology and development of R. rosea on corn meal agar, reported the sporangia as being variable in shape and size, colorless when small but becoming yellow to golden upon maturity. The mature sporangium possesses many branched or unbranched rhizoids and one or more discharge tubes. The posteriorly uniflagellate spores are approximately 3.3 to 5.3 µ in diameter and are produced abundantly within the sporangium. The zoospores contain one or more dark brown globules and escape from the sporangium through a discharge tube, swim for up to four hours, encyst, and ultimately enlarge to form a sporangium. As the thallus matures, golden-yellow to brown pigment is produced and three or more branched rhizoids (some of which may extend 650 µ) develop near the base of the sporangium. Stanier (1942) studied the development of R. rosea on a synthetic medium containing mineral base, agar, and ammonium sulfate (0.1%) and glucose (0.5%) as nitrogen and carbon sources respectively. He noted that the sporangia on the glucose-agar medium were 40-50 µ in diameter with five to ten relatively thick extensively branched rhizoids whereas sporangia on the cellulose-agar were 2 to 3 times larger and produced as many 50-100 separate, thin rhizoidal outgrowths with very little branching.

Stanier (1942) has also reported the results of his nutritional experiments with Rhizophlyctis rosea. In his screening of carbon sources, he obtained good yields with cellulose, cellobiose, and glucose, but very little growth with xylose, mannose, sorbose, levulose, maltose, sucrose, lactose, starch, and chitin. In assessing the availability of nitrogen sources, glucose was used as the sole source of carbon. The nitrogen compounds tested were ammonium sulfate, potassium nitrate, alanine, aspargine, glutamic acid as well as complex materials such as yeast extract and peptone. Growth occurred with all these nitrogen sources except potassium nitrate.

Johanson (1944), in her studies of a *Rhizophlyctis rosea* isolate from Brazilian soil, observed that the discharge papilla of the mature sporangium possessed a thick membranous structure at its base. She introduced the term "endooperculum" for such a structure and made it the basis for separating the chytrid from *Rhizophlyctis*. She placed the isolate in a newly created genus, *Karlingia*, under the name *Karlingia rosea*. Karling (1947) confirmed endooperculation in *R. rosea* and also verified its presence in other "rhizophlyctoid" chytrids such as *Rhizidium* (Lindau, 1900), *Diplophlyctis* and *Amoebochytrium* (Zopf, 1884) and *Nowakowskiella* (Couch, 1939). Haskins (1948) made an extensive study of many strains designated as *Karlingia* by Johanson (1944) and noted that in some strains zoospore development occurred without any evidence of endooperculation. Subsequent observations by Haskins and Weston (1950) of species of *Rhizophlyctis* indicated endooperculation to be a variable characteristic with variation occurring not only between species but even in individual sporangia of the same clone. Sparrow (1960) believes that endooperculation is an unstable character and under certain conditions it may be formed in any chytrid, particularly those whose sporangia form discharge papillae (*Rhizophlyctis*, *Diplophlyctis*, and *Amoebochytrium*). Sparrow (1960) considers *Karlingia* (Johanson, 1944) to be synonymous with *Rhizophlyctis*. However, he (Sparrow, 1960) has suggested that if endooperculation is found to be a constant character in some rhizophlyctoid, chytrids, Johanson's (1944) segregation of the genus *Karlingia* may be justified.

Haskins and Weston (1950) examined various physical and chemical factors affecting the growth and pigmentation of *Rhizophlyctis rosea*. They noted that both light and oxygen had profound effects on pigment synthesis. Their study did not, however, distinguish between the separate effects of light and aeration on pigmentation. They observed that, with potassium nitrate as the sole nitrogen source, a brighter orange pigment was developed than in thalli grown with ammonium sulfate. With respect to carbon sources, cellobiose resulted in the production of more intense pigmentation than glucose. Their report did not clarify whether "more intense coloration" resulted simply from a greater amount of growth since one may get the illusion of more pigmentation from having more cells. They did not determine the amount of pigment on a per cell basis.

Sparrow (1937),in his article on "some chytridiaceous inhabitants of submerged insect exuviae",detailed the morphology of *Rhizophlyctis petersenii* growing on the empty cases of midges and dragonflies. He described the thallus as consisting of a spherical, subspherical, or ellipsoidal (occasionally irregular) sporangium varying from 50-170 µ in diameter and having 1-10 stout rhizoids extending up to 500 µ from various regions of the sporangium. The mature sporangium possesses a prominent, discharge tube (50x20 µ) with a refractile tip. The spherical zoospores are 5.2 µ in diameter and contain several colorless refractile granules in addition to a small orange-brown globule near the flagellum attachment. Resting spores were observed by Sparrow (1937) in several instances but early stages in the development of the thallus were not seen. Haskins (1939) studied the morphology and development of *R. petersenii* Sparrow cultivated on cellophane. He noted that sporangia often are over 350 µ in diameter and develop one to ten exit tubes. When mature they produce numerous zoospores. The resting zoospores produce the germ tube and in 2 to 3 days develop to form new thalli. In a study devoted mainly to *R. petersenii*, Haskins (1946) noted sporangial development from a swelling on the germ tube and not from the direct expansion of the encysted zoospore. Haskins emphasized this as a characteristic of the *Entophlyctis* type of development and placed it in the genus. Because of the distinct bright orange color of the mature sporangium, he (1946) named the fungus *Entophlyctis aureus* (sp. nov.).

The literature concerning *Entophlyctis aureus* is based only on reports of its ontogeny on insect exuviae and cellophane strips. The taxonomic status of *E. aureus* (Haskins, 1946) as a species novum has been disputed by Karling (1947) and by Sparrow (1960). They consider *E. aureus* a species of *Rhizophlyctis* (probably *R. rosea*). Karling (1947) reported that *R. petersenii* Sparrow was chitinophilic and produced an endooperculum. This characteristic is exhibited by some strains of *R. rosea* and, therefore, concluded that *R. petersenii* is actually *R. rosea* (*Karlingia rosea* Johanson) and not a new species of *Entophlyctis* as claimed by Haskins (1946). Sparrow (1960) has supported Karling's (1947) contention.

Morphological and developmental criteria used in the taxonomy of Entophlyctis and Rhizophlyctis.

The presumed difference between Entophlyctis and Rhizophlyctis is based primarily on the supposedly endobiotic development of Entophlyctis (Fischer, 1892; Schroeter, 1897; Karling, 1928, 1930) in contrast with the epibiotic nature of Rhizophlyctis thalli (De Barry and Woronin, 1865; Fischer, 1892: Minden, 1915; Karling, 1947). Furthermore, sporangia arise from the enlargement of the germ tube in Entophlyctis, whereas in Rhizophlyctis they form by the expansion of the encysted zoospore (Karling, 1928, 1930; Ward, 1939: Stanier, 1942; Haskins, 1946). These supposed generic differences are open to question since some species of both genera may occasionally develop in either way (E. confervae-glomeratae Karling, 1930; Barr, 1971a; Rhizophlyctis spinosa Karling, 1947: E. aureus Haskins, 1946; Karling, 1947; Booth, 1971).

Whiffen (1944) has suggested that "chance alone" determines whether thalli are intramatrical (endobiotic) or extramatrical (epibiotic). Miller (1968) too has stated that endobiotic or epibiotic thallus development varies depending upon the kind of substrate used and the environmental conditions under which the fungus is maintained. Karling (1947) reported that, in Rhizophlyctis spinosa, thalli grew either epibiotically or endobiotically when heat killed Spirogyra were used as a substrate,and Barr (1971a) demonstrated the endobiotic thallus development of E. confervae-glomeratae with an isolate grown on heat killed Allomyces arbuscula or Pilobolus kleinii. However, Barr (1971a) also determined that when pine pollen was used as bait, the fungus grew epibiotically, but if the pollen was sparsely distributed and the cultures contaminated with bacteria, then the chytrid developed endobiotically. Emerson (1950) argued that the use of endobiotic versus epibiotic development as a taxonomic criterion becomes meaningless when the organism is grown on nutrient media. He suggested that taxonomic work on the aquatic fungi should be based upon pure culture studies in defined media under controlled enviromental conditions. He also emphasized that such studies should include large number of isolates collected from many localities. Barr (1969a) has noted that morphological variation in isolates of Rhizophydium and Phlyctochytrium were far less marked on standardized nutrient media than on pine pollen.

Whiffen (1944) considered type of thallus development to be a reliable criterion for the classification of monocentric chytrids. She prefered to group these fungi using the criterion of whether the sporangium developed from the expansion of the encysted zoospore or by the enlargement of the zoospore germ tube. Karling (1947), Sparrow, (1947, 1960), Koch (1957), Salkin (1970) do not accept this as taxonomically valid since thalli of Entophlyctis and Rhizophlyctis can develop in either way (Karling, 1928; Booth, 1971; Barr, 1971a). Booth (1971) in describing the development of ten isolates of Entophlyctis reported that isolates 1 to 5 developed thalli by germ tube swelling, whereas 50% of the thalli of isolates 6, 8, and 10 developed by germ tube enlargement and the rest by the expansion of the encysted zoospore. The thalli of isolate 7 developed by germ tube swelling, but occasionally the encysted zoospore enlarged to form the sporangium. In his studies on the development of E. confervae-glomeratae, Barr (1971a) also noted that sporangia were formed from the expansion of the germ tube although sometimes the resting zoospore enlarged to become the sporangium. Booth

(1971) considers E. texanà, E. confervae-glomeratae and E. aureus doubtful taxa since they share characters of both Entophlyctis and Rhizophlyctis. Willoughby and Townley (1961), in describing E. lobata, found that the encysted zoospore produced a very small germ tube and consequently the developing sporangia and zoospores were in such close proximity that a definite germ tube was often difficult to observe. It appears that E. lobata forms a bridge between the true entophlyctoid chytrids such as E. apiculata and doubtful taxa such as E. aureus.

Sparrow (1942, 1960), Johanson (1944), and Karling (1947) have postulated that operculation of the sporangium could be used as a valid criterion for chytrid classification. Sparrow (1942, 1960) and Johanson (1944) recognize two parallel series in chytrids, the Inoperculatae, in which the zoospores are discharged following the deliquescence of a portion of the sporangial wall, and the Opeculatae, in which the sporangium produces a discrete operculum through which the zoospores emerge. Several workers (Haskins, 1948; Haskins and Weston, 1950: Willoughby and Townley, 1961: Koch, 1968; and Miller, 1968) have concluded that operculation is an unstable character and should not be given undue taxonomic importance. Booth (1971) maintains that a definite study of operculation and inoperculation should first be made in order to decide what constitutes an "operculum", since the very concept of this structure varies with different investigators. Terms such as "quasioperculation" (Umphlet and Koch, 1969) "false operculum" (Johnson, 1969), "endooperculation" (Johanson, 1944), and "exooperculation" (Sparrow, 1942) have not been defined with precision.

Other features such as shape, size, color, number of rhizoidal outgrowths from the sporangia, number of lipoid globules contained in thalli and or zoospores, as well as the length and type of attachment of the flagellum have been used to identify and distinguish species of Entophlyctis and Rhizophlyctis, these features vary considerably within different isolates of the same species. Among the factors responsible for these variations are the effects of different substrates, ecological conditions, and temperature (Bostick, 1968: Barr, 1969a: Hasija and Miller, 1970). These structural and developmental variations are not limited to the Entophlyctis-Rhizophlyctis complex, but are, in general, a complicating factor in chytrid taxonomy. Koch (1957) in his paper on Phlyctochytrium, Sparrow (1960) in describing the genus Rhizophydium, and Salkin (1970), in an investigation of Allochytrium, have all questioned the validity of some of the classic morphological criteria used to delineate a chytrid taxon precisely because the characters vary with cultural conditions.

From the preceding discussion it should be apparent that the use of structural and developmental criteria alone to define relationship between members belonging to the Entophlyctis-Rhizophlyctis group of chytrids are inadequate. Therefore, to clarify the taxonomic status of a chytrid, it may be necessary to resort to other techniques in addition to morphological and developmental studies.

Need for new techniques

Because of the scarcity of genetic studies, it is difficult to establish a clear species concept in chytrids. The formation and study of hybrids has been an important approach in establishing taxonomic relationships in higher fungi. Since chytrid reproduction is usually limited to the development of asexual zoospores, there is a scarcity of genetic data. The existence of sexual reproduction for most chytrids is still an open question. Sexuality has been noted in some members of Phlyctidiaceae (*Phlyctidium eudorinae*, Gimesi, 1924), Synchytriaceae (*Micromyces longispinosis*, Couch, 1931), and Rhizidiaceae (*Polyphagus euglenae*, Bartsch, 1945). It has not been feasible to construct chytrid hybrids such as Emerson and Wilson (1954) were able to do with *Allomyces*, a member of a related order (Blastocladiales). Mayr's (1942, 1969) definition of species "as groups of inbreeding natural populations that are reproductively isolated from other such groups" is not applicable to organisms in which sexual reproduction does not occur. Mandel (1969) has defined an asexual species as a collection of organisms which can be distinguished from other groups on the basis of structure and nutritional requirements as well as by biochemical, serological, and ecological characteristics. He has suggested that a natural classification of asexually reproducing organisms may result from studying the largest number of characters practicable in such groups. The phenotypic features which have generally been the basis for taxonomic distinctions reflect the genetic differences only incompletely. Therefore, an additional contemporary approach has been to uncover the non-morphological features of an organism which can be of taxonomic value. Such characters are particularly useful in the classification of morphologically simple organisms such as chytrids, yeasts, and bacteria. Sneath and Sokal (1962), Sokal and Sneath (1963) and Mandel (1969) advocate the Adansonian approach to systematics (numerical or empirical taxonomy). Numerical taxonomists record as many characteristics as possible and then group taxa according to the proportion of shared features. A broad spectrum of morphological, physiological, biochemical, and ecological features are examined and analyzed for similarities and differences. Adansonian biologists give equal weight to all characters (Sneath and Sokal, 1962: Sokal and Sneath, 1963: Mandel, 1969). Hatheway (1962), Cronquist (1964), Mayr (1964), do not accept the equivalency of all features and assign greater weight to characteristics which are consistently present in organisms and that are not readily affected by ecological changes. Thus, for mycologists, the structure of the reproductive organs are considered more significant than the number and kind of pigmented globules present in zoospores, whereas phycologists may stress the kind of pigments produced in different groups of algae. Modern taxonomists emphasize that comparison of related taxa should be based on as large a number of features that can be determined in a particular group by techniques currently available.

Physiological characteristics

Physiological criteria are used in bacterial, dermatophyte, and yeast taxonomy and the separation of yeast taxa is often based on nutritional tests (Wickerham and Burton, 1948; Blakely, 1951; Georg and Camp, 1957; Barnett, 1960, 1966; Lodder and Van-Rij, 1967). The suggestion that physiological studies might prove of value in fungal taxonomy was expressed as early as 1932 by Ciferri. He proposed that species be defined on several different bases including morphological, physiological and ecological ones.

Cantino (1950, 1955) was the first to emphasize the possible importance of physiological studies in the classification of chytrids and other water molds. He accepted Lwoff's hypothesis (Lwoff, 1936; Knight, 1945) that metabolic deficiencies have arisen in the evolution of microorganisms by the loss of functions originally present in the more primitive ancestral forms. On the basis of nutritional data, Cantino (1955) argued that chytrids may be considered the most primitive of the water molds. He inferred that the utilization of nitrate nitrogen and the synthesis of all vitamins would be among the endowments of primitive chytrids. Cochrane (1958) believes that it is highly unlikely that the inability to use nitrate can serve as a taxonomic criterion since the capacity can be lost by mutation. Further, even within a genus individual species and different isolates of the same species vary in their ability to utilize nitrate.

The pattern of vitamin deficiencies may also be irregular within species (Cochrane, 1958). Bonner (1946), Georg (1952), and Pontecorvo _et_ _al_., (1953) reported that strains of _Aspergillus_ and different isolates of _Penicillium_ varied in their vitamin requirements. Since marked differences within strains of a species is common, it is difficult to accept the proposition that vitamin requirements can yield valid criteria to separate taxa or provide evidence of relationship.

Patterns of carbon nutrition have been used as indicators of taxonomic relationship in groups of fungi where morphological differences are not clearly delineated (Cochrane, 1958). Thus, for example, Wickerham and Burton (1948) used different sugars, sugar alcohols and organic acids for the classification of *Hansenula*. Blakely (1951) used the sugar alcohols, sorbitol, ribitol, mannitol, erythritol and galacitol for identifying species of *Brettanomyces*. Barnett and Kornberg (1960) separated *Saccharomyces* species on the basis of malate, succinate, and citrate tests.

Existing accounts of chytrid physiology viewed from a taxonomic standpoint are relatively few and the classification of these fungi is based mainly on morphological characters. In a pioneering attempt to apply physiological data to a chytrid taxonomic problem, Crasemann (1954) determined nutritional differences in two related but morphologically distinguishable organisms--*Chytridium* sp. and *Macrochytrium botrydioides*. The two chytrids are operculate, monocentric and eucarpic. *Chytridium* species are widely distributed and are commonly found growing on fresh water algae, whereas *Macrochytrium* is monotypic, probably the largest of the monocentric chytrids (Sparrow, 1960), and grows on rotting fruits and vegetable debris. In *Chytridium*, the rhizoidal system consists of a single unbranched tap root-like structure and the resting spores are endobiotic. *Macrochytrium* possesses a thick rhizoidal axis profusely branched at the base and its resting spores are epibiotic. Crasemann (1954) studied the nutritional characteristics of the two fungi in pure culture and noted differences in the utilization of inorganic nitrogen as well as various carbon sources. Her experiments with diverse carbon compounds suggested that patterns of xylose, maltose, fructose and galactose utilization might also be used as adjunct criteria to distinguish the two chytrids. Her studies, however, did not include different isolates of the two chytrids so that it is difficult to evaluate her findings.

Nutritional data (Barr., 1969b) have also been used in connection with the classification of *Phlyctochytrium* and *Rhizophydium*, two of the most commonly reported chytrids. The morphological structures used to separate these taxa are not sharply defined, and vary under different conditions of growth. Members of the genus *Rhizophydium* are ubiquitous and Sparrow (1960) has suggested that extensive morphological work will be necessary before the limits of the genus can be established. *Rhizophydium* is distinguished from the closely related but less frequently encountered members of *Phlyctochytrium* primarily by the latter's possession of a subsporangial swelling--the apophysis-- and usually by their production of a multiaxiate (several axes) rhizoidal system. *Rhizophydium* normally has a single rhizoidal axis originating from the sporangium. The sporangium lacks an apophysis. Several workers have questioned the validity of these features for differentiating organisms of the *Phlyctochytrium*-*Rhizophydium* complex (Koch, 1957; Karling, 1967; Miller, 1968; Booth, 1971). However, Koch (1957) and Paterson (1963) have observed more than one rhizoidal axis in isolates belonging to the genus *Rhizophydium*. Additionally, Booth (1971) detected nonapophysate thalli of *Phlyctochytrium* *cheatiferum* growing on synthetic medium as well as on *Pinus* *sylvestris* pollen.

Goldstein (1960b) in his studies on the nutrition of *Phlyctochytrium* *punctatum* Koch and *Rhizophydium* *sphaerotheca* Zopf, reported that they differed markedly with respect to a variety of nutritional requirements. In addition to the physiological differences, he also provided micrographs showing the apophysis in *P. punctatum*, and the uniaxiate (single axis) rhizoidal system of *R. sphaerotheca*. Barr (1969a, 1969b), in a comparative study of the morphology and physiology of two species of *Rhizophydium* (*R. sphaerotheca* and *R. capillaceum*) and three isolates of *Phlyctochytrium* (*P. californicum*, *P. plurigibbosum*, and *P. acuminatum*), reported that *Rhizophydium* and *Phlyctochytrium* form two distinct physiological groups, whereas species within a genus show minor differences. In an attempt to find morphological distinctions under identical conditions of growth, he cultivated the aforementioned chytrids on a synthetic medium. Microscopic examinations of the thalli revealed that the rhizoids of the isolates of *Rhizophydium* were delicate and thin, whereas *P. californicum*, *P. plurigibbosum*, and *P. acuminatum* had a tubular rhizoid slightly enlarged near the base of the sporangium. He did not, however, observe a distinct apophysis among these members of *Phlyctochytrium* as was demonstrated by Goldstein (1960b) for *P. punctatum*. In *P. plurigibbosum*, he did note a small swelling at the base of the sporangium, but such swellings were also occasionally seen in *R. sphaerotheca* and *R. capillaceum*. Barr (1969a) on the basis of his morphological studies, has suggested that difference in rhizoidal structure serve as better distinguishing feature than apophysis formation since the occasional development of apophyses in some isolates of *Rhizophydium* does not justify placing them in the genus *Phlyctochytrium*. Barr (1970), in discussing the physiology of a recently described *Phlyctochytrium*, (*P. reinboldtae*) noted that the organism was very similar in its physiology to *P. punctatum* (Goldstein, 1960b) and *P. plurigibbosum* (Barr, 1969b). He speculated that if more physiological data were available on other isolates of *Phlyctochytrium* such as *P. papillatum* and *P. dictolum*, they might show nutritional traits similar to *P. punctatum* and *P. plurigibbosum* and could perhaps be considered as belonging to a physiologically related group of species.

From the scanty experimental literature available on chytrids, it is difficult to assess the reliability to physiological traits as aids in their classiffication and identification. Even so, attempts have been made to utilize the meager physiological data that are available as aids in establishing phylogenetic relationships (Cantino, 1950, 1955; Crasemann, 1954; Cantino and Turian, 1959; Barr, 1969).

This dissertation is concerned with the experimental taxonomy of chytrids belonging to the genera *Entophlyctis* and *Rhizophlyctis*. These genera are closely related and the structural and developmental criteria used to distinguish them are based on variable features. Although the morphology of the *Entophlyctis*-*Rhizophlyctis* group of chytrids has received considerable study (Ward, 1939; Stanier, 1942; Haskins, 1946; Sparrow, 1939, 1960; Karling, 1928, 1931, 1947, 1967; Miller, 1968; Booth, 1971; Barr, 1971a), investigations of their physiology have been limited in number and scope (Stanier, 1942; Haskins and Weston, 1950; Barr, 1971b). The growth requirements of relatively few of these primitive fungi are known, and such scanty data that are available have not been examined from a comparative taxonomic standpoint. The purpose of this investigation is to extend our knowledge of the group's physiology as well as to assess to value of nutritional characteristics in their taxonomy. Furthermore, the work will provide data leading to the development of a chemically defined medium for the cultivation of *Entophlyctis* sp., *E. aureus*, *R. harderii*, and *R. rosea*.

Materials and Methods

Organisms and their sources

The axenic cultures used in this investigation were obtained from several sources. Entophlyctis sp. and Rhizophlyctis harderii Uebelmesser were supplied by Dr. Tom Booth of the University of British Columbia, Vancouver, Canada. An axenic culture of Rhizophlyctis rosea DeBary & Woronin was obtained from Centraalbureau Voor Schimmelculture, Oosterstraat 1, Baarn, Holland (Netherlands). An isolate of Entophlyctis aureus Haskins in pure culture was provided by Professor Solomon Goldstein.

Media

Three basic media were used for physiological experiments and a uniform set of experimental methods was employed throughout this study. Unless otherwise indicated, all media were prepared in 10 ml aliquots in 18 ml Pyrex glass tubes provided with plastic screw caps. They were sterilized by autoclaving at 125 C, 15 pounds pressure for 15 minutes. Hydrogen ion concentrations were measured after sterilization. All liquid nutrient media contained 0.1% agar (Bacto) to provide a proper density to retain cells at the top of the liquid medium, since preliminary experiments revealed that the four chytrids showed little growth under microaerophilic conditions.

Medium 1.

Medium 1 (Table 1) was routinely used to maintain the cultures of the chytrids. One gram of agar (Bacto) was dissolved in one liter of hot glass-distilled water and thereafter components of the medium (Table 1) were added. The mixture was stirred for approximately ten minutes on a hot plate/magnetic stirrer. The hydrogen ion concentration of the medium was adjusted to a pH of ± 7.2 and 10 ml of the medium was dispensed into 18 ml clean and dry Pyrex glass tubes with plastic screw caps. The ingredients in medium 1 were autoclaved together since this procedure was not found to exert either inhibitory or stimulatory effects on growth.

TABLE 1

Composition of medium 1 per liter of distilled water

Glucose	2.00 g
Bacto-tryptone	5.00 g
$K_2HPO_4 \cdot 3H_2O$	0.50 g
$MgSO_4 \cdot 7H_2O$	0.25 g
Chytrid mineral mix[a]	5 ml
$FeCl_3 \cdot 6H_2O$[b]	1 ml
$CaCl_2$[c]	1 ml
Agar (Difco)	1.00 g
Glass distilled water made up to a liter	

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[a] Contains 30 mg H_3BO_3 ; 95 mg $CuSO_4 \cdot 5H_2O$; 25 mg H_3MoO; 250 mg $MnSO_4 \cdot H_2O$; 395 mg $ZnSO_4 \cdot 7H_2O$; 5 mg $CoCl_2 \cdot 6H_2O$; 5 mg $CuCl_2 \cdot 2H_2O$; distilled water 500 ml

[b] 2 mg/ml stock solution

[c] 10 mg/ml stock solution

Subcultures of the chytrids were routinely made every sixth day by transferring 0.5 ml of the culture to the fresh nutrient medium. Medium 1 was also used in the determination of growth curves and in temperature and pH experiments.

Medium 2 (Table 2)

This medium was essentially the same as medium 1 except that bacto-tryptone was replaced by enzyme hydrolyzed, vitamin-free casein hydrolysate. The medium was utilized to culture the organisms for three serial sub-transfers before their vitamin requirements were determined.

TABLE 2

Composition of medium 2 per liter of distilled water

Glucose		2.00 g
5% vitamin-free casein hydrolysate (enzymatic; Difco)		100 ml
$K_2HPO_4 \cdot 3H_2O$		0.5 g
$MgSO_4 \cdot 7H_2O$		0.25 g
Chytrid mineral mix[a]		5 ml
$FeCl_3 \cdot 6H_2O$	(2 mg/ml)	1 ml
$CaCl_2$	(10 mg/ml)	1 ml
Agar (Difco)		1 g
Distilled water made up to a liter		

[a] Same as 'a' in Table 1, page 25.

Medium 3 (Table 3)

This defined medium was used for assaying vitamin, nitrogen, and carbon requirements. It was also used in growth, temperature, and pH experiments.

TABLE 3

Composition of medium 3 per liter of distilled water

Ammonium nitrate[a]		4.0 g or 0.05M
Glucose[b]		4.0 g or 0.05M
$K_2HPO_4 \cdot 3H_2O$		0.5 g
$MgSO_4 \cdot 7H_2O$		0.25 g
Chytrid mineral mix[c]		5 ml
$FeCl_3$ $6H_2O$	(2mg/ml)	1 ml
$CaCl_2$	(10 mg/ml)	1 ml
Agar		1 g
Vitamins[d]	0.2 ug; 20 ug; 100 ug per 10 ml	

[a] 4 g ammonium nitrate was employed in vitamin experiments and 0.05M in the carbon assimilation tests.

[b] 4 g of glucose was provided as the carbon source in vitamin experiments and 0.05M in the survey of utilizable nitrogen compounds.

[c] Same as 'a' in Table 1, page 25.

[d] Appropriate vitamins were added to the above medium for assaying exogenous requirements.

In an effort to determine the optimal nutrient concentrations, carbon (glucose) and nitrogen (ammonium nitrate) were incorporated at the following molarities; 0.01M, 0.05M, and 0.1M in all possible combinations. Maximum growth in the four chytrids occurred at a concentration of 0.05M glucose and 0.05M ammonium nitrate (Table 4). Unless otherwise noted, carbon and nitrogen compounds tested were used at this concentration. It cannot be asserted that the optimal concentration of other carbon and nitrogen compounds would be identical;

TABLE 4

Effect of different concentrations of glucose and ammonium nitrate on growth of *Entophlyctis* sp., *E. aureus*, *R. harderii*, and *R. rosea*.[a]

	0.01M C[b] 0.01M N[c] Klett[d]		0.01M C 0.05M N Klett		0.01M C 0.1M N Klett		0.05M C 0.01M N Klett		0.05M C 0.05M N Klett		0.05M C 0.1M N Klett		0.1M C 0.01M N Klett		0.1M C 0.05M N Klett		0.1M C 0.1M N Klett	
	Exp. 1	Exp. 2	Exp. 1	Exp 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Entophlyctis sp.	7	8	11	13	30	33	33	36	28	31	25	29	26	28	27	25	30	32
E. aureus	9	11	12	14	12	12	31	31	30	34	22	26	28	25	29	24	24	27
R. harderii	11	12	11	13	12	15	24	26	32	36	23	25	24	25	22	23	19	21
R. rosea	8	9	9	11	8	9	23	26	31	29	24	23	24	25	18	19	14	17

[a] All chytrids in stationary culture for two weeks at 22 C. Initial pH for all cultures was 7.5

[b] Refers to carbon source, glucose

[c] Refers to the nitrogen source, ammonium nitrate

[d] Mean Klett reading derived from two experiments (Exp.1 & Exp.2), three replicates per experiment

nevertheless, it provided a reasonable basis for making comparisons. A 0.1M concentration of glucose and ammonium nitrate resulted in a drop in pH (between 4-6) in poor yields of the four chytrids. Among aquatic phycomycetes, inability to utilize carbon and nitrogen at elevated concentrations is perhaps due to a combination of factors such as decline in pH, increased osmotic pressure, and increased excretion of toxic metabolites into the medium (Foster, 1949; Cochrane, 1958).

Carbon sources and vitamins were separately sterilized by filtration or by autoclaving and added aseptically to the basal medium. Carbon compounds were filter-sterilized using Nalgene filter units containing a 0.45 u grid membrane. Because vitamins are known to decompose under heat sterilization at neutral and alkaline pH's, the vitamin solutions were separately autoclaved at pH 4.5 and added aseptically to the basal medium. Three ranges of vitamin concentrations (0.2 µg/10 ml, 20 µg/10 ml, 100 µg/10 ml) were used in studying used in studying growth factor exogenous requirements as well as when studying the effect of higher doses of vitamins on the growth of the four organisms.

Chemicals

The chemicals employed in this study were of reagent grade. The majority of the organic compounds were obtained from the Nutritional Biochemicals Corportion. Agar and Bacto-tryptone were purchased from Difco Laboratories. The source of inorganic chemicals was Fischer Scientific Corporation.

Glassware was machine-washed twice using detergent (Borax) and warm water followed by three rinses in distilled water. The vessels were then permitted to drain dry. Pipets used for inoculation were autoclaved at 125 C, 15 lbs. pressure for 15 minutes and then oven-dried at 180 C for 2-4 hours.

Inoculum

Zoospore suspensions for use as inoculum were obtained as follows. Stock cultures of the chytrids were grown on slants of medium 1 solidified with 1.5% agar. After an incubation period of 8 days at 22 C, 3-4 ml of sterilized distilled water was poured over the slants so that after an hour a layer of water remained at the surface of the solidified medium. The water facilitated the discharge of the sporangia. Rolling the liquid over the agar surface several times during the hour resulted in good harvests of zoospores. The thick zoospore suspension was diluted with 5 ml of sterilized basal salt medium (BSM, Table 5).

Table 5

Composition of basal salt medium (BSM)

$MgSO_4$	$10^{-3}M$
$CaCl_2$	$10^{-3}M$
NH_4NO_3	$2.5x10^{-3}M$
KCl	$8x10^{-3}M$
$K_2HPO_4.3H_2O$	$5x10^{-3}M$

The density of the zoospore suspensions was determined by counting the cells with an hemocytometer. Subsequently, 1 ml of the basal salt medium containing 10^8 cells was used as inoculum per tube. Preliminary experiments designed to test the standardization of the inoculation procedure revealed that growth was slow with a light inoculum (0.5×10^8 cells) and was more rapid with a higher (1.5×10^8 cells) inoculum. Using the latter zoospore concentration resulted in a drop in pH in the culture especially in the defined medium (Table 6).

Measurement of growth

Since the fungi utilized in the present investigation have simple thalli, do not possess hyphae, and consequently do not clump together, turbidimetric method was used for the measurement of growth. The turbidity of a suspension depends on the size and concentration of particles and, therefore, a photometric measurement corresponds to the mass of the material present in a suspension. The photo-electric colorimeter (Klett-Summerson, model 9003) using a blue filter (number 42) was used for measuring growth. The Klett readings were calibrated against serial concentrations of cells as well as against cellular nitrogen at different concentrations. Figure 1 illustrates the correlation. The calibration curve was plotted by measuring the Klett response to four serial concentrations of prepared suspensions. In addition, the instrument was also calibrated against total nitrogen at different concentrations. The two methods gave satisfactorily parallel results. Klett readings were linear over a wide range except at a very high or low turbidities.

TABLE 6

Effect of quantity of inoculum on growth of _Entophlyctis_ sp., _E. aureus_, _R. harderii_, and _R. rosea_.[a]

Inoculum	Medium 1								Medium 3							
	Entophlyctis sp.[b] Klett		_E. aureus_ Klett		_R. harderii_ Klett		_R. rosea_ Klett		_Entophlyctis_ sp. Klett		_E. aureus_ Klett		_R. harderii_ Klett		_R. rosea_ Klett	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
0.5×10^{8} cells (Zoospores)	60	68	56	59	49	52	45	49	19	18	17	19	18	21	15	18
10^{8} cells (Zoospores)	100	116	82	87	70	76	33	29	29	26	30	29	30	33	29	27
1.5×10^{8} cells (Zoospores)	123	111	88	86	91	83	31	28	26	24	25	26	28	25	28	23

[a] All chytrids in stationary liquid culture for two weeks at 22 C. Initial pH for all cultures was 7.5.

[b] Mean Klett readings derived from experiments 1 & 2, three replicates in each experiment.

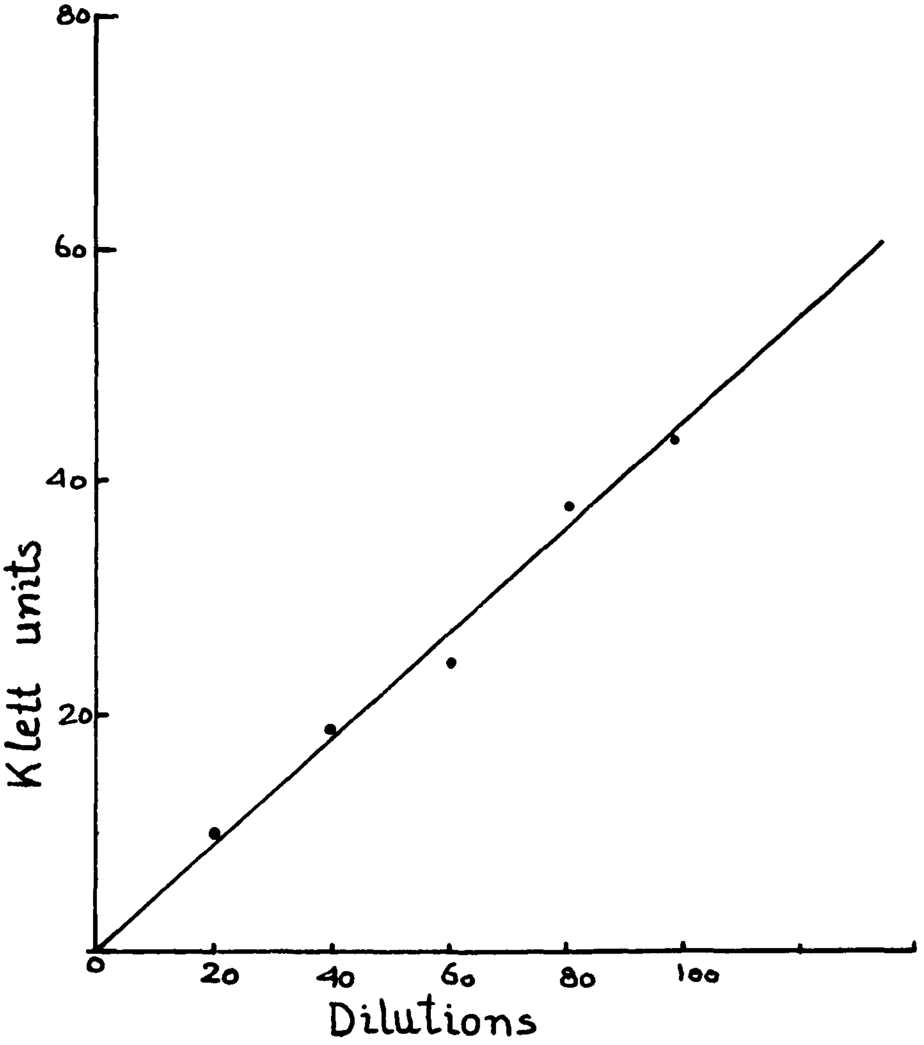

Fig. 1. Relationship between culture (inoculum) dilution and Klett readings.

Results and discussion

Growth curve

Figures 2 and 3 show the growth curves in the four chytrids in medium 1 and medium 3. Although, the relative amount of growth differed in the four chytrids, maximum growth occurred in 4 to 5 days when the organisms were grown in medium 1 and 7 to 8 days in the defined medium. Growth was accompanied by a drop in pH of the medium.

Temperature requirements

In the temperature experiment optimum yields were recorded between 19-28 C in medium 1 and 19-25 C in medium 3 with a two week incubation period (Figures 4 and 5). The four organisms showed very little growth below 10 C or above 30 C. The temperature-growth curve exhibits a rapid decline above 25 C in the defined medium whereas the same effect is observed beyond 30 C when the chytrids are grown in the complex medium (medium 1).

The effect of temperature on growth is a variable feature and different isolates or clones may exhibit different temperature optima. The shape of the temperature-growth curve is also somewhat affected by the time of incubation and supply of nutrients in the culture medium (Fries, 1953; Cochrane, 1958; Barr, 1971b). Barr (1971b) in his studies on the physiology of *Entophlyctis confervae-glomeratae*, noted that with an incubation period of two weeks, isolate 81 grew best at 25 C whereas isolate 9 showed maximum yields at 20 C. Furthermore, he (Barr, 1971b)

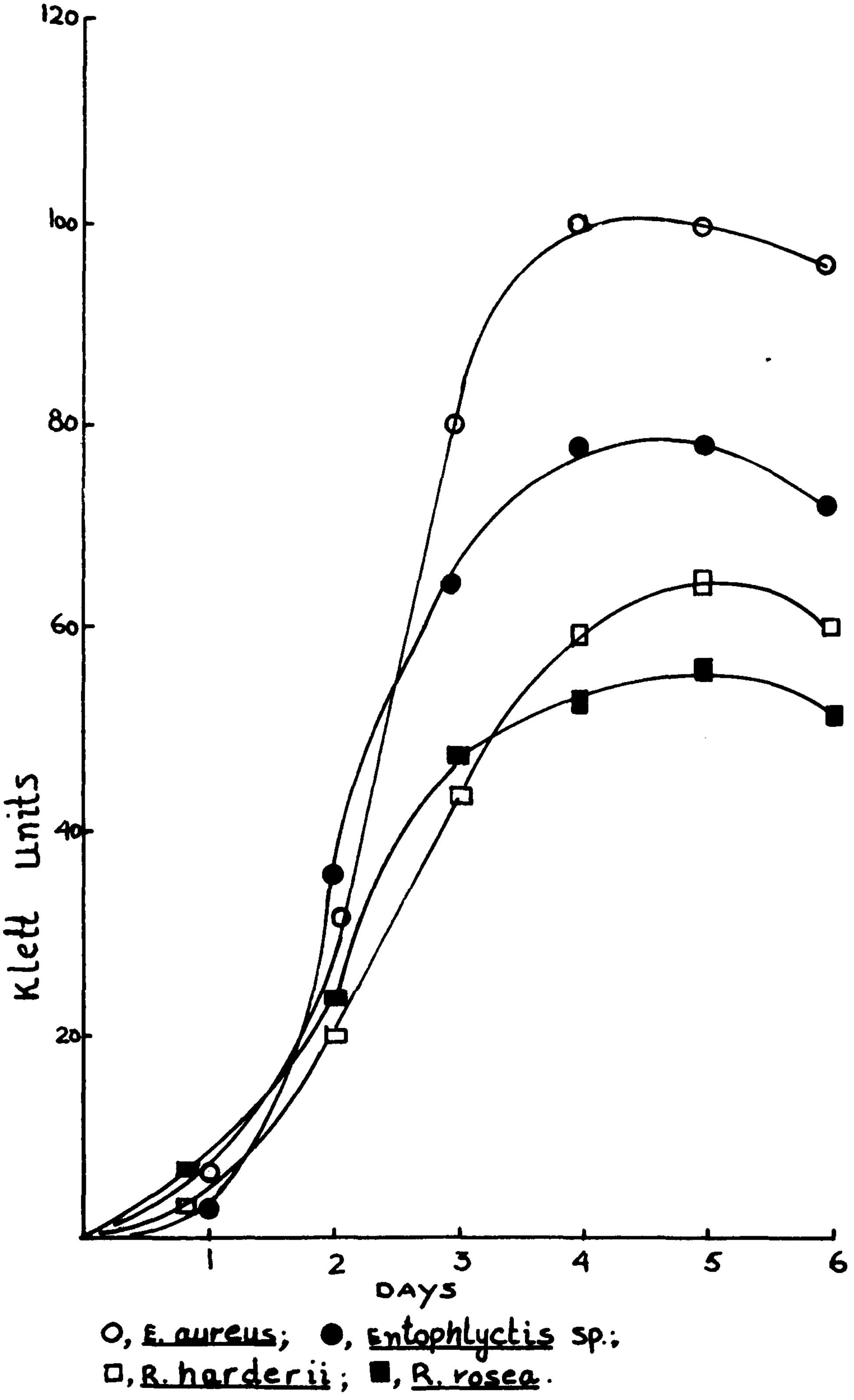

Fig., 2. Growth in medium 1.

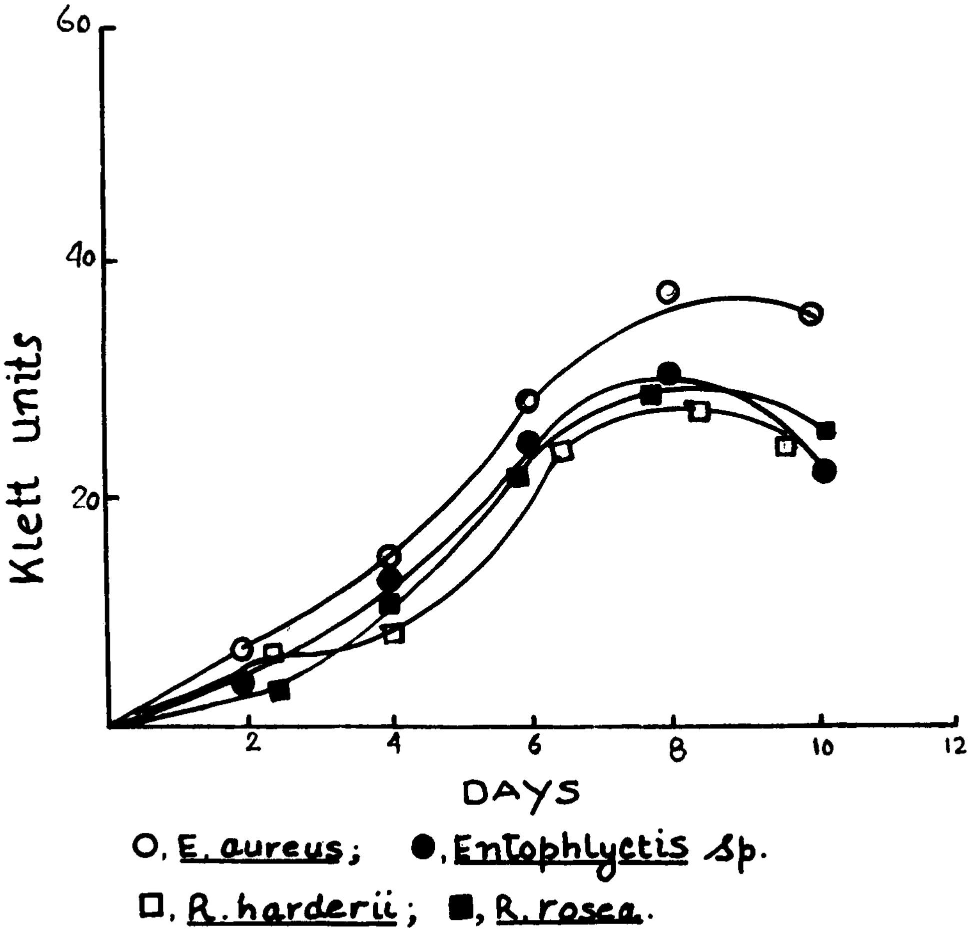

Fig., 3. Growth in medium 3.

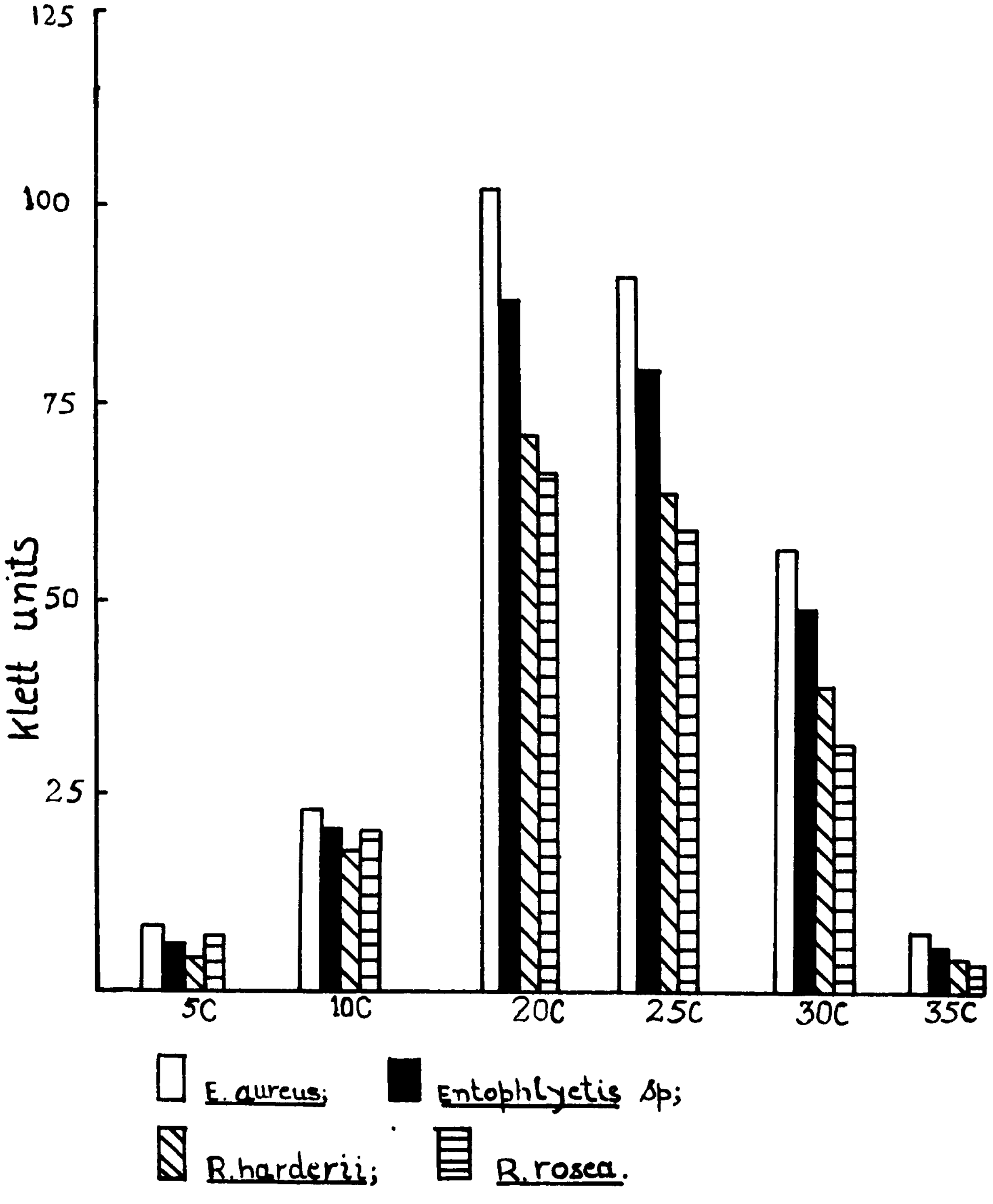

Fig. 4. Growth at different temperatures in medium 1.

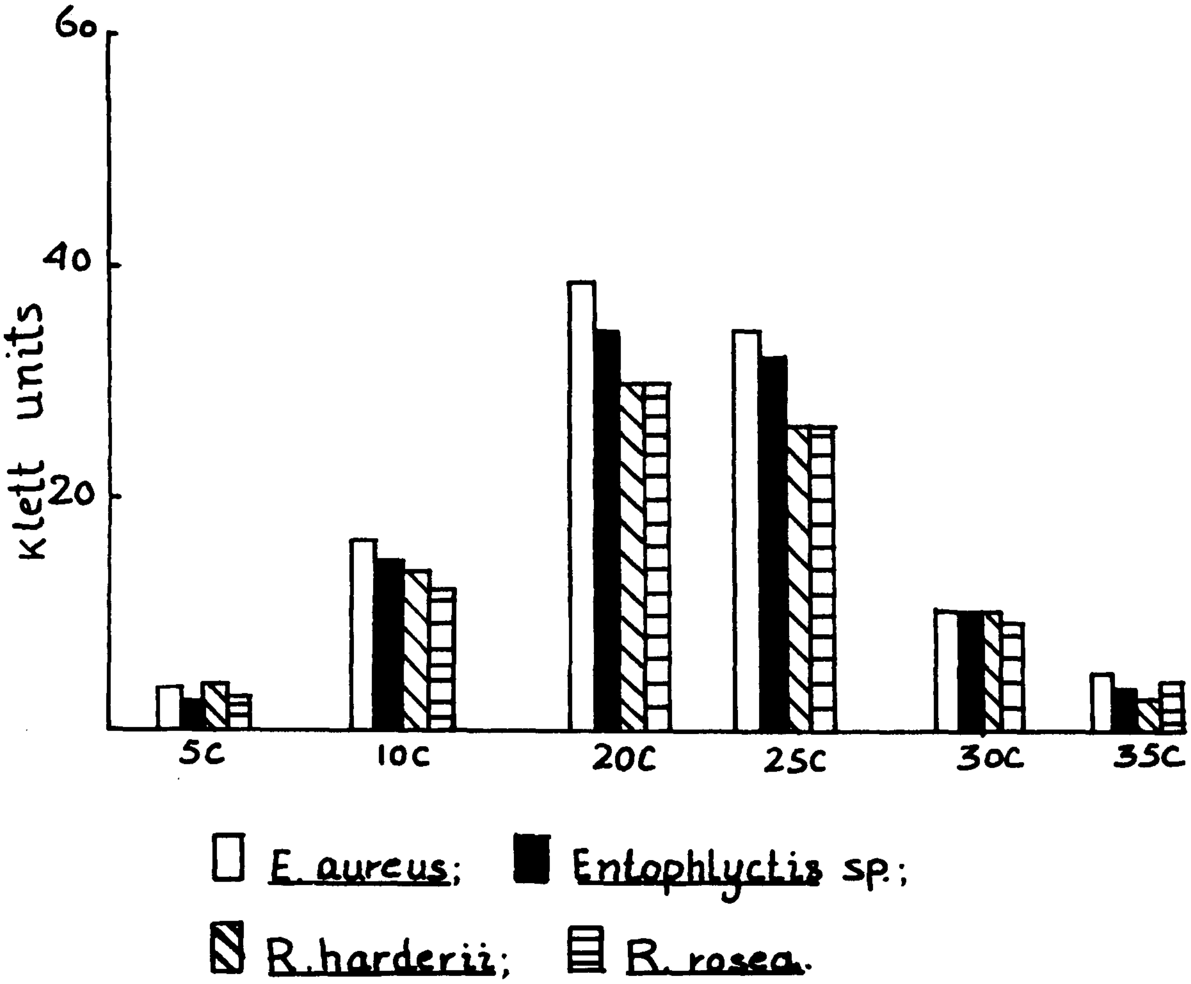

Fig. 5. Growth at different temperatures in medium 3.

observed that although isolate 9 attained maximum yields at 20 C when incubated for two weeks, a three week period was required at 15-20 C.

pH effect

Medium 1 and medium 3 were adjusted to different pH levels by adding 2N HCl or 2N NaOH. Figures 6 and 7 show the effect of pH on growth at 22 C. The range of intial pH that supported growth in the four chytrids was between 6 to 8.5. Entophlyctis aureus showed optimum growth between the pH of 6.5 to 8.0, whereas the pH range for optimum growth of Entophlyctis sp. was 7.5 to 8.5. Rhizophlyctis harderii yielded best growth at pH 8.0 to 8.5 and Rhizophlyctis rosea showed maximum growth at a pH of 7.5. Phosphate buffer was not used in pH tests since it was found to be toxic to the chytrids. The sensitivity of chytrids to inorganic phosphate has been noted by several workers (Crasemann, 1954; Goldstein, 1960b: Barr, 1971b). Crasemann (1954) reported that the growth of Chytridium sp. and Macrochytrium botrydioides was impaired by concentration of phosphate above 0.003 M. Barr (1971b) found phosphate and tris buffers toxic to isolates of Entophlyctis confervae-glomeratae.

Exogenous vitamin requirements

Because some members of this group have been reported (Cantino, 1955; Cochrane, 1958; Fries, 1965) to be dependent upon their environment for more than one vitamin,it was decided to examine the response to such growth factors in a drop out series. The vitamin requirements of the four fungi were tested with medium 3 to which no vitamins were added,

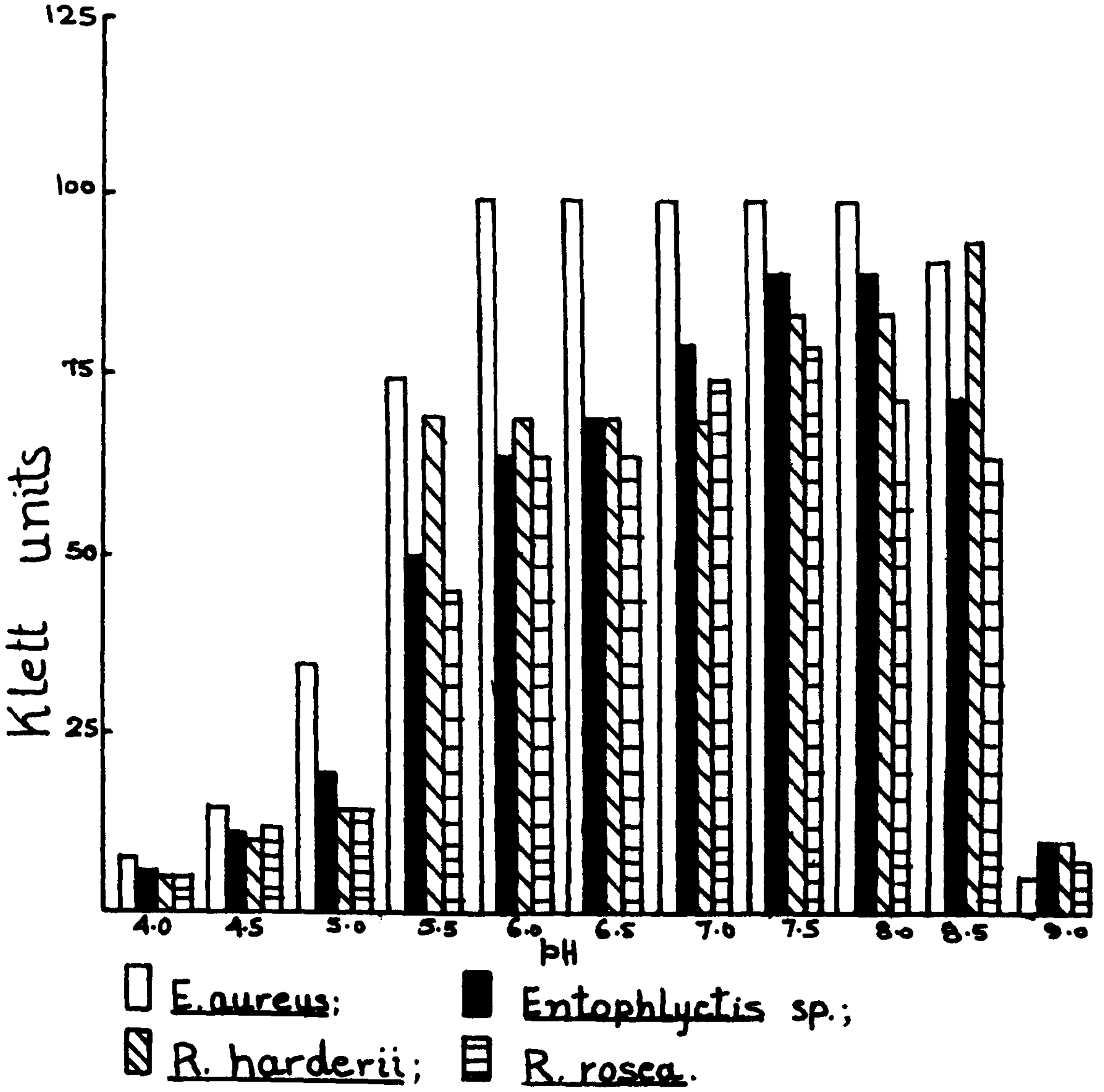

Fig. 6. Effect of pH on growth in medium 1 at 22 C.

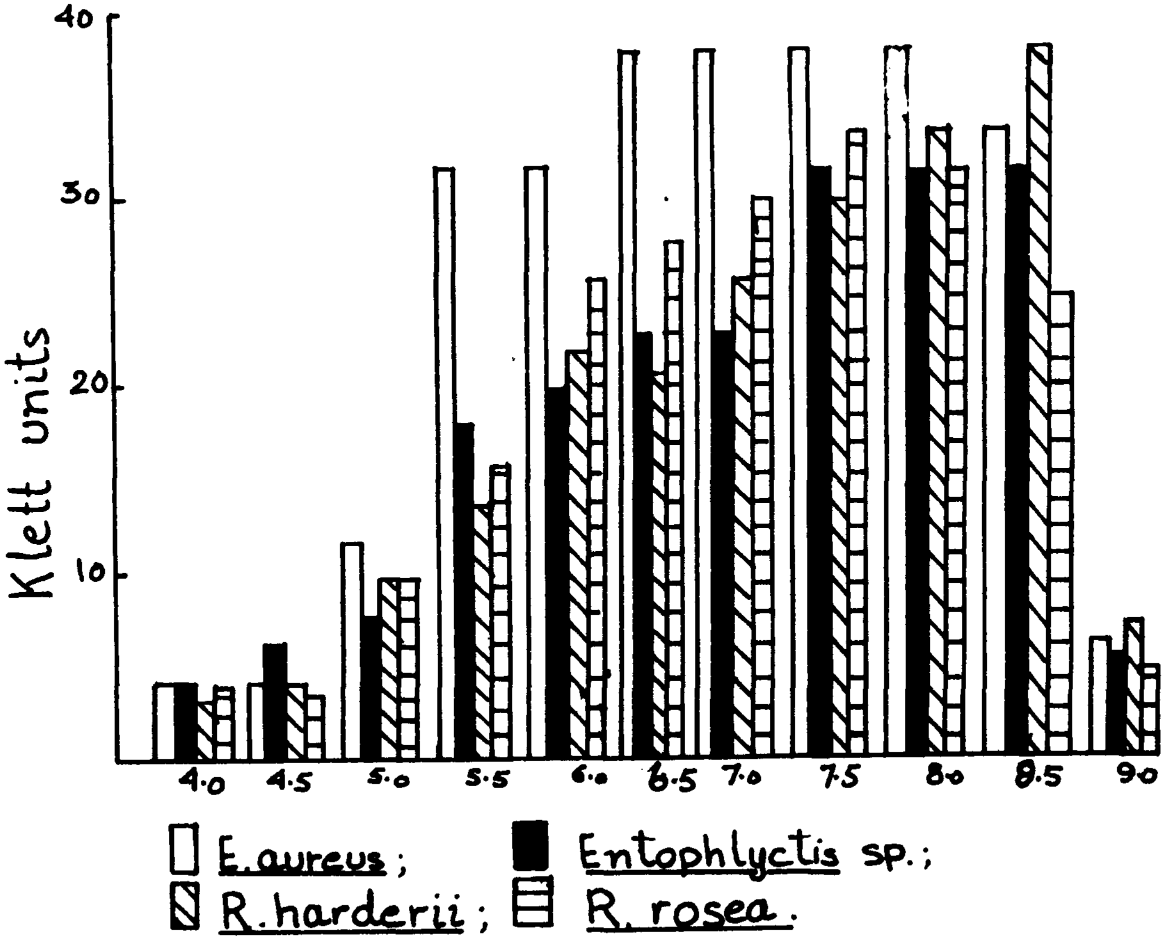

Fig. 7. Effect of pH on growth in medium 3 at 22 C.

medium 3 plus a supplement of all the 11 vitamins listed in the Table 7, and in a drop-out series of this nutrient solution such that only one vitamin was ommitted from each batch in the series. Since vitamins may be required in very minute amounts, precautions were taken to avoid carrying over traces of vitamins with the inoculum. The chytrids were grown through four transfers in a vitamin-free medium (medium 2, Table 2, page 26) and zoospores for inoculum were harvested from the fourth generation. A vitamin solution (1 ml) was added aseptically to 9 ml of medium 3 and the final pH of the medium before inoculation was ± 7.5.

The results given in Tables 7,8, and 9 indicate that the chytrids grew without an exogenous supply of vitamins. However, this does not preclude the possibility that maximal yields may have been attained more rapidly as a consequence of incorporating additional growth factors in the medium. Barr (1971b) in his studies on Entophlyctis confervae-glomeratae also reported that addition of thiamine (0.2 ppm) to the defined medium had no effect on final yields. Vitamins added at a concentration of 100 ug/10 ml were inhibitory to growth, a phenomenon previously reported for other fungi (Ryan, Kunin, Ballentine and Maas, 1953; Chiao and Peterson, 1956; Cochrane, 1958; Esposito et al. 1962). The mechanism of inhibition is not known. Certain thiamine-auxotrophic species of Rhizopus (Schopfer, 1935, Fusarium (Elliot, 1949; Esposito et al. 1962), and the yeast, Saccharomyces carlsbergensis (Chiao and Peterson, 1956) respond to added thiamine with curtailed growth. Reed et al. (1959) demonstrated that the growth of Saccharomyces cerevisiae is inhibited by concentrations of p-aminobenzoic acid higher

TABLE 7

The effect of vitamins on growth of <u>Entophlyctis</u> sp., <u>E. aureus</u>, <u>R. harderii</u>, and <u>R. rosea</u>.[a]

Vitamin added[b]	<u>Entophlyctis</u> sp. Klett[c] readings		<u>E. aureus</u> Klett readings		<u>R. harderii</u> Klett readings		<u>R. rosea</u> Klett readings	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
None[d]	30	28	27	29	30	29	28	29
All vitamins listed below	28	28	24	28	28	29	25	28
All but thiamine	27	29	25	28	27	29	26	28
All but biotin	29	29	28	28	24	29	27	29
All but nicotinamide	26	24	28	29	27	29	26	31
All but p-amino benzoic acid	24	20	25	29	29	28	28	27
All but ascorbic acid	27	29	23	20	25	26	23	28
All but riboflavin	30	26	28	27	27	26	25	28
All but calcium pantothenate	24	28	23	28	25	29	24	29
All but folic acid	29	26	27	26	21	26	23	24
All but pyridoxine	27	28	21	28	28	26	25	24
All but lipoic acid	26	26	24	27	27	27	24	26
All but Vit. B_{12}	29	27	28	29	25	28	23	28

[a] All chytrids in stationary liquid culture for two weeks at 22 C. Initial pH of all cultures was 7.5

[b] 2 μg/10 ml of each vitamin listed in the text.

[c] Mean Klett readings of experiments 1 & 2, three replicates per experiment

[d] Glucose and ammonium nitrate (0.05M each) served as carbon and nitrogen sources in all test media

TABLE 8

The effect of vitamins on growth of *Entophlyctis* sp., *E. aureus*, *R. harderii*, and *R. rosea*.[a]

Vitamin[b] added	*Entophlyctis* sp. Klett[c] readings		*E. aureus* Klett readings		*R. harderii* Klett readings		*R. rosea* Klett readings	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
None[d]	28	28	26	29	31	27	27	28
All vitamins listed below	22	20	21	19	28	27	24	20
All but thiamine	24	21	23	20	25	28	21	20
All but biotin	21	20	20	20	23	22	19	18
All but nicotinamide	25	28	22	19	23	19	21	17
All but p-amino benzoic acid	23	22	21	19	20	18	22	24
All but ascorbic acid	25	28	24	29	18	20	21	18
All but riboflavin	22	20	21	18	22	20	23	21
All but calcium pantothenate	19	21	19	20	21	19	23	21
All but folic acid	23	24	21	20	22	18	21	20
All but pyridoxine	22	26	21	19	21	20	20	19
All but lipoic acid	20	20	19	20	22	21	24	28
All but Vit.B_{12}	19	20	22	18	21	19	21	19

[a] All chytrids in stationary liquid culture for two weeks at 22 C. Initial pH of all cultures was 7.5

[b] 20 µg/10 ml of each vitamin listed in the text

[c] Mean Klett readings of experiments 1 & 2, three replicates per experiment

[d] Glucose and ammonium nitrate (0.05M each) served as carbon and nitrogen sources in all test media

TABLE 9

The effect of vitamins on growth of *Entophlyctis* sp., *E. aureus*, *R. harderii*, and *R. rosea*.

Vitamin[b] added	*Entophlyctis* sp. Klett[c] readings Exp. 1	Exp. 2	*E. aureus* Klett readings Exp. 1	Exp. 2	*R. harderii* Klett readings Exp. 1	Exp. 2	*R. rosea* Klett readings Exp. 1	Exp. 2
None[d]	27	28	27	28	29	27	27	28
All vitamins listed below	12	12	11	13	14	11	11	13
All but thiamine	13	12	13	12	11	11	13	12
All but biotin	12	11	12	11	14	12	12	11
All but nicotinamide	10	12	14	12	11	11	12	11
All but p-amino benzoic acid	13	12	15	18	12	10	11	11
All but ascorbic acid	14	16	16	19	13	18	14	12
All but riboflavin	14	16	13	12	11	11	12	12
All but calcium pantothenate	16	16	14	12	13	16	14	18
All but folic acid	13	18	12	12	12	12	12	11
All but pyridoxine	12	12	10	11	14	16	11	11
All but lipoic acid	11	12	10	11	14	16	13	17
All but Vit. B_{12}	14	12	15	17	12	12	14	16

[a] All chytrids in stationary culture for two weeks at 22 C. Initial pH for all cultures was 7.5

[b] 100 μg/10 ml of each vitamin listed in the text

[c] Mean Klett readings of experiments 1 & 2, three replicates per experiment

[d] Glucose and ammonium nitrate served as carbon and nitrogen sources in all test media

than 25 µg/ml. Shirakawa (1955) in his studies on Diplocarpon rosea showed that biotin and pyridoxine added singly or together restricted yields.

Ryan, Kunin, Ballentine, and Maas (1953) showed that the growth of a mutant strain of Neurospora crassa decreased when the concentration of biotin in the medium exceeded 0.025 µg/ml. However, the inhibitory effect of excess biotin was eliminated when pantothenate was added to the medium. Chiao and Peterson (1956) reported that the inhibitory effect of thiamine on Saccharomyces carlsbergensis could be reversed by the addition of pyridoxine. Esposito et al. (1962) noted that thiamine-induced growth inhibition in Fusarium was reversed by the addition of biotin or by vigorous aeration. The mechanism of such interaction is not known.

The presence of other metabolites in the medium as well as culture conditions (pH, temperature, aeration, etc.) may also influence the requirement for a growth factor (Cochrane, 1958; Fries, 1965). Bartnicki-Garcia and Nickerson (1961) reported that Mucor rouxii, which is prototrophic, requires thiamine and niacin when cultured under anaerobic conditions. Lichstein and Begue (1960) noted that Saccharomyces cerevisiae grew well at 30 C in a synthetic medium without pantothenic acid but required this vitamin for growth at 38 C. Strigini and Morpurgo (1961) in their experiments on Aspergillus nidulans and Neurospora crassa observed that biotin was not needed if fructose instead of glucose served as the carbon source. Mathew (1952) noted that Pellicularia koleroga utilized thiamine or biotin for growth on sucrose , but required thiamine exclusively for growth on glucose. In *Trichophyton equinum*, Georg (1949) reported that the requirements for niacin could be replaced by the addition of tryptophan in the medium.

It should also be mentioned that the need for vitamin may change during the life cycle of a fungus. Mandels (1955) reported that spores of *Myrothecium verrucaria* needed biotin for germination, whereas the fully developed mycelium showed no requirement for biotin. In the dimorphic *Histoplasma capsulatum*, it is only the yeast-like phase which requires thiamine and biotin (Pine, 1957). Fergus (1952) in *Penicillium digitatum* and Strigini and Morpurgo (1961) in *Aspergillus nidulans* have reported that initial growth may be strongly promoted by added vitamins.

Attempts have been made to use vitamin requirements as a physiological parameter for the delineation and identification of species of fungi, particularly where morphological characters are few or unreliable, e.g., in *Trichophyton* (Georg and Camp, 1957), *Rhodotorula* (Hasegava and Banno, 1963), and *Exobasidium* (Sundstrom, 1960, 1964). Cantino (1955) proposed that nutritional characteristics could serve to clarify the phylogenetic relationships of genera and orders of water molds. Cantino (1955) and Crasemann (1954) argued that chytrids are the most primitive of the uniflagellate Phycomycetes since they have retained what is presumably an ancestral capacity to reduce inorganic sulfur. Many of the chytrids synthesize all their essential vitamins. In addition, the majority have the capacity to use both oxidized and reduced forms of inorganic nitrogen compounds for growth. On the basis of these facts Crasemann (1954) proposed an evolutionary scheme in which chytrids are viewed ancestral to members of the order Blastocladiales. The latter, they consider, to be more nutritionally advanced in the sense proposed by Lwoff (1936) and Knight (1945). Thus, blastocladiaceous fungi have lost the capacity to reduce sulfur, can not grow with inorganic nitrogen as the sole source of that element and require the pyrimidine moiety of thiamine. They are more specialized and more dependent upon their environment than are the Chytridiales.

Cochrane (1958) is critical of Cantino's scheme and notes that vitamin deficiencies and inability to use nitrate nitrogen do not offer substantive clues to separate a taxon or to yield valid evidence of phylogeny. He believes that vitamin requirements and inability to grow with nitrate nitrogen occur without descernible patterns within species and genera. Marked differences may exist between the vitamin demands of strains comprising a single species as in Fusarium (Robbins and Ma, 1941), Trichophyton (Mackinnon and Arlagavettia-Allende, 1948), Lenzites trabea (Lilly and Barnett, 1948), Glomerella cingulata(Strubble and Keitt, 1950), and similarly there may be diverse responses by different species within a genus, e.g., Ophiostoma and Ceratostomella (Robbins and Ma, 1942a and 1942b), Ustilago (Blumer, 1940), Marasimus (Lindenberg, 1944). In the light of this intra- and interspecific variation, Cochrane (1958) argues that it is unlikely that vitamin requirements can offer important clues to taxonomic and phylogenetic relationships.

Nitrogen requirements

A survey was made of the nitrogen requirements of the four chytrids. The nutrient solution employed was medium 3 in which one of several nitrogen sources was substituted for ammonium nitrate. Individual nitrogen sources were added at a concentration of 0.05M with 0.05M glucose as the carbon source. The nutrient solution contained no growth factors. Glucose was separately sterilized by filtration and added aseptically to the autoclaved nutrient solution. The complete test medium was dispensed in 10 ml aliquots into 18 ml Pyrex glass tubes. The results cited in Table 10 represent the arithmetic mean of two trials performed at different times and three replicates were used in each experiment.

Of the nine inorganic nitrogen salts tested, six supported good growth in the four chytrids. Sodium and potassium nitrate were utilized by the four organisms suggesting that the fungi possess a nitrate reduction pathway. The organisms did not grow in sodium nitrite. The ammonium salts that supported good growth included the acetate, chloride, nitrate, and sulfate. However, they did not utilize ammonium carbonate and ammonium phosphate. On the basis of present investigation alone these results are not fully explainable. However, Morton and Mac Millan (1954) and Brian _et_ _al_. (1947) have reported similar findings with _Scopulariopsis brevicaulis_. It is possible that the permeability of ammonium ion or other nutrients is somehow blocked by the carbonate and phosphate ions. It may well be that the latter at a concentration as high as 0.05M inhibits the functioning of the hexose monophosphate shunt pathway, a phenomenon demonstrated by Kravitz and Guarino (1958) in ascites tumor cells.

The data of Table 10 show the rate of utilizability of the common

TABLE 10

Effect of nitrogen sources on growth of *Entophlyctis* sp., *E. aureus*, *R. harderii*, and *R. rosea*.[a]

Nitrogen sources (0.05M)	*Entophlyctis* sp. Klett readings[b]		*E. aureus* Klett readings		*R. harderii* Klett readings		*R. rosea* Klett readings	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
None[c]	5	6	6	7	5	4	6	4
Inorganic nitrogen								
Ammonium acetate	25	26	20	20	15	18	14	17
Ammonium carbonate	0	2	2	3	5	5	7	5
Ammonium chloride	30	29	25	25	33	36	28	29
Ammonium nitrate	30	29	28	29	30	32	30	30
Ammonium phosphate	2	4	2	4	2	3	2	3
Ammonium sulfate	25	26	22	21	25	29	18	17
Potassium nitrate	24	29	18	17	15	17	16	17
Sodium nitrate	23	26	21	23	18	17	21	17
Sodium nitrite	3	5	4	4	5	4	7	7

[a] All chytrids in stationary liquid culture for two weeks at 22 C. Initial pH of test media 7.5.

[b] Mean Klett readings of two experiments (Exp.1 & 2), three replicates per experiment.

[c] Filter sterilized glucose (0.05M) present as carbon source in all test media.

TABLE 10 Continued

Nitrogen sources	*Entophlyctis* sp. Klett readings Exp. 1	*Entophlyctis* sp. Klett readings Exp. 2	*E. aureus* Klett readings Exp. 1	*E. aureus* Klett readings Exp. 2	*R. harderii* Klett readings Exp. 1	*R. harderii* Klett readings Exp. 2	*R. rosea* Klett readings Exp. 1	*R. rosea* Klett readings Exp. 2
Amino acids:								
L-alanine	21	21	24	28	53	56	24	22
D-arginine	57	62	22	21	39	38	2	5
L-arginine	55	62	20	20	40	38	2	3
L-aspargine	26	29	16	18	58	66	53	55
D-glutamic acid	28	29	25	26	31	28	15	18
L-glutamic acid	28	28	27	27	35	36	20	21
L-glutamine	60	65	18	20	40	45	20	20
Glycine	34	37	14	13	26	29	34	39
L-histidine	4	5	4	5	5	5	6	7
L-hydroxyproline	34	30	25	27	30	30	30	31
DL-isoleucine	21	19	28	27	28	29	38	34
L-isoleucine	23	24	35	38	25	24	32	35

TABLE 10 Continued

Nitrogen sources	*Entophlyctis* sp. Klett readings Exp. 1	 Exp. 2	*E. aureus* Klett readings Exp. 1	 Exp. 2	*R. harderii* Klett readings Exp. 1	 Exp. 2	*R. rosea* Klett readings Exp. 1	 Exp. 2
D-leucine	45	42	25	22	5	7	40	40
L-leucine	50	56	27	28	50	48	40	41
D-lysine HCl	10	13	8	8	15	13	25	29
DL-lysine HCl	18	17	23	24	20	22	25	29
D-methionine	20	22	30	30	10	13	10	12
DL-methionine	20	21	24	22	4	4	14	13
L-methionine	40	39	26	26	4	5	30	29
DL-phenylalanine	5	5	20	19	5	4	25	29
L-proline	39	34	20	21	43	48	22	21
D-serine	5	5	10	12	4	5	5	7
DL-serine	20	21	14	14	20	22	16	18

TABLE 10 Continued

Nitrogen sources	*Entophlyctis* sp.		*E. aureus*		*R. harderii*		*R. rosea*	
	Klett readings		Klett readings		Klett readings		Klett readings	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
L-serine	37	37	29	30	35	38	30	28
D-threonine	9	7	10	8	7	7	3	5
DL-threonine	37	38	17	18	35	39	39	38
L-threonine	8	7	6	5	4	5	4	5
D-valine	25	27	12	14	5	5	25	28
L-valine	25	29	54	58	55	62	25	27

amino acids by the four fungi. Wherever possible the D, L, and DL isomers of a given amino acid were used in the assay. Since the L isomers of amino acids constitute natural proteins, only this racemic form is generally incorporated into proteins. The utilization of D-amino acids depends upon the presence of a flavoprotein enzyme, D-amino acid oxidase. This enzyme has broad substrate specificity and acts at different rates on a number of amino acids (Cohen, 1954; Meister 1957). D-amino acid oxidase has been studied in *Neurospora crassa* (Horowitz, 1944) *Aspergillus niger* (Bender and Krebs, 1950), *Penicillium* sp. (Emerson, Puziss, and Knight, 1950).

From the results it may be seen that the different amino acids were not equally effective in supporting growth in the four chytrids. Whereas *Entophlyctis aureus* gave fair yields when furnished with L-valine, L-isoleucine, L-methionine, and L and D-glutamic acid, *Entophlyctis* sp. grew well when provided with L-glutamine, D and L-arginine, glycine, D and L-leucine, L-methionine, and L-proline. L-aspargine, L-glutamine, glycine L-histidine, D-lysine HCl, DL-methionine, D-serine, D and L-threonine and D-valine were poor sources of nitrogen for *E. aureus* and Entophlyctis sp. showed only minimal growth in L-histidine, D-lysine HCl, DL-phenylalanine, D-serine and L-threonine.

Entophlyctis aureus preferentially utilized L-isomers of serine and valine. Growth in D-serine and D-valine was poor and yields in DL-serine and DL-valine were less than half of those obtained with the L-isomers suggesting that only they were utilized. *Entophlyctis* sp. used L and D forms of valine to the degree indicating the presence of a D-amino acid oxidase. However, with regard to the utilization of serine, *Entophlyctis* sp. followed the pattern for *E. aureus*.

A comparison of the two *Entophlyctis* chytrids revealed that *Entophlyctis* sp. gave better yields when grown in glutamine, arginine, leucine, and proline whereas *Entophlyctis aureus* preferentially utilized valine, isoleucine, and methionine. DL-phenylalanine supported fair yeilds in *E. aureus* but was poorly assimilated by *Entophlyctis* sp. The response of *Entophlyctis* sp. to arginine and glutamine is noteworthy. Glutamine has been found to be superior to glutamic acid as a nitrogen source in *Rhizophydium sphaerotheca* (Goldstein, 1960) and *Tricholoma gambosum* (Norkrans, 1953). Glutamine has been shown to stimulate glycolysis (Meister, 1957) and there is also evidence that it may serve in arginine synthesis (Orly, Hood, and Lyman, 1954). It is, therefore, possible that the superior value of glutamine for this chytrid may reside in these functions.

The amino acids that supported good growth in *Rhizophlyctis harderii* included L-aspargine, L-alanine, L and D-arginine, L and D-glutamic acid, L-glutamine, glycine, L-leucine, L-serine, L-valine and L-proline, whereas L-histidine, D-leucine, D and L-methionine, DL-phenylalanine, D-serine, D and L-threonine and D-valine were poor sources of nitrogen for the chytrid. *Rhizophlyctis rosea* showed maximal growth in L-aspargine, glycine, DL-isolecucine, D and L-leucine, DL-phenylalanine, and L-serine. It did not grow in D and L-arginine, L-histidine, D-methionine, D-serine and D and L-threonine. A comparison of the two *Rhizophlyctis* chytrids revealed differences in their responses to arginine, D-leucine, L-methionine and D-valine. Whereas D and L arginine resulted in fair yields of R. harderii, it did not grow in L-methionine, D-valine, or D-leucine. However, L isomers of leucine and valine supported good growth of the chytrid suggesting that only the L forms were utilized. R. rosea did not grow in D and L-arginine but gave fair yields when grown in D-leucine, D-valine and L-methionine. D and L forms of leucine and valine were equivalent for R. rosea possibly indicating the presence of the amino acid oxidase enzyme.

The growth response of the four chytrids to threonine is interesting. D and L forms of threonine did not support growth in the four fungi. However, the use of DL-threonine resulted in fair yields of the four organisms. It may be that the commercial (Nutritional Biochemical Corp.) preparation of DL-threonine used in the present investigation was contaminated with some unidentified growth supporting material.

Summarizing the results of nitrogen experiments, the four water molds readily metabolized inorganic nitrogen salts in addition to several amino acids. Maximum growth was achieved in the complex medium (medium 1). Members of Entophlyctis grew best in L-valine and L-glutamine respectively, whereas the two Rhizophlyctis species gave maximal yields with L-aspargine. With the two Entophlyctis representatives D-methionine supported fair growth 20 or more Klett units, but it was poor source less than 12 Klett units of nitrogen for Rhizophlyctis members. A comparison of amino acids utilization made in Table 11 showed that E. aureus grew well in nutrient solutions containing D and L arginine, D-methionine, and L-valine whereas R.rosea did not grow in arginine and utilized D-methionine and L-valine poorly. The best yields of R.rosea were obtained with L-asparg.

but E. aureus grew poorly with the amino acid.

Table 11

A physiological comparison of amino acids utilization by Entophlyctis sp., E. aureus, E.confervae-glomeratae[a], R.harderii, and R. rosea.

Ability to utilize	Entophlyctis sp.	E. aureus	E.confervae-glomeratae[a]	R. harderii	R. rosea
D-arginine	+[b]	+	0[c]	+	-[d]
L-arginine	+	+	0	+	-
L-aspargine	+	+	+	++[e]	++
L-glutamine	++	+	+	++	+
D-leucine	++	+	0	-	++
D-lysine HCl	±[f]	±	0	-	-
D-methionine	+	+	0	±	±
L-methionine	++	+	0	-	+
DL-phenylalanine	-	+	0	-	+
D-valine	+	±	0	-	+

[a] Results from Barr, 1971b.

[b] + = fair yields (Klett units of 20-40 with two weeks incubation at 22 C)

[c] 0 = not tested

[d] - = no growth

[e] ++ = good yields (Klett units of 40 and more, two weeks incubation,at 22 C)

[f] ± = poor yields (Klett units of less than 12,two weeks incubation at 22 C)

Carbon nutrition

Ammonium nitrate was employed as the sole source of nitrogen in the carbon assimilation tests. The carbon compounds were filter-sterilized and added aseptically to the autoclaved fraction of medium 3. A wide variety of carbon-containing compounds including organic acids, sugars, sugar alcohols, and amino sugars were tested. Since adaptive utilization of substrates occurs in fungi (Crasemann, 1954; Cochrane, 1958), especially with respect to carbon sources, two incubation periods (2 & 6 weeks) were used. The data in Table 12 may be summarized as follows.

Acids

Formic, acetic, glycolic, citric, fumaric, oxalic, succinic, and tartaric acids were employed as sole carbon sources and none of these compounds supported growth in the four chytrids even by the end of a six week incubation.

Sugar alcohols

Mannitol and sorbitol are two hexitols derived from the reduction of fructose and glucose. These two sugar alcohols did not support growth in the four fungi. Very few lower phycomycetes have been found to grow with sugar alcohols and the few that use them, do so poorly (Cantino, 1949; Cochrane, 1958).

Pentoses

Three pentose sugars, arabinose, rhamnose, and xylose were tested and none of the chytrids grew during a two week incubation. However,

TABLE 12

Effect of carbon sources on growth of Entophlyctis sp., E. aureus, R. harderii, and R. rosea.[a]

Carbon sources[b]	Entophlyctis sp.		E. aureus		R. harderii		R. rosea	
	Klett readings[c]		Klett readings		Klett readings		Klett readings	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
None[d]	2	2	1	2	1	2	1	1
Organic acids[e]	0	0	0	0	1	0	2	0
Pentoses								
Arabinose	20[f]	19[f]	1	2	1	2	1	2
Rhamnose	1	1	1	2	2	1	2	2
Xylose	18[f]	20[f]	1	1	1	1	16[f]	18[f]
Hexoses								
D-galactose	15	16	15	16	18	18	1	2

[a] All chytrids cultivated for at least 2 weeks at 22 C in stationary culture. Initial pH of test media 7.5

[b] Carbon sources sterilized by filteration.

[c] Mean Klett readings of two experiments (Exp. 1 & 2), three replicates per experiment.

[d] In all instances ammonium nitrate (0.05M) served as the sole source of nitrogen

[e] Organic acids tested include, acetate, citrate, formate, fumarate, glycolate, oxalate, succinate, and tartrate (Sodium salts).

[f] Represents growth at 6 weeks since no growth was detectable at 2 weeks.

TABLE 12 Continued

Carbon sources	*Entophlyctis* sp. Klett readings		*E. aureus* Klett readings		*R. harderii* Klett readings		*R. rosea* Klett readings	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
D-glucose	28	30	24	29	27	27	25	29
D-fructose	32	37	28	28	1	2	1	2
D-mannose	24	25	19	19	19	19	17	19
Sorbose	1	1	1	1	2	1	2	2
<u>Disaccharides</u>								
Cellobiose	2	4	2	4	19	19	18	18
Lactose	1	1	3	4	16	19	9	11
Maltose	50	54	40	42	42	46	31	38
Melibiose	2	2	2	2	1	2	2	2
Sucrose	1	2	1	1	2	2	1	2
Trehalose	2	2	3	4	1	3	2	2
<u>Trisaccharides</u>								
Raffinose	15	19	14	16	1	2	1	2
<u>Sugar alcohols</u>								
Mannitol	0	0	1	0	0	1	1	1
Sorbitol	1	1	0	1	1	1	0	1

by the end of six weeks, Entophlyctis sp. grew in arabinose and xylose, whereas Rhizophlyctis rosea grew only in xylose. It can not be stated whether these results were a consequence of enzyme induction or whether growth was due to the selection of mutants during the extended incubation. Barr (1970), in his studies with Phlyctochytrium reinboldtae, noted that with glycerol virtually no growth was detected after a 3 week incubation period, but good yields were obtained by six weeks. Sistrom and Machlis (1955) reported that Allomyces macrogynus, a member of the Blastocladiales, did not grow in mannose and fructose during a two week incubation, but grew gell with these substrates if the cultures were maintained for six to nine weeks. The final yields were comparable to that obtained on glucose. In general, if a compound is utilized following a long lag phase, enzyme induction is postulated, however, the selection of a mutant already present in the inoculum or arising during the long incubation can not be precluded.

Hexoses

D-glucose, D-fructose, D-mannose, D-galactose, and sorbose were tested and D-glucose and D-mannose supported good growth in all four chytrids. Glucose and mannose have been noted to be the most readily utilizable hexoses for the chytrids, Entophlyctis confervae-glomeratae (Barr, 1971b), Phlyctochytrium reinboldtae (Barr, 1970), P. punctatum and Rhizophydium sphaerotheca (Goldstein, 1960b) and Cladochytrium replicatum (Goldstein, 1960b).

D-fructose supported the growth of Entophlyctis sp. and E. aureus but not of Rhizophlyctis harderii and R. rosea even by the end of eight weeks. There are a number of reports that many chytrids and higher fungi fail to utilize fructose (Ajello, 1948; Crasemann, 1954).

Entophlyctis sp., *E. aureus*, and *Rhizophlyctis harderii* grew well with D-galactose but *R. rosea* did not utilize the sugar. The latter gave meager yields even after eight weeks.

Sorbose, a ketohexose differing from fructose in the configuration of carbon atom 5, did not support growth of any of the four chytrids. The sugar appears not to be utilizable by most fungi and has been found to be toxic to some (Barnett and Lilly, 1951; Lilly and Barnett, 1956; Cochrane, 1958).

Inability to utilize a substrate may result from the failure of the substrate to enter the cell, or because an organism lacks one or more enzymes necessary to integrate it into a metabolic pathway,or the substrate may be toxic. It is unlikely that related organisms would have marked differences in major metabolic pathways, although minor differences might exist. The failure of *Rhizophlyctis rosea* to utilize D-galactose suggests that the sugar does not penetrate the cells,or that the chytrid does not have the enzyme to phosphorylate galactose, or that it lacks the enzyme to convert the sugar into glucose-6-phosphate. The inability of both species of *Rhizophlyctis* to utilize D-fructose may result from inability of the sugars to penetrate the cells. Both species of *Entophlyctis* use aldose and ketose sugars, but the two members of *Rhizophlyctis* fail to grow on 6-carbon ketose sugar, namely D-fructose. Additional species and strains of these chytrids have to be tested before this physiological criterion is considered to be taxonomically significant at the genus level.

Disaccharides

Disaccharides are composed of two monosaccharides and may be characterized by the nature of the sugar units involved and the type of glycosidic bonds that are present. Six disaccharides, maltose, cellobiose, trehalose, melibiose, sucrose, and lactose were tested.

Maltose occurs in nature as a product of starch hydrolysis and consists of two glucose molecules having a 1-4-glucosidic linkage. It supported growth of the four chytrids. Indeed, growth was better than in glucose. Its superiority to glucose may be due to a concentration factor or possibly to the fact that maltose is likely to be contaminated with growth factors (Cochrane, 1958).

Like maltose, cellobiose is also composed of two glucose residues but there is a β-1-4-glucosidic linkage between them. Of the four chytrids tested, Rhizophlyctis harderii and Rhizophlyctis rosea utilized the sugar but Entophlyctis aureus and Entophlyctis sp. did not. However, Barr (1971b) has reported that two strains of Entophlyctis confervae-clomeratae grow in cellobiose, and hence this disaccharide can not be used to differentiate the genera.

Lactose on hydrolysis yields D-galactose and D-glucose. Only Rhizophlyctis harderii produced fair yields in lactose. Rhizophlyctis rosea grew poorly in the disaccharides. Since both species of Entophlyctis utilize D-glucose and D-galactose, failure to grow in lactose indicates either that the fungi are impermeable to the sugar or that if it does enter the cells, it is not hydrolyzed. Barr (1971b) has noted that Entophlyctis confervae-glomeratae does not utilize lactose. More species and strains of Entophlyctis and Rhizophlyctis need to be tested before this disaccharide may be considered useful for differentiating these taxa at the generic level.

None of the chytrids grew with sucrose, although sucrose is generally a good source of carbon for higher fungi and Entophlyctis confervae-glomeratae does indeed grow with it (Barr, 1971b). Yet, failure to utilize sucrose appears to be common in the Chytridiales (Lilly and Barnett, 1953; Cochrane, 1958).

Melibiose, an α-glycoside, which upon hydrolysis yields galactose and glucose, did not support growth of the chytrids. Again, this may result from a failure to penetrate the cells or be due to the absence of hydrolyzing enzymes.

Trehalose is an α-D-glucosido-glucoside and yields two molecules of glucose upon cleavage. None of the chytrids examined grew in the disaccharide. Since maltose also consists of two molecules of glucose and the four chytrids grow well with maltose, it would seem that the glucosidase which splits maltose is specific for that sugar or that trehalose is not translocated into the cells.

Raffinose is a trisaccharide and may be hydrolyzed either into galactose and sucrose or into fructose and melibiose. Only Entophlyctis sp. and E. aureus showed growth in raffinose. It is interesting to speculate on the manner in which they may cleave the sugar. It would appear that the trisaccharide is split into fructose and melibiose since only *Entophlyctis* sp. and *E. aureus* grew in fructose and none of the chytrids used melibiose. It would seem that *Rhizophlyctis harderii* was unable to hydrolyze raffinose into galactose and sucrose since it is capable of utilizing galactose. Although the nutritional data are suggestive, failure of the trisaccharide to penetrate the *R. harderii* cells can not be ruled out.

A comparison of the results of the carbon nutrition tests indicates that *Entophlyctis* and *Rhizophlyctis* constitute two physiological groups. There appear to be markedly more carbon nutritional differences between *Entophlyctis aureus* and *Rhizophlyctis rosea* than between *Entophlyctis* sp. and *E. aureus*. *Entophlyctis* sp. and *E. aureus* are closer in their carbon nutrition to *Entophlyctis confervae-glomeratae* (Barr, 1971b) than they are to *R. rosea* or *R. harderii*. A comparison of the carbon nutrition characteristics of these fungi is made in Table 12. *Entophlyctis* sp. and *E. aureus* used six of the fifteen sugars tested (Table 12) and differed from *E. confervae-glomeratae* (Barr, 1971b) in their pattern of trehalose and raffinose utilisation. In contrast *Entophlyctis* sp. and *E. aureus* did not use trehalose but assimilated raffinose. *E. confervae-glomeratae* utilized cellobiose and sucrose albeit poorly after six weeks (Barr, 1971b), whereas *Entophlyctis* sp. and *E. aureus* did not grow at all with the two sugars. *Entophlyctis* sp. differed from *E. aureus* in the utilization of arabinose and xylose. Although both chytrids did not grow with arabinose or xylose during a two week incubation, *Entophlyctis* sp. exhibited adaptive utilization of the two pentose sugars as evidenced by some growth being attained in six weeks. However, *E. aureus*

TABLE 13

A physiological comparison of carbon nutrition of *Entophlyctis* sp., *E. aureus*, *E. confervae-glomeratae*[a], *R. harderii*, and *R. rosea*.

Carbon sources (0.05M).	*Entophlyctis* sp.	*E.aureus*	*E.confervae-glomeratae*[a]	*R.harderii*	*R.rosea*
Arabinose	-[b] (+[c] at 6 weeks)	-	0[d]	-	-
Xylose	- (+ at 6 weeks)	-	-	-	- (+ at 6 weeks)
D-fructose	+	+	+	-	-
D-mannose	+	+	0	+	+
D-galactose	+	+	0	+	-
Sorbose	-	-	0	-	-
Cellobiose	-	-	±[e]	+	+
Trehalose	-	-	+	-	-
Sucrose	-	-	±(at 6 weeks)	-	-
Lactose	-	-	-	+	±(at 6 weeks)
Raffinose	+	+	-	-	-

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[a] Results from Barr, 1971b

[b] - = can not utilize

[c] + = can utilize

[d] 0 = not tested

[e] ± = poorly utilized

did not grow in these compounds even after an extended incubation (eight weeks). *E. confervae-glomeratae* (Barr, 1971b) was not tested with arabinose and xylose.

Rhizophlyctis harderii and *Rhizophlyctis rosea* were nutritionally similar in their response to the fifteen sugars tested (Table 12). *R. harderii* grew well with D-glucose, D-mannose, D-galactose, maltose, cellobisoe, and lactose, whereas *R. rosea* was unable to utilize D-galactose and grew poorly in lactose during an eight week incubation. *R. rosea* showed adaptive utilization of xylose at six seeks but the phenomenon was not observed with *R. harderii* even after an extended incubation.

A comparison of *Entophlyctis aureus* and *Rhizophlyctis rosea* indicated that the two chytrids differed in their utilization of xylose, D-fructose, D-galactose, lactose, and raffinose. The former gave good harvests when grown with D-fructose, D-galactose, and raffinose while the latter did not grow in them even during an eight week incubation. Cellobiose supported the growth of *R. rosea* but *E. aureus* did not utilize the disaccharide. During a two week incubation both failed to grow in xylose, however, *R. rosea* gave fair yields by six weeks. Lastly, although *R. rosea* grew only scantily in lactose, *E. aureus* did not utilize this sugar at all.

The carbon nutrition data reported here suggest that nutritional studies may aid in clarifying the taxonomy of the *Entophlyctis-Rhizophlyctis* group of chytrids. Comparative nutritional studies of additional species within a genus as well as the use of different isolates of the same species from diverse habitats might prove to be of value. The taxonomic implications of the nutritional differences observed between Entophlyctis and Rhizophlyctis isolates, would be strengthened if additional strains and species of these chytrids yielded intraspecific differences markedly less disparate than interspecific patterns detected.

Conclusion

It is of interest to interpret the phylogenetic position of these chytrids with reference to previously proposed systems based upon nutritional characteristics (Crasemann, 1954; Cantino, 1955; Cantino and Turian, 1959). All were endowed with those physiological features which have been presumed to characterize the order Chytridiales. Thus, they were prototrophic with respect to vitamin requirements, assimilated nitrate nitrogen, and apparently utilized inorganic sulfur supplied as sulfate.

The assignment of chytrids to the genera Entophlyctis or Rhizophlyctis is morphologically based (Sparrow, 1937, 1960; Haskins, 1946; Karling, 1947; Booth, 1971; Barr, 1971a). Developmental studies indicate that the constancy of morphological features used to distinguish Entophlyctis from Rhizophlyctis is dubious. Assertions as to the morphological plasticity and variability of organisms of the two genera are to be found in the recent work of Miller (1968), Booth (1971), and Barr (1971a). In Sparrow's (1960) key to the Chytridiales, Entophlyctis and Rhizophlyctis are separated on the basis that the thallus of the former is endobiotic and develops from the enlargement of the zoospore's germ tube, whereas in the latter an epibiotic thallus grows directly from the expansion of an encysted zoospore. Barr (1971a), Booth (1971) and Karling (1947) have reported variations in thallus development of members belonging to *Entophlyctis* and *Rhizophlyctis*.

Although the morphology of the *Entophlyctis*-*Rhizophlyctis* group of chytrids has received considerable study (Ward, 1939: Stanier, 1942: Haskins, 1946: Sparrow, 1937, 1960; Willoughby and Townley, 1961: Cook, 1966: Karling, 1928, 1931, 1947, 1949, 1967: Miller, 1968; Booth, 1970: Barr, 1971a), investigations of their physiology have been limited in number and scope (Stanier, 1942: Haskins and Weston, 1950; Barr, 1971b). The purpose of this investigation was to extend our knowledge of the group's physiology as well as to assess the value of nutritional characteristics in their taxonomy. Species were selected for this study with a view towards determining whether the establishment by Haskins (1946) of *Entophlyctis aureus* as a species novum, could be supported on nutritional grounds. The morphological basis of the taxon has been challenged (Karling, 1947; Sparrow, 1960). The presumed relationships of these taxa based on the results of nutritional studies are given in Figure 8. Figure 8 depicts the clustering of the four fungi according to their hypothesized relationship based on nutritional data (Table 13). To facilitate discussion the nutritional differences have been assigned weighted units based upon variations in substrate utilization. For example, *Entophlyctis aureus* differs from *Rhizophlyctis rosea* in the utilization of D & L arginine, D-lysine HCl, xylose, D-fructose, D-galactose, raffinose, and cellobise and, therefore, eight units apart.

TABLE 14

A physiological comparison of nitrogen and carbon sources utilization by *Entophlyctis* sp., *E. aureus*, *E. confervae-glomeratae*[a], *R. harderii*, and *R. rosea*.

Compound tested.	*Entophlyctis* sp.	*E. aureus*	*E. confervae-glomeratae*[a]	*R. harderii*	*R. rosea*
Nitrogen sources:					
D-arginine	+[b]	+	0[c]	+	-[d]
L-arginine	+	+	0	+	-
D-leucine	+	+	0	-	+
D-lysine HCl	±[e]	±	0	-	-
D-methionine	+	+	0	±	±
L-methionine	+	+	0	-	+
DL-phenylalanine	-	+	0	-	+
D-valine	+	±	0	-	+
Carbon sources:					
Arabinose	- (+ at 6 weeks)	-	0	-	-
Xylose	- (+ at 6 weeks)	-	-	-	-(+ at 6 weeks)
D-fructose	+	+	+	-	-
D-galactose	+	+	0	+	-
Cellobiose	-	-	±	+	+
Trehalose	-	-	+	-	-
Sucrose	-	-	-(± at 6 weeks)	-	-
Lactose	-	-	-	+	±
Raffinose	+	+	-	-	-

[a] Results from Barr, 1971b

[b] + = can utilize

[c] 0 = not tested

[d] - = can not utilize

[e] ± = poorly utilized

The former utilized D & L arginine, D-lysine HCl, D-fructose, D-galactose, and raffinose, whereas the latter used cellobiose and did not grow with D & L arginine, D-lysine HCl, D-fructose, D-galactose, and raffinose.

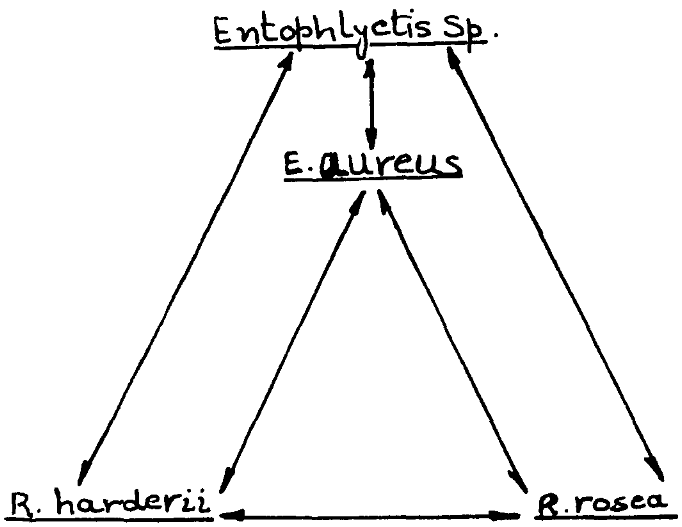

Figure 8. Hypothesized relationship of several members of the Entophlyctis-Rhizophlyctis complex based on nutritional studies.

Similarly, since E. aureus and R. harderii differ in the utilization of D-leucine, D-lysine HCl, L-methionine, D-valine, D-fructose, cellobiose , lactose, and raffinose and, therefore, they are eight units apart. Entophlyctis sp. and E. aureus are nutritionally identical in the utilization of amino acids and carbohydrates except that the former utilized DL-phenylalanine, showed adaptive growth with arabinose and xylose at six weeks, and hence they are considered three units apart.

Entophlyctis sp. differs from Rhizophlyctis rosea by ten units since the former grew with D and L arginine, arabinose, D-fructose, D-galactose, and raffinose, whereas *R. rosea* did not utilize these substrates. In addition, DL-phenylalanine, cellobiose and lactose did not support growth of *Entophlyctis* sp. but produced yields of *R. rosea*. *R. harderii* also differs from *Entophlyctis* sp. in the utilization of ten substrates. D-leucine, D-lysine HCl, L-methionine, D-valine, arabinose, xylose, D-fructose, and raffinose support the growth of *Entophlyctis* sp. but not *R. harderii*. In contrast, *R. harderii* grew well with cellobiose and lactose and *Entophlyctis* sp. did not.

Rhizophlyctis *rosea* differed from *Rhizophlyctis* *harderii* in the utilization of D & L arginine, L-methionine, DL-phenylalanine, D-valine, D-galactose and xylose and, therefore, they are placed 7 units apart. The latter grew well with D-galactose and did not utilize xylose even at six weeks, whereas the former did not grow with D-galactose but showed adaptive utilization of xylose.

The insufficiency of data pertaining to *Entophlyctis* *confervae-glomeratae* (Barr, 1971b) does not permit meaningful placement of the organism within this proposed scheme.

From Figure 8 it would appear that *Rhizophlyctis* rosea and Rhizophlyctis harderii are more closely related to *Entophlyctis* *aureus* than they are to *Entophlyctis* sp. *E. aureus* may represent a transitional form showing kinship to other members of the genus *Entophlyctis* as well as to isolates of *Rhizophlyctis*. The taxonomic worth of the nutritional criteria can be evaluated by comparison with data derived from morphological studies. Reports that in *E. confervae-glomeratae* (Barr, 1971a) and *E. aureus* (Karling, 1947) sporangia frequently develop from the direct expansion of the encysted zoospore support the inference of their relationship to *Rhizophlyctis*.

The question arises as to the extent affinities inferred from nutritional data coincide with conventional morphological evidence. As mentioned earlier (p.12) the distinction between *Entophlyctis* and *Rhizophlyctis* is based on differences in thallus development (endobiotic and epibiotic respectively, Sparrow, 1960). An additional morphological criterion is whether the sporangium arises from germ tube enlargement as in the former or by direct expansion of the zoospore as in the latter (Whiffen, 1944). Yet the sporangia of *Entophlyctis confervae-glomeratae* (Barr, 1971a) and *E. aureus* (Karling, 1947) may occasionally develop either way. This either suggests a close relationship between the two genera or that the criteria are not adequate for making distinctions. The nutritional results derived from the present study are in general consistent with results derived from morphological studies. Although the more extensive testing of diverse members of these genera would be desirable, this study does provide support to the value of an experimental approach to chytrid taxonomy. In addition, the data gained from these investigations were instrumental in the development of defined media for their culture.

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