THE REACTIONS OF *CLAVICEPS PURPUREA* TO VARIATIONS OF ENVIRONMENT[1]

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INTRODUCTION

Claviceps purpurea (Fr.) Tulasne is an outstanding species among parasitic fungi. Forming its conspicuous sclerotial stage in the heads of rye and other grains, it very early came under observation. Long before its developmental history was known, it was looked upon with suspicion, and later was proved to be the cause of disastrous epidemics of gangrenous disease.

In its sclerotial stage, *C. purpurea* elaborates a violent poison which later, mixed with grain or flour, attacks human beings and animals alike. Reference to epidemics of this dreadful affliction are found in the literature as far back as the time of Caesar, and outbreaks of the much-feared "sacre feu" were frequent down through the centuries among the European peasantry. People gradually came to associate the terrible scourge with the years when an intensely cold winter was followed by a very rainy summer, conditions that favor the development of this fungus.

Claviceps purpurea passes through a most interesting cycle of growth. The horny, dark-violet sclerotia fall to the ground when the rye head is mature. In the spring those which survive the winter send up stromata whose small, globose heads later contain the perithecia. The asci normally ripen as the new rye crop comes into bloom. Thread-like ascospores are ejected in great numbers, and are borne, chiefly by insects, to the open flowers. By infection of the ovary, further normal development of the rye kernel is prevented. Instead there ensues abundant conidial (sphacelial) formation. A sweet liquid, "honey dew," heavily laden with the conidia, forms during this early stage and exudes from the flowers in drops or even in tiny streams. After this period of vigorous conidial fructification, the mycelial mass so formed gradually changes into the dry, hard, violet to blackish resting-stage, in which form it may retain vitality for at least a year.

Not until the eighteenth century was the full life history studied. Tulasne (38) first followed the fungus through its development under natural conditions, and his work was later confirmed by Brefeld (7), Lindau (22), and other observers. Brefeld was the first to attempt controlled

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culture of *C. purpurea* upon laboratory media. As for so many other fungi, he used moist bread as a substratum upon which to sow the ascospores. He describes rather briefly the conidial (sphacelial) growth which ensued in the cultures. His work was confirmed by Engelke (11) who grew the conidial stage upon both liquid and solid media. Like Brefeld, he started with the ascospore stage, and his resulting cultures were wholly of conidial type.

Meyer (25) also grew *C. purpurea* in pure culture, but he planted conidia from the honey dew, instead of ascospores. His results are in agreement with those of the preceding workers, but he gives a considerably more detailed description of the organism in culture. He used a most complex liquid medium in an attempt to duplicate the chemical composition of young rye heads, but did not succeed in stimulating sclerotial development.

Stäger's work on *C. purpurea* (30, 31, 32, 33, 34, and 35) was confined mainly to its biology and distribution. He champions the hypothesis of "biological races" of this species, *e.g.*, on *Lolium*. However, he also grew the organism in culture and found that the conidia, like the sclerotia, may remain viable for at least a year, a fact later confirmed by Bonns (6), and many times found to be true in the experiments of the writer, who has had conidial cultures live four and one-half years from the time of planting.

McFarland (24) likewise carried on field-inoculation studies, in the course of which he isolated twelve "strains" which he grew on numerous media. He criticizes Stäger's work adversely, and believes Stäger announced the existence of a biological race on *Lolium* without sufficient data. McFarland found no evidence that such biological races occurred upon cereals and common grasses. He rightly stressed the necessary experimental condition that *glumes must be open* in the host plant if either natural or controlled infection is to occur.

To Vincens (39) and to Killian (16) we owe such knowledge as we have of the sexual development by means of antheridial and ascogonial structures, but further cytological work needs still to be done.

The sclerotial stage, commonly designated "ergot," occupies an important place among therapeutic agents, so it has been the subject of considerable investigation with a view to utilizing its commercial value. Professor Ludwig Hecke (14, 15), in his experiments on inoculating blooming rye in the field, reached the conclusion that the fungus could be grown on an industrial scale with reasonable profit. Quite recently, Bonns (6) made an attempt to culture this organism in the laboratory because of its commercial importance, but he failed to secure sclerotia and found that the conidium-bearing mycelium was practically lacking in medical value.

That no previous worker had achieved the goal of securing sclerotia in artificial cultures indicated that the problem presented unusual difficulty. Nevertheless, a review of these various contributions clearly showed the

desirability of a thorough physiological study under carefully controlled conditions, of course with the hope of determining the essential factors for production of sclerotia provided by the living host. Such a study has been undertaken by the writer during the past several years, with the results herein reported.

TECHNIQUE

Isolations. In the beginning, isolations were made from sclerotia obtained from many regions: Germany, Russia, Portugal, Spain, Holland, and France, as well as several localities in the United States.

In saprophytic culture, these strains were found to be closely similar when conditions were identical. Three were chosen for comparative study under various nutritional conditions. After more than a year of study, when it had become evident that there was no significant difference among them, the work was limited for convenience to a single culture which had been isolated by the writer in 1922 from Spanish ergot of high quality. From time to time new isolations have been made from fresh sclerotia, with which this culture has been carefully compared. In 1926, after it had been saprophytically maintained for more than three years, it was tested on rye (*Secale cereale* (L.) Beauv.) and on quack grass (*Agropyron repens* L.) for any sign of diminished parasitism. Hecke's technique (14, 15) was used in this outdoor work as far as possible, but the plants were covered with parchment caps before anthesis in order to prevent or at least decrease pollination. When flowers were opening they were sprayed with a conidial suspension and recapped. The rye readily became infected, forming large, normal sclerotia, from four to thirteen per head. The quack grass, likewise, was heavily infected with typical sclerotia. It is felt, therefore, that the culture has maintained full virility during the time of experimentation.

In the laboratory, cultures were grown in glass containers of common types: large test tubes (35 × 240 mm.); capsules of from 25 cc. to 250 cc. capacity; liter Erlenmeyer flasks, so slanted as to give a large surface and abundant supply of food and moisture. Parent strains were isolated and maintained under room conditions upon Thaxter's hard potato agar. Upon this substratum they produced only a thin mycelial mat, with numerous small conidia over the entire surface. If they are properly transferred, not more than six times per year, no degeneracy is to be noted. Cultures maintain full character on this base for an indefinite time, and are readily transferable to such other media and conditions as may be chosen for investigation.

In general, planting was done by means of a conidial suspension, prepared from stock cultures not less than a month old to ensure maturity of the growth. Frequently, however, for the purpose of comparison or merely for convenience, tissue transplants were made directly to the surface of the new medium. Ensuing growth was the same whichever method was used. Suitable control cultures for all of the experiments were planted upon

either potato agar or Leonian's medium (20, 21, modified as will be noted later), or upon both of these, as seemed advisable for the purpose in view.

Nutritional Studies. Preliminary study showed that many cereal agars and mashes offer acceptable substrata for good growth of *Claviceps purpurea.* As shown in table I, nearly all of those media tested permit good vegetative development. Cultures retain vitality over a long period of time, but the author has never obtained, either in cereal mashes or in cereal agars, any semblance of such a cortex layer as was reported by Bonns (6), although it has occurred upon various other media as will appear later. In the mashes, growth penetrated throughout the whole substance, not just over the surface. Moreover, on the heavy cereal agars, where the mat of growth often became deeply wrinkled as the food supply was depleted, the convolutions remained uniformly tender throughout the long life of the culture, except only in the little knots of denser growth herein discussed as *pseudosclerotia* (p. 56).

Oatmeal, rye meal, cornmeal, soy bean meal, ground rye, and ground wheat were used (*a*) in the form of mashes and (*b*) in solid form by the addition of agar. Except for the fact that in the mashes the penetration of mycelium occurred throughout the whole substance, growth was not different from that obtained upon the corresponding solid medium. Hence it is felt that one tabulation may well suffice for both series of results.

Several "synthetic media" were also tested for comparison with cereals: the formulae of Engelke (11), of Coons (8), and of Leonian (20, 21). As table 1 shows, only Leonian's proved equal to the cereals for promotion of growth.

Meanwhile a more closely controlled study was under way in a search for readily available sources of carbon. For each series, control cultures were grown upon Leonian's agar medium for comparative purposes. In testing the various sugars as a source of carbon, the following basic formula was used both in liquid form and as an agar medium, sugar being added in varying amounts:

Calcium nitrate	1.0 g.
Dihydrogen potassium phosphate	1.25 g.
Magnesium sulfate	0.625 g.
Water	1000 cc.
Test sugar	1, 2, 3, 5, 8, and 10 percent

Maltose, dextrose, levulose, and sucrose were tested in the above amounts, final judgment being based upon not less than ten cultures for each percentage. Cultures were allowed to run until a static condition was reached, usually about five weeks, before final records were made. The higher content of sugar did not prove to be favorable in any case. Growth was decidedly better at 2 percent and 3 percent than at 1 percent, but it was no better at 5 percent or 8 percent than at 3 percent. At 10 percent, cultures did not progress much beyond germination. Even where some initial

growth occurred, the cultures soon lost vigor, and final development was below that of all the other cultures, even those at 1 percent.

TABLE 1. *Comparative Growth of Claviceps purpurea upon Various Media* *

Medium	Mycelial Development	Conidial Development	Further Development
Hard potato agar	I	I	Lacking
Oatmeal	III	III	Numerous pseudosclerotia formed on drying
Cornmeal	III	III	As above
Rye meal	III	III	As above
Soja bean meal	I	I	Lacking
Ground rye	III	III	As on oatmeal
Ground wheat	III	III	As on oatmeal
Engelke's medium	I	I	Lacking
Coons's medium	I	o to I	Lacking
Leonian's medium	III	III	As on oatmeal

* In the above table, relative amount of growth is indicated by the Roman numerals: I indicates the minimum, III the maximum development here obtained of the factor concerned.

Maltose proved to be decidedly the most favorable sugar of those tested for growth of this organism. Not much difference was apparent between dextrose and levulose, but sucrose appeared quite poor as a carbon source for *C. purpurea*. In none of the entire series could it be said that growth was more than half as heavy as it is upon the cereals. Since maltose is probably used by the organism only after digestion to dextrose, it perhaps may be assumed that the clear advantage of maltose over dextrose lies in the fact that digestion takes place *pari passu* with utilization. Twice as much growth could thus be insured with maltose since it provides twice as much carbon as does dextrose in a solution of the same osmotic pressure. In any case, since the superiority of maltose over the other sugars was unmistakably demonstrated, it was definitely chosen as the standard sugar for subsequent use.

Selection of a Standard Medium. Several of the more common nitrates were tested, but none of them proved to be capable of developing growth as good as that normally obtained upon peptone. Meanwhile, the growth of the organism upon Leonian's agar suggested that a modification of his formula might be of advantage in attaining the desired heavy increase of growth. This nutrient mixture was therefore tested both with and without the malt extract ingredient which Leonian found so useful in his studies. While it proved to be very satisfactory for vegetative growth and conidial formation, better results were secured when the malt extract was retained. After much experimentation, this formula appeared to be the most promising of all those tested and later, *using double quantities of the nutrients and 6 percent agar*, it became the standard medium used in all other phases of the investigation unless otherwise stated.

Physiological Considerations

Description of Typical Culture

Previous descriptions of *C. purpurea* in saprophytic culture have been based upon growth in or on a variety of media, which probably explains the prevailing differences of opinion in regard to hyphal arrangement. In order, therefore, to have a standard for the mycelial and conidial developmental history, a thorough microscopical study was made of cultures growing upon this chosen medium. In the early stages, gelatinous growth spreads slowly in all directions from the point of inoculation. Hyphae are relatively few at this time, but within forty-eight hours conidia are abundant. They show wide variation in size, ranging from 2.2 by 1.3 to 8.5 by 3.2 microns. Daily examination of material taken from points equidistant from the original inoculum show that conidia of maximum size are produced in about fifty hours after planting, or in about twenty-four hours after conidial formation is well started. Conidia are typically one-celled, hyaline, ellipsoidal, often with bi-polar "oil-drops," as noted by Meyer (25), which are doubtless the same as the polar granular areas observed in other species, such as *C. paspali.* Germinating conidia typically show mycelial development initiated at or near these drops, but quite often the hyphae are seen to start from the lateral portions of the conidium also.

This gelatinous mass of growth soon becomes firmer by the increase of mycelial development, and the plicate area is usually 3.5 to 4.0 cm. in diameter within a week. Subsequent growth is relatively slower. If left in the dark, little or no upright hyphal development ensues, though a good horizontal mat is formed. If, however, the cultures are placed in direct or strong diffused light, they respond very quickly by producing aërial mycelium over the entire surface to a height of 1.0 to 1.5 mm., while the horizontal mat normally becomes about 1.0 mm. thick. As will appear later, this upright growth has been markedly increased in both thickness of mat and height of the hyphae when cultures were put under specially favorable conditions. No grouping of hyphal threads such as Meyer (25) described has ever been noted in the horizontal web, nor have coremium-like bundles ever been observed in the normal mycelium. The upright hyphae branch repeatedly but do not attach to each other in any way. In four to five weeks a static condition has occurred. Further development is not observable for some time, but bits of the fungus from all parts of the culture are fully viable and develop typically if placed upon fresh medium.

After a further period of several weeks, when the culture has been planted from ten to fifteen weeks, characteristic bodies begin to develop in the mat. It seems beyond doubt that these are the structures called by Engelke "microsclerotia." Unfortunately, he merely noted their occurrence, giving no description or illustration with which comparison could be made. They constitute one of the most interesting phenomena encountered,

occurring consistently in old, drying cultures. When one is teased apart or crushed under the glass, it shows a mass of closely packed hyphae, with marked anastomosis of the hyphal strands. Conidia of this growth seem especially rich in the oil drops. Except in these two points, however, such growth does not appear to differ from that of the normal mat. These knots of growth round up into pulvinate to hemispherical structures, embedded in the loose hyphae of the mycelial mat. They are usually very small, not often more than 2 mm. in diameter.

Various sizes of these structures were examined histologically, from small, forming ones to the older, dried, sclerotioid ones. They stain fairly well in haematoxylin-alum (Mayer's haemalum). In sections of the older stages, as shown in the photomicrographs (Pl. VII, figs. *a*, *b*), vacuolated areas are numerous, but in no stage could any sign of differentiation of tissues be found. In general appearance such a section is quite strongly suggestive of the inner (prosenchyma) tissue of a dried sclerotium; but the vacuolated areas are larger and more numerous than in the true sclerotial tissue, and there is nothing comparable to the pseudoparenchyma or rind portions of the natural sclerotium. It cannot, therefore, be said that these are real sclerotia, even of "micro" type, hence the writer has preferred to designate them by the term pseudosclerotia. The term *pseudosclerotium* as herein used designates a small, hemispherical, sclerotioid body, composed of dense layers of hyphae covering an inner, vacuolated tissue, and embedded in the mycelial web (Pl. VII, fig. *d*). Whether such a structure may be considered the primordium of a sclerotium that did not meet with conditions which would permit full development, the writer is not prepared to maintain. It is certain, nevertheless, that such bodies are a part of the natural development of a thrifty culture, grown saprophytically under normal conditions, and that they are affected favorably by the influences which permit the maximum development of this fungus in culture. Typically, they appear only after the culture has aged and become relatively dry, but their appearance can be hastened and their size can be increased by special treatment of the mycelial mat where they originate, as will be shown later. When such bodies are freed from clinging mycelium by dropping them into alcohol and washing in water before transferring to fresh nutrient, they readily develop mycelium and conidia just as does a bit of the tissue from the center of a true sclerotium, and subsequent growth corresponds closely to ordinary conidial cultures.

Special Stimulants

Ergot Extract. Several special stimulants were tried in the hope of inducing the excellent vegetative growth to make further development and differentiation. An aqueous extract of ergot was prepared from high grade Spanish Ergot, by the British Pharmacopoeia method. This was sterilized by passage through a clay filter (Mandler) and added to the standard medium, one part in twenty-five. The fungus on this medium developed

a heavy, firm mycelium, 1.5 to 2.5 mm. thick, with aërial hyphae 2.5 to 3.0 mm. tall, which showed a tendency to form groups or hyphal bundles quite similar to coremia. Conidia were greatly decreased in number, but those present were of typical appearance, though somewhat larger than usual. As usual, pseudosclerotia were formed as the cultures dried after maturity and were slightly larger on the average, but no further differences were found from the usual growth. Meanwhile, however, because of the noticeably heavier growth of mycelium, a variation of the experiment was made by doubling the strength of the B.P. Extractum Ergotae, and adding it to the medium one part in ten. This decidedly stimulated the rate of growth, and also shortened the period preceding formation of pseudosclerotia which now appeared in less than three weeks without the preliminary drying of the medium which heretofore had preceded them. As time passed, however, it was found that the stimulating effect was confined to the early stages of development. In three or four months, these cultures showed no further sign of differentiation than did the control cultures without the extract. The experiment was repeated several times and always the heavier, quicker development was clear-cut, but it proved to be only a matter of favorable nutrient spurring the initial growth, and not of effective stimulation to any greater final development.

Vitamin Extract. It is well known that animal reproduction is greatly influenced by the presence or absence of Vitamin E. Because of this important property, it was decided to test the effect of such an extract upon the development of *C. purpurea.* The extract is of an oily nature, and great care is necessary to incorporate it in the medium and prevent it from merely forming a thin film over the surface. It can be done successfully, however, by cautiously adding it when the medium is just ready to gel. The material was used in both crude and concentrated, or purified, form and in every case it strongly stimulated the fungus and caused an early development of the pseudosclerotia. No further development could be induced, however, even when the amount was increased from two to five and then to ten drops per twenty cc. of the medium.

Sprouted Rye. Well-filled grains of rye were selected, passed through an air blast to remove dust, washed thoroughly in tap water, dipped for one minute into 1–1000 bichlorid of mercury solution, washed several times in sterile, distilled water, and allowed to sprout, five grains being placed in each test-tube. When the sprouts were about 2 cm. long, a conidial suspension was sprayed into the tubes. The fungus grew rapidly, the very tough, heavy mat of mycelium soon checking the growth of the young shoot. This mat differed in three ways from that grown on standard medium: (*a*) it was very tough; (*b*) it developed very few conidia; (*c*) it never developed any of the pseudosclerotia so characteristic of the aging growth upon nearly all media. The method was varied by pouring cooled, nutrient agar over the sprouted rye and planting it with the fungus in the usual way.

The same tough, heavy growth was obtained as on the rye alone, but no tendency toward greater sclerotinization was ever obtained.

Physical Factors

It is recognized that in the most carefully regulated experimentation dealing with physical factors, more or less overlapping of influence is unavoidable. So far as it could be done, however, each factor was studied as a unit, keeping all other influences at their optima. The salient matters chosen for detailed study were the effects of temperature, moisture, light, pressure, transpiration, and oxidation.

Temperature

C. purpurea is able to make normal saprophytic development over a rather wide temperature range, from 18° to 28°–30° C. Hence room temperature has proved entirely suitable during the course of the work. When transplants were placed immediately in an environment below 10° C., germination occurred slowly and subsequent development was very much retarded and scanty in amount, although the organism remained alive for several months. During this time, any cultures removed to room conditions quickly revived, and growth then proceeded in normal manner. If transplants were placed at once in an oven kept at 37° C., the conidia germinated very quickly. If then removed to room conditions, typical development ensued. If left at the high temperature, however, cultures did not progress much beyond germination. At best, a thin film of distorted growth was formed which in the fourth week failed to give living subcultures. Between 10° C. and the optimum, ranging from 20° C. to 30° C., vigor and rapidity of growth are in direct ratio to the temperature.

Thrifty young cultures, started under room conditions and a week later subjected to the extremes, showed noteworthy results. Those transferred to the cold were quickly checked and made little or no subsequent growth while left in the cold but, if they were returned to the laboratory at any time within the succeeding eight weeks, the cultures again became active and made typical development. Comparable, thrifty, week-old cultures transferred from room conditions to 37° C., however, quickly showed harmful effects. Growth was checked in a few hours. Within a week the hyphal strands showed marked wilting and the general appearance of the culture became very atypical. Even when removed to room conditions, growth was not resumed in these cultures. For several weeks, however, portions transferred to fresh medium and kept under room conditions made normal growth.

It may thus be said quite definitely that temperature is not a closely limiting factor for this fungus. Even at the very unfavorable temperature of 37° C., when development is greatly inhibited, it requires prolonged exposure to kill the culture. Likewise, although it is clear that the conidia

cannot develop much beyond germination at a low temperature, they can nevertheless survive periods of unfavorable temperature for a considerable time and be ready for rapid growth when again put under favorable conditions,—a characteristic that, under field conditions, probably is important for the survival of the organism.

Moisture

Very early in the experimental work, it was noted that an abundant supply of moisture is necessary for typical growth of *C. purpurea*. In its natural habitat the organism has ready access to the plant juices, and the sphacelial stage develops its myriads of conidia in a constant drip of "honey dew." Liquid media, however, did not prove to be favorable for growth. Sturdy mycelium was obtained only upon solid or semi-solid substrata. Ordinary percentages of agar, even to three percent, dried out too rapidly to permit normal vegetative development, but when the percentage was increased to six percent or more, thus insuring a moisture supply slowly set free throughout a long period of months, even of years, a much heavier growth was obtained than on any liquid medium. The factor of hardness might be thought to enter here, but it is of negligible significance compared to moisture supply. Hyphae of *C. purpurea* never penetrate the agar to any appreciable extent. Once the growth is mature, the entire mycelial mat can be stripped intact from the surface without great difficulty. After these facts had been ascertained, the general method was to supply adequate, constantly available moisture to the fungus by means of a medium with an agar content of at least six percent.

Pressure

In an attempt to induce the organism to grow against pressure within the agar mass, solid materials were added at the time of inoculation. In one experiment, bits of rye straw, soaked in spore suspension, were stirred into the nutrient agar just before the gel set. Those which chanced to be upon the surface developed normal mycelium and conidia, but no growth took place within the mass. In another case, bits of glass tubing about 3 by 8 mm. were similarly used, care being taken to have the lumen filled as far as possible with spore suspension as each tube was dropped into the warm agar. Evidently the air in the aqueous suspension within the tubing was sufficient to support initial growth in and around the tubing, for very soon the agar was split in all directions so that pressure was no longer a factor. The resulting growth, although of normal type, differed in no way from that of an ordinary agar surface culture.

Sterilized kernels of rye were used for a third effort to put the growing fungus under pressure, half of them being first dipped into melted hard paraffin. A teasing needle was forced about two-thirds of the way down from the tip, to make an opening. Into this canal a small amount of spore

suspension was injected by means of a fine Luer syringe with Yale needle. Each kernel, as it was inoculated, was pressed, base down, into a hard agar gel that contained no nutrients, being used merely to hold the grains upright. All kernels developed heavy cushions of mycelium at the mouth of the canals which, in the non-coated ones, proceeded no farther. The paraffined ones, however, proceeded in approximately a month to round up this mycelial weft into bundles of close-packed hyphae very rich in oily material. These cylindrical structures measured about 0.5 by 2.5 mm. and, on exposure to light, readily assumed the characteristic carrot red (R) [2] color, but they made no farther progress toward sclerotial formation.

Light

No indication of heliotropism has ever been seen in this fungus. Hyphae show no tendency to curve in any particular direction, even when the mycelium is at its greatest height.

The influence of light upon color production has been rather fully discussed by the writer in a previous paper (23). As there stated, its effect upon the mycelium of *C. purpurea* was to change the growing mat from whitish to a beautiful "carrot red." It should be emphasized here that throughout the work, whenever it seemed advisable, cultures were grown in triple series: in the dark, in diffused light, and in direct sunlight. When grown entirely in the dark, no aërial mycelium formed on any of the many nutrients used, nor did even a faint trace of the characteristic red color appear. The culture surface remained smooth, of greasy or waxy appearance, although conidia were abundant on the mycelial web. When cultures received a moderate amount of diffused light; *e.g.*, when kept in a cupboard with glass doors but at some distance from the source of light, they were able to develop characteristic aërial hyphae, "tilleul buff" in color, but they never formed the red color. When grown upon a table in diffused light somewhat stronger than that which reached the cupboard, aërial mycelium formed and became faintly pink, but the coloration proceeded no farther. When cultures were kept continuously for six weeks under a 100-watt daylight bulb at a distance of from twelve to fifteen inches, only a faint pink color was developed. Temperature was checked and found to be well within the optimum range at all times.

Earlier work of the writer (23) established the fact that color production in this organism is directly dependent upon the presence of the shorter rays of the spectrum. To extend this experiment a study was subsequently made to determine the effect of ultra violet rays upon cultures exposed to full radiation from a Cooper-Hewitt mercury-vapor lamp. Five series of cultures were run with varying conditions of exposure.

Series I tested the effect upon cultures about two weeks old growing on standard agar in small capsules. These were exposed directly at a distance

[2] Ridgway, Robert. Color standards and color nomenclature. XIV: 7′ : b.

of 50 cm. from the lamp, using 220 volts, 5.3 amperes, for 15 seconds, 30 seconds, and one minute, respectively. They were then returned to room conditions and, when no effect was found after a three-day interval, all were again exposed as before. Under these conditions growth was checked in direct ratio to the time of exposure. A third, similar exposure seemed to have checked permanently any extension of the mycelium, but the cultures were not killed. In from two to four weeks after they were returned to room conditions, a thin mat had covered the entire available surface. The cultures exposed 30 seconds and 60 seconds developed a slight pinkish color after the first exposure, but this never became intensified and it disappeared after the second exposure. In Series II, the above time intervals remained the same and a two-minute exposure was added. As before, the growth of the vegetative mat was much hindered as compared with the controls, but in no case was the culture killed. Within three weeks after they were replaced under room conditions, sparse growth covered the entire surface of the medium. The slight color induced by the first exposure of the 30 second, one minute, and two minute cultures soon faded.

In Series III, the effect of a single long exposure was tested instead of three short ones as in the two preceding series. Thrifty cultures two weeks old, growing on cornmeal agar in Petri dishes, were exposed at 21 cm. distance to radiation at 4.3 amperes, 150 volts, for 5, 10, and 15 minutes, respectively. Surprisingly, none were killed, but the 15 minute culture was able to make very little subsequent growth. Feeble as it appeared, however, good transplants could be made from all parts of its surface. The temperature of the air over the cultures exposed so long reached as high as 58 degrees C., thus again showing that this fungus is able to survive a very high temperature for an exposure of at least several minutes. In Series IV, the same type of cultures were used as for Series III. The 10 and 15 minute time intervals were repeated, and exposures for 20 and 25 minutes were also included. The lethal period lies between 15 and 20 minutes. Death may be due in large measure, however, to abnormally high temperature, as the heat could not be overcome readily with the apparatus at hand. Series V was planned to test the effect of the rays upon germination of the conidia planted upon standard medium. The lamp was operated at 650 watts, 5 amperes, 130 volts, at a distance of 25.4 cm. Direct exposures were made for 30 seconds, one minute, and two minutes. All conidia were killed by an exposure of one minute, but practically all withstood 30 seconds. The growth of the vegetative mat was slowed down for several weeks, but otherwise was normal and eventually equalled that of the controls.

The results obtained from ultra violet radiation, applied by a mercury-vapor lamp, may therefore be summarized as follows:

a. Actively growing cultures one to two weeks old survive three exposures of two minutes each at a distance of 50 cm.

b. Such cultures survive a single long exposure for from 15 to 20 minutes at a distance of 21 cm.

c. Conidia are able to germinate, with subsequent growth, after 30 seconds exposure at a distance of 25.4 cm.

d. Color induced by these exposures was faint and temporary; it never approximated that produced by sunlight, and it never withstood the second exposure.

e. Radiation had no effect upon the development of sclerotia.

Conidia of *Claviceps purpurea* are thus somewhat more resistant than those of *Glomerella cingulata* (Stoneman) Spaulding and von Schrenk for which the dose is 15 seconds, as reported by Stevens (36, 37). This time interval he also found fatal to the aërial mycelium, although the colonies survived a much longer exposure; *viz.*, 4 to 15 minutes. However, the striking stimulus to formation of fruit bodies which he repeatedly obtained has never occurred in the writer's cultures.

The remarkable color produced in the hyphal walls upon exposure to direct sunlight; *viz.*, carrot red, naturally lead to an investigation of the nature of the pigment. Numerous solvents were tried in an attempt to isolate the coloring matter from fresh or dried mycelium (table 2). The deeply colored fungus mat was stripped from the surface of the hard agar medium and carefully freed from any clinging fragments of the nutrient. Part of it was used at once in the fresh, moist condition. Part was quickly dried in a desiccator over calcium chlorid, a method which allows the full color to be retained in the mycelium. In this dried form, such a colored mycelial mat can be kept indefinitely.

Small amounts of the material, 15 mg. per cc. of liquid, were placed in the selected solvents at room temperature, records being taken at intervals of from 20 minutes to 24 hours. In the case of dried mycelium it was found impossible to extract the pigment satisfactorily with any of the solvents tested. At the end of 24 hours practically all fragments retained their full color. They were then heated on a water bath for 20 minutes at 60° C., but the color was not affected. The tubes containing alcohol and acetone were then brought to the boiling point, but with very slight extraction of the color of the fungus in either solvent.

The pigment from fresh, moist mycelium was likewise extracted with difficulty by most of the common solvents. As reported previously (23) absolute alcohol and acetone were the two most effective reagents in dissolving the pigment although, as table 2 shows, several others also removed it to a greater or lesser degree. It is noteworthy, too, that when the thoroughly dried mycelium was soaked for a short time in water and then tested with the various solvents, it behaved in every way like the fresh mycelium, hence it does not appear that the drying process changed the nature of the pigment. Therefore, the difference in extraction of the pigment from moist versus dry mycelium resolves itself into a question of penetration of the solvents which apparently is much less in the case of the dried material.

TABLE 2

Reagent	Effect	
	Fresh Mycelium	Dried Mycelium
1. Acetone	Color extracted in 1 hour	No effect in 24 hours
2. Alcohol, ethyl, absolute	Color extracted in 1 hour	No effect in 24 hours
3. Alcohol, ethyl, 75%	No effect in 24 hours	No effect in 24 hours
4. Alcohol, ethyl, 50%	Effect very slight in 24 hours	Very slight effect in 24 hours
5. Alcohol, amyl	No effect in 24 hours	No effect in 24 hours
6. Alcohol, methyl	Color extracted in 1 hour	No effect in 24 hours
7. Benzol	No effect in 24 hours	No effect in 24 hours
8. Carbon disulphid	Color quickly changed to rose-pink permanently with no extraction	No effect in 24 hours
9. Carbon tetrachlorid	No effect in 24 hours	No effect in 24 hours
10. Chloroform	No effect in 24 hours	No effect in 24 hours
11. Ether	Effect very slight in 24 hours	Very slight effect in 24 hours
12. Ethyl acetate	Color extracted in 1 hour. Mycelium shrunken, brown	No effect in 24 hours
13. Ethylene chlorid	Color extracted in 1 hour	Very slight effect in 24 hours
14. Formaldehyde, 40%	No effect in 24 hours	No effect in 24 hours
15. Furfural	No effect in 24 hours	No effect in 24 hours
16. Glacial acetic acid	Color gone in 1 hour bleached. Liquid still water white	Only partly bleached in 24 hours. Completely in about 3 days
17. Petroleum ether	Very slight extraction in 24 hours	
18. Toluol	No effect in 24 hours	No effect in 24 hours
19. Xylol	No effect in 24 hours	No effect in 24 hours
20. Alcohol-ether-chloroform in equal volumes	Color extracted in 1 hour	About ½ extracted in 24 hours oily layer on surface.
21. Pyridine	Color extracted in 1 hour	No effect in 24 hours

The oily residue obtained by evaporation in those cases where the pigment was dissolved was very suggestive of carotin in solution, but it failed to give the characteristic test for carotin when treated with a solution of antimony trichlorid. It does, however, give a characteristic test for ergosterol when brought into contact with the antimony trichlorid: pink, changing to a permanent blue. In mycelium comparable in every way except that it had not been exposed to light and therefore was not colored, the ergosterol test was very faint; hence it seems quite evident that the content of this lipoid had been increased by exposure to light.

Transpiration

An extensive study was made of the effect of inducing an increased amount of transpiration by supplying a forced current of dried air to the fungus. Cultures two to three weeks old were used, growing upon standard medium in deep agar slants in liter Erlenmeyer flasks. The set-up was aspirated by means of a Chapman filter pump. Indrawn air was sterilized by passage through a 1–1000 solution of bichlorid of mercury, dried by passage through several tubes of sterile calcium chlorid, drawn to the bottom of the slant by a long intake tube, then out through a short tube in the neck of the flask. This arrangement worked satisfactorily with the cultures attached. The intake was constantly dry, the exit tube constantly moist,—at times even quite wet. With frequent changes of the calcium chlorid

tubes, thus keeping the incoming air thoroughly dried, the moisture in the medium evaporated much more rapidly than is usually the case in similar flask cultures with ordinary cotton plugs. The vigor of the cultures was not impaired by this rather drastic treatment. They continued to thrive and developed a thick mat of growth appreciably heavier than that of the controls.

When the set-up was so placed that the fungus was exposed to light, the reaction was characteristic; the carrot red color quickly appeared, then became obscured by a new overgrowth which in turn became deeply red. This succession occurred several times, the period between the appearance of colored mycelium varying more or less according to atmospheric conditions. The age of the cultures did not appear to be important.

After such constant aëration for three weeks, the medium had dried to approximately one-eighth the original volume, and the usual pseudosclerotia began to appear, embedded in the mycelial mat which had become deeply convoluted during the drying process. The experiment was allowed to run until no further change could be noted; *i.e.*, the spread of the mycelium had reached its maximum, and evaporation had apparently ceased. The flask was then broken and the adhering medium, now dense and leathery, was carefully whittled off as closely as possible, leaving the fungus mat intact. As the photograph shows (Pl. VII, fig. *c*), the surface was thickly dotted with the embedded pseudosclerotia which were approximately twice as large as those normally found in aged cultures. Many of them were as large as 3 mm. in diameter. Numerous ones were removed for histological study, but they differ from those previously described only in the larger size. It is concluded, therefore, that there are some significant changes produced in the vegetative condition of the organism as a result of the increased transpiration; but that either the reaction could not be carried far enough, or the combination of other stimuli, internal or external, was unfavorable for the production of sclerotia.

Oxygenation

In studying the relations of oxygen to *C. purpurea*, cultures were placed under a variety of conditions with respect to this element.

Hydrogen Peroxid. This substance was found to be inimical to the growing fungus under any arrangement for its use that was attempted. If placed in direct contact with the mycelium, the threads were quickly blackened. By means of a Glaseptic Nebulizer,[3] an extremely fine spray was added to the atmosphere above cultures growing in glass capsules, but in this case, too, the mycelium was soon darkened and further growth was inhibited. Increasing the amount of the hydrogen peroxid spray

[3] "Glaseptic Nebulizer" is the trade name of an atomizer, kindly provided by Parke, Davis & Co., Detroit, Mich., which gives an exceedingly fine spray and requires only a very small amount of the experimental material.

correspondingly increased the amount of injury, and at no point could any favorable stimulation be observed.

Enclosed Oxygen. Cultures in capsules and test-tube slants were placed in Novy jars which were then evacuated by means of an air pump and refilled with varying proportions of air and of oxygen from commercial tanks. The oxygen was added in 30 percent, 50 percent, and 80 percent amounts; the remaining percentage was air. An atmosphere of commercially pure oxygen was also used, evacuating the jar and allowing the tank pressure to fill it. It soon became evident that an enclosed atmosphere of high oxygen content does not favor the vegetative processes in this fungus. The mycelial mats in the jar containing approximately 44 percent oxygen and 56 percent nitrogen did not fall much below the controls, but when exposed to light they showed decidedly less coloration as compared with the controls. In the atmosphere of 60 percent oxygen, 40 percent nitrogen, there was a decrease in rate of growth and the color was also much paler than that of the controls. In 84 percent oxygen, 16 percent nitrogen, harmful effects were very marked; further growth was inhibited. With 100 percent oxygen, the conidia lost their power to germinate. Cultures that were first grown for a week under room conditions before placing in the jar of pure oxygen, were soon inhibited and were dead in approximately two weeks from the time of exposure.

Flowing Oxygen. Simultaneously with the foregoing tests, experiments were in progress on the effect of a continuous stream of oxygen at atmospheric pressure over the surface of cultures. For this a slightly more complicated apparatus was necessary than that used in the transpiration work, as shown in the photograph (Pl. VIII, fig. *c*), but it was similarly arranged.

A. Moist Oxygen Current. Gas from the tank was forced by its own pressure through a deep tube of 10 percent sulfuric acid, then through several tubes of sterile, distilled water, and finally over the surface of a thrifty week-old culture growing on deep agar slant in a liter Erlenmeyer flask. Gas intake was regulated by means of bubble count, about 100 per minute, so that no undue collection of moisture occurred as compared with the controls, but the nutrient material naturally did not dry as rapidly as that in the controls. The entire set-up was placed in the same southern exposure at a fourth-floor window facing on the open campus, and allowed to run continuously for four weeks.

A decided acceleration of both mycelium development and color formation occurred in the cultures thus treated. A heavier mycelial mat formed, and the characteristic carrot red color was produced from 24 to 48 hours earlier in the treated cultures than in the controls. Thus it regularly happened that treated cultures became fully colored and the color was obscured by the new overgrowth before the controls had developed their full coloration. The repeated cycle of tilleul buff-carrot red-tilleul buff was

always more marked in the treated cultures than in the controls. In the culture treated with oxygen the red color appeared three times with intervals between when newly formed mycelium obscured it before it, in turn, became colored. In the controls this cycle occurred only twice in the same period of time. Due to abundant moisture, the mycelium spread rapidly down the sides of the medium in the treated culture as the agar receded from the container. In this mycelium and in that on the thinner part of the slanted agar in the flask, pseudosclerotia were very numerous, deep red in color, and varied in size from 1.0 to 3.1 mm. in diameter. Not only did they appear nearly a week earlier, but they were also considerably larger than were those in the controls.

Despite this remarkable stimulation in the early stages of the treated cultures, it later became evident that through long exposure to the moist current of oxygen the fungus had lost its ability to develop the characteristic color. At the end of the fourth week it had seemingly run its course; the whole surface was now grayish while the control was still in its prime. The flasks were therefore detached and returned to the cupboard. Two weeks later, however, a most remarkable resumption of growth had occurred in the culture which had been treated: whereas the surface had been of a pasty, gray appearance when the culture was removed from the window, it now showed very marked, vigorous, upright hyphal development over the whole surface, with heavy mats extending down the sides. When again placed in direct light, the deep, rich carrot red color quickly appeared over the entire surface. This color continued for several weeks thereafter, and the mat became deeply plicate by the rapid development of hyphae as is shown in the photograph (Pl. VIII, figs. *a*, *b*).

B. Dry Oxygen Current. To test the effect of a stream of dry oxygen, the preceding apparatus was varied only by passing the stream of sterile, washed gas through a series of tubes of sterile calcium chloride before it entered the culture flasks. All other conditions duplicated those used with the moist gas. Results were somewhat different when the dry current was used. Instead of the treated culture leading, it now lagged about twenty-four hours behind the development of the controls. Succession of color and overgrowth was less marked from day to day under the dry gas than it had been under the moist current, and the controls passed through the color-overgrowth-color cycle about twenty-four hours earlier than did the treated culture. It is believed that the delay may be attributed to the effect of drying, rather than to that of the oxygen *per se*, for at the end of the experiment the treated culture was more vigorous than the controls. No harmful effect was apparent here, such as had occurred under the prolonged moist current; indeed, the culture had spread considerably wherever the medium contracted from the glass, and this mycelium, as well as that on the thinner portion of the slanted surface, was thickly dotted with pseudosclerotia showing characteristic color among the thrifty upright hyphal clumps. On

the whole, the dry oxygen seemed more favorable for increased mycelial development than did the moist oxygen. The apparatus was allowed to run several weeks, until the agar had practically disappeared. The mycelial mat had shrunk to about one-fifth the original area, and was correspondingly thickened and cortex-like. In cross-section, it showed a structure quite similar to the pseudoparenchyma of the natural sclerotia. In this mycelium the bodies were firmly embedded. Both of the foregoing series, with moist and with dry oxygen, were checked and substantiated the findings as given. They were also repeated, varying (*a*) the amount of food available to the fungus, and (*b*) using tissue transplants instead of spore suspension; but results in each case corresponded closely to the detailed account given above.

C. Oxygen Plus Carbon Dioxid. Since it had been found (*a*) that streaming pure oxygen stimulated vegetative development, and (*b*) that enclosed oxygen gave an unfavorable environment, a test was planned to study the effect produced by a stream of 50 percent oxygen. The same set-up was used, except that it was attached to a tank of carbon dioxid as well as to the tank of oxygen. The gases were mixed in closely approximate amounts (volumes), adjusted by means of bubble count, about 100 per minute. Development under this mixture was not different from that of the control during the first week. Then the treated culture lost its color, not from the usual formation of new overgrowth, but by fading or a real bleaching. The mat spread no farther, the surface appeared abnormal, of a grayish hue which remained the same throughout and presented a striking contrast to the control which was passing through the repeated color-overgrowth-color succession. No permanent injury had been done, however, for again after a two weeks rest from the current, the treated culture had resumed growth and continued to thrive for several weeks thereafter, giving characteristic color reactions upon exposure to light.

Throughout the experimentation with oxygen, the same facts stood out and may be summed up as follows:

1. In cultures grown in streaming oxygen, the growth of the mycelium is decidedly accelerated and color formation is promoted.

2. The characteristic pseudosclerotia are formed much sooner than under any other conditions, and are appreciably larger.

3. Further differentiation of tissue is lacking; and, somewhat later, control cultures reach a stage of development approximately equal to that of the treated cultures.

4. Oxygen gas, when available, is used by this fungus in large amounts for more rapid metabolism as shown by the marked increase in mycelium under such conditions, where a web two to three millimeters thick is formed, as compared to the average of one millimeter.

5. The accumulation or presence of carbon dioxid has a somewhat inhibitory effect upon the fungus, as shown in the experiment with flowing

gas, 50 percent oxygen and 50 percent carbon dioxid, and as also shown, presumably, in the experiment with enclosed oxygen.

Homothallism

Much successful work has been reported recently on the production of reproductive organs through the crossing of mycelium derived from single spores or from mycelial strains in such fungi as the rusts, several species of *Phytophthora* (2, 3, 4, 5), and *Monilia sitophila* (Mont.) Sacc. (9). Since *Claviceps purpurea* had proven to be unresponsive to the stimuli provided for inducing the formation of sclerotia, it seemed possible that in the case of this organism also heterothallism might figure in an important way in producing the stromata on which the perithecia are borne.

However, rather strong indications of homothallism have resulted from the present study, in attempting to isolate plus and minus strains. For preliminary work, the sclerotia used had been obtained from ten sources of commercial "Ergot." Three were from Spain, two from Germany, one from Portugal, one from Switzerland, and three from the United States. Bits of tissue were isolated aseptically from each of these, and from each of the resultant cultures a single-spore culture later was isolated. These ten cultures were then repeatedly crossed in the forty-five combinations. No antagonism was found. In every case, the hyphae from each source intermingled as they spread over the surface, forming a mat of normal appearance that could in no way be distinguished from that of either of the originals. These ten single-spore mycelial cultures have been maintained for more than a year, with paired cultures made at intervals of about three months, but no indication of sexual grouping has ever occurred.

From germinating sclerotia, mature asci were obtained for a similar study of single-ascospore cultures. These fine, thread-like, hyaline spores, which measure about $1.0 \times 14\ \mu$, are exceedingly difficult to separate from each other. A single ascus can be drawn up by means of an extremely fine capillary pipette, but when the slender spores are freed from the ascus and sprayed over the nutrient surface, it has proved impossible by any means yet devised to prevent their clumping in groups of two, three, or more spores. They are, moreover, so fragile that, even when a single ascus has been isolated in a little water, any attempt to separate the spores resulted invariably in broken or disintegrated spores. In no case, working with seventy-nine individual asci, was a successful isolation of a decisive number of single spores attained from a single ascus. The technical difficulties here can scarcely be over-emphasized though it is fully realized that, unless they be surmounted, no satisfactory conclusion can be drawn as to the possible heterogeneity of the ascospores.

In the course of this work a large number of cultures accumulated, for the ascospores grow readily when sprayed upon the agar surface. Some were from groups of ascospores, others were from single spores, but all

appeared identical both macroscopically and microscopically. Several of these cultures were used as the basis for further crossing experiments. Cultures I, II, III, IV were from the same ascus and consisted, respectively, of one, one, two, four ascospores. Cultures V, VI, VII, VIII, and IX were from another single ascus and consisted, respectively, of one, one, one, two, three ascospores. Thus seven cultures known to be from single ascospores *from three different asci*, and five cultures comprising two or more ascospores from the same ascus were maintained for a sufficient length of time to test and repeat the sixty-six possible combinations. Here, as with the conidial cultures, it was clearly seen that no combination resulted in any evidence of inhibition or acceleration. Cultures grew normally, whether both were inoculated in the same area of the medium or planted at opposite sides and allowed to meet. In no case could any tendency toward sexual grouping be discerned. The mat formed by hyphae meeting from separate inoculations differed in no way from that developed where crossing was made in the same area; nor could either of these be seen to differ from an ordinary saprophytic culture of a single ascospore or a single conidium.

Field Culture of Ergot

During the summer months of 1923 and 1924, a study was made of the practicability of growing ergot in field rye. In the fall of 1922, preparations were made by seeding three one-fourth acre plats with Rosen rye at intervals of two weeks, hoping thus to secure three blooming periods. All plats grew well, but in the spring not much difference was to be noted in their general appearance. The field planted earliest came into bloom first but the other two were very little behind it, so that all three had to be inoculated together during most of the flowering period, June 6 to June 14. To prepare the inoculum, spore suspensions from mature cultures of *C. purpurea* were grown in mass upon trays of agar medium for from four to five days. This gelatinous, conidial growth was then washed from the surface, filtered through sterile gauze, and made into a heavy suspension with sterile, distilled water. In the field this suspension was added to a tank of water and, by means of a pressure pump, sprayed in a fine mist over the blooming heads of rye. The whole spraying equipment was mounted upon a horse-drawn wagon. Sterile plates of standard medium were exposed each time to the mist at the beginning, midway, and at the end of the field spraying. They developed heavy growth over the entire plate, with surprisingly few contaminants showing. Spraying was repeated every second day until five inoculations had been given. The weather was troublesome, showers occurred on two of the five days during or soon after the spraying. However, a considerable infection was evident as the "honey dew" began to appear June 21, from which normal conidial cultures were obtained. Immature sclerotia were forming abundantly by June 29 and were beginning to show color when a definitely unfavorable period of weather occurred.

An extremely hot, dry interval was followed by a violent hailstorm July 5, which badly flattened the rye. It revived fairly well within the next few days, but during July 6–11 there were three disastrous wind and rain storms which left the rye badly lodged. Many of the developing sclerotia had been killed. Subsequent development was much less than had been indicated in June, but a fair amount of infection still persisted. The sclerotia, however, were rather stunted and mis-shapen, and the final yield was scarcely worth the trouble of harvesting by hand. While material results for the season were disappointing, yet it had been shown that mass inoculation could produce heavy infection.

The following summer considerable improvement was made in technique, but matters in general were handled as before except that a new, better sprayer was available, steam was added to make the water tepid, and glucose was added to the spray mixture with the idea that it might be useful as an adhesive agent. Four one-quarter acre plats were used: *a*, Rosen rye; *b*, Old Type rye; *c*, Spring rye; and *d*, Burt oats, an open-glume type of oats, said to be susceptible to *Claviceps purpurea*. Plats *a* and *b*, having been put in in the fall, came into bloom first, and were sprayed twice daily from June 17 to June 20, inclusive, a total of eight times. Plats *c* and *d* came into flower a few days later, and were sprayed twice daily June 24 to June 30, inclusive, except on June 28 when a heavy thunderstorm prevented. Throughout much of the time when the fungus should have been developing, the weather continued hot and dry with the result that very little infection was obtained in any of the plats. It was therefore concluded that such method of ergot production is not likely to be of practical use in America, where the cost of hand labor is prohibitive as compared with, for example, the conditions under which Hecke carried on his experiments. As stated elsewhere (p. 52), large, well-formed ergots can be produced by means of hand inoculation, even when conditions are not especially favorable, but this could not prove practicable on a large scale.

Pharmaceutical Characteristics of Mycelial Extracts

The active principles of ergot are chiefly the specific alkaloids (ergotoxine and ergotamine), histamine, and tyramine. The relative amount of each present in an extract of ergot is judged by physiological methods, of which the cock's comb test is official in the United States Pharmacopoeia. In general it may be said that the specific alkaloids are well shown by the cock's comb test; the oxytocic test, using isolated guinea-pig uterus muscle, indicates more closely the histamine content; while the blood-pressure test will indicate the presence of all three but, because of the antagonistic action of histamine, is not a reliable quantitative index of activity.

Rather numerous tests were made of extracts prepared from *C. purpurea* grown upon various media, in order to compare such saprophytic growth with the natural parasitic type. Some of the samples were subjected to

all three of the tests but, since the cock's comb test is official and is considered by many pharmacologists to be the most reliable for indicating the actual value of an ergot extract, many were tested only by that method.[4] The many details of the preparation of each sample need not here be given. Suffice it to say that each represents a Fluid Extract made from saprophytic growth of a culture of *C. purpurea*, approximating as closely as possible the strength of the official U.S.P. preparation. Where results are indicated by figures, it is to be understood that 100 percent is typified by U.S.P. Fluid Extract.

TABLE 3. *Physiological Tests of Saprophytic Mycelium of Claviceps purpurea*

Sample Number	Culture Number	Cock's Comb Test	Oxytocic Test Test	Blood Pressure Test
1	168	30–40 percent of Standard	400 percent of Standard	Not characteristic
2	169	50 percent of Standard	40 percent of Standard	
3	171	About 60 percent of Standard	40–50 percent of Standard	Rather characteristic
4	169	About 50 percent of Standard	100 percent of Standard	Marked depressor
5	169	50–40 percent of Standard	Fair	Equal to Standard
6	168	About 40 percent of Standard	Fair	Nearly Standard
7	177	About 75 percent of Standard	None	Not characteristic
8	177	Typical. 60–75 percent of Standard	Marked activity	Marked depressor Moderate depressor
9	177	Fair. 25–40 percent of Standard	Fair	Not characteristic
10	177	75 percent of Standard		
11	181	40 percent of Standard		
12	169	50 percent of Standard		
13	181	65 percent of Standard		
14	181	50 percent of Standard		

The Standard referred to in the above table is the reaction produced by the official U.S.P.X. Extract of Ergot. A study of the above data will show that Culture 177, used throughout this work, gave the highest yield of the characteristic alkaloids. The percentage is found to vary according to the medium used; *e.g.*, the only difference in nos. 7, 8, 9, 10 was the medium upon which the fungus was grown.

A study of table 3 will show that the extracts varied considerably in their content of the active principles, but several showed a typical reaction approximately equal to three-fourths that of a standard ergot extract; *e.g.*, Nos. 7, 8, 10, and 13. The studies were carried far enough to show considerable encouragement in this method of securing the active principles from the mycelium. It could be employed successfully in case it should become necessary to depend upon artificial culture of the organism for

[4] For physiological testing of the samples listed in table 3, the author is indebted to L. W. Rowe of Parke, Davis and Company.

medicinal purposes. The significant fact is that this is the first record of a successful attempt at securing the extract from any part of the fungus other than from the sclerotium. Because of practical considerations, nothing more was done on this phase of the work.

General Discussion

In the foregoing studies, *Claviceps purpurea* under saprophytic maintenance has been found responsive in marked degree to several factors of environment, such as control of food or temperature, and especially so with regard to oxygen, moisture, and light. Control of vegetative development by high optima of these factors has met with unprecedented success, a heavy, cortex-like layer having been secured at will. In section, this layer is strongly suggestive of the structure of a cross-section of the natural sclerotium, but it has not yet been induced to round up into definite sclerotia.

The effects of light upon fungi vary greatly in different species, from a factor of great importance to one of no moment. Generally speaking, prolonged exposure to strong light has been regarded as detrimental for successful vegetative development. Early mycologists believed light to be of much importance in the development of morphological structures in fungi, and its absence was held accountable for the formation of great masses of sterile mycelium in cellars and caves. Brefeld (7) was the first one to study the question scientifically, and found that while some species can accomplish feeble fructification in darkness, many others remain sterile unless given at least a brief exposure to light. Even so slight a duration as two hours he found sufficient to incite the formation of reproductive bodies which then made normal progress in the dark. Klebs (18, 19) found light to be of the utmost importance in the development of fungi and he stressed the need for investigating the effect of this factor upon each organism studied. Coons (8) reported that light is a strong determining factor for pycnidium production in *Plenodomus fuscomaculans* (Sacc.) Coons and, like Brefeld, he found that a short exposure is sufficient to bring about the desired result. Robinson (28), working with *Pyronema confluens* (Pers.) Tulasne, states that no reproductive structures occur except in the presence of some illumination, although the minimum of light energy required is small. Angell (1), in a study of pigment production in *Macrosporium porri* Ell., considers light to be wholly without influence. In the writer's work on the ergot fungus, the best development was obtained while the organism was exposed to the full influence of direct sunlight. Bonns (6) does not state the conditions under which he grew *C. purpurea*, except that his cultures were left at room temperature; but he mentions the occasional appearance of color, so it is probable that they were not grown in the dark. Kirchhoff (17) grew this organism entirely in darkness, and held light to be unnecessary for typical development both of mycelial growth and of his

so-called "artificial sclerotia." His result is decidedly at variance with the work of the writer who has found that light exerts a profound influence in such cultures, favorably affecting the growth and producing a most striking color development in the mycelial walls. No other stimulus caused these effects, whereas they were invariably induced by exposure to sunlight even for so brief a period as one hour. It does not appear that such color is due primarily to ultra-violet rays; indeed, it seems evident that the blue rays are the more effective for chromogenesis. Color was not produced by exposure to the ultra-violet rays from a Cooper-Hewitt mercury-vapor lamp, with subsequent removal to darkness, but a culture exposed to direct sunlight no more than one hour would later develop the characteristic color even when removed to the dark chamber. It was of considerable interest to note that no purple color, as reported by Bonns (6) and by Kirchhoff (17), has ever occurred in the cultures of the writer. Shades from "coral pink" to the characteristic, brilliant "carrot red" were readily obtainable according to the intensity of the light given, but no trace of purple ever was seen.

The lipoid material containing the pigment appears to be very rich in ergosterol. When it was extracted from the colored mycelium, as an oily, amber-colored liquid, and tested with antimony trichlorid, the characteristic evanescent pink, followed by a permanent blue color was obtained (10). Mycelium in every way comparable to this, except that it had not been exposed to light and hence was not colored, was similarly extracted and tested. It gave a faint reaction for ergosterol, but it was very slight as compared to that obtained from the colored mat.

Among the essential conditions for all forms of life, oxygen must be regarded as outstanding. There is, of course, a wide variation in the optimum requirement for different organisms, even for different stages of development of the same organism, but there are few, if any, species in existence that can be considered strictly anaerobic. In its relation to fungi, oxygen has been the subject of a great many studies. Among others, Angell (1) reported that oxygen is essential for pigmentation in *Macrosporium porri* Ell. Fellows (12) stated that *Ophiobolus graminis* Sacc. is not much affected until the oxygen content falls below 6 percent, when growth is greatly reduced. Leonian (20) studied the oxygen requirements of 20 species of Sphaeropsidales. Decreased oxygen supply suppressed fruiting in 3 of the species, reduced it in 11 species, and had no effect upon the other 6 species.

In the case of *C. purpurea* oxygen is a prime necessity. In any proportion, even to 100 percent, the fungus can utilize it, providing there is free circulation. Enclosed oxygen or enclosed air is unfavorable for good development of this organism, possibly because of carbon dioxid accumulation; but vigor of growth, thickness of mycelial mat, height of aërial mycelium, size of conidia, and size of pseudosclerotia were all more marked

in cultures given prolonged exposure to an atmosphere of flowing oxygen. While elaborate studies, such as those of Novy (26) and Novy and Soule (27) could not well be carried on with a fungus of this type, nevertheless it has been established that the organism thrives only with ample aëration. It is precisely this necessity for abundant aëration that made one phase of the study unattainable. Any attempt to grow the organism under mechanical pressure failed; cultures were unable to survive such conditions. This leaves unsettled the theory of Bonns (6) that cortex-like portions of the mycelial mat fail to round up into sclerotia only because they are developed without pressure; a plausible idea, but difficult to prove in this case.

So abundant has been the mycelial development obtained in these cultures, so constant have been the characteristics of such growth and its behavior over long periods of time, that the author feels certain *Claviceps purpurea* will yet be induced to carry through its full life cycle as a saprophyte. It is probable that such completion depends upon giving to the pseudosclerotia the environmental conditions comparable to those encountered by the natural sclerotium forming in the living host. It is not easy for one thoroughly familiar with this organism to believe that these pseudosclerotia are merely casual in the development of the mycelium. They are a constant, well-defined phenomenon the further study of which is fully warranted. However, nothing herein said is to be taken as a claim that they are, in truth, sclerotia, for such an opinion is not held. To make a claim that cannot be substantiated in every detail merely clouds the issue. Such an unwarranted assumption is made by Kirchhoff (17) in stating that he has obtained, in culture, artificial sclerotia of *C. purpurea*. The structures which Kirchhoff designates by this term are not new, nor are they at all uncommon. Hard, cortex-like, sclerotioid tissues are a fairly constant feature of the growth upon a variety of media, and just such portions as Kirchhoff describes can frequently be dissected, not only from the edges of the mycelium but in other parts of the surface. Several years before Kirchhoff's work was published, Bonns (6) recorded his opinion that the formation of such relatively hard crusts represents "a stage analogous to that found in the sclerotium," but he did not suggest that they be considered really sclerotia. It is recognized that size or shape alone are not features that determine the reproductive bodies developed in saprophytic culture. In the case of this fungus it is scarcely to be expected that they would conform closely in shape to the sclerotia formed in the rye flower. Nevertheless, they should be able to form the typical stroma, perithecia, and asci. Until there can be produced at will, by the growth of *C. purpurea* in culture, sclerotia that will later develop perithecia with viable ascospores, no one can maintain in fairness that he has succeeded in the production of "artificial sclerotia." To this extent, the writer takes definite issue with the claim so put forth recently by Kirchhoff.

No previous work has been done on the problem of heterothallic strains occurring in this species. As would be expected, matings between single-spore cultures from conidia of widely separated sources gave no significant results and merely made typical development. Even with the paired cultures from ascospores decisive conclusions cannot be drawn from the cultures so far obtained, yet the evidence points in the direction of homothallism. Although five single spores have never been secured from the same ascus, nevertheless all eight spores from an ascus have frequently been in a group of matings; *e.g.*, cultures from four, two, one, one ascospores, respectively, from a single ascus, were mated with each other and with similar cultures of one, one, two, three ascospores from another ascus. Neither in single-spore matings nor in the matings of the group cultures was there ever any indication of heterothallism. It may be possible eventually to isolate decisive numbers of single ascospores from a convincing number of single asci. Numerous crossings of such single-ascospore strains might then yield mycelium that will produce viable sclerotia. But these fragile, filiform spores are so easily destroyed in the manipulation necessary to isolate them that the difficulty in this case is much greater than with many types of ascospores or basidiospores such as those which were studied by Hanna (13), by Sass (29), or by Dodge (9).

The laboratory production of ergot of characteristic properties has been found to be quite feasible. To a somewhat less extent, this is true of field culture of *C. purpurea*. It is problematical, however, whether such laboratory production will be practical for medicinal application. The possibility of so utilizing cultivated ergot interlocks closely with conditions of European commerce and, being an economic question, need not here be discussed.

Summary

1. The studies of *Claviceps purpurea* herein reported have shown that under controlled experimental conditions, both physical and physiological, an unprecedented mycelial growth of this fungus can be obtained in saprophytic culture.

2. A nutrient synthetic agar was selected and used as a standard medium for securing a maximum mycelial development of this organism.

3. Of the stimuli tried, oxygen was found to have the greatest effect upon mycelial growth, also for increased size of the conidia and for earlier appearance and greater size of the pseudosclerotia.

4. The optima of heat, light, and moisture were determined, and reactions to other physical stimuli such as mechanical pressure and aëration are reported.

5. Sunlight produces a marked chromogenic effect upon the mycelium of this fungus by causing an intense coloration, Carrot red (R.). This greatly exceeded the faint trace of color produced by means of any other stimulant tried.

6. It has been shown in this and in a previous work by the writer (23) that the favorably stimulating rays for color production lie in the blue-violet region of the spectrum, and that the ultra-violet rays, although not markedly injurious to *C. purpurea*, have no special effect upon growth or reproduction.

7. Light has been found to increase the ergosterol content of the mycelium of this fungus growing in saprophytic culture.

8. No true sclerotia have been developed in culture, but the production of definite mycelial knots herein designated as pseudosclerotia is a constant phenomenon in properly nourished mycelium. It is suggested that these structures are possibly primordia of true sclerotia which have failed to reach their full development through lack of some essential conditions.

9. A partially sclerotioid mycelium is readily obtained on standard medium. Such tissue, however, does not develop the morphological characteristics of true sclerotia nor does it pass through a stage analogous to the stroma formation and subsequent germination of the natural sclerotia.

10. The experiments with paired cultures give strong indication of homothallism in *C. purpurea*.

11. *It has been demonstrated for the first time that this fungus develops in saprophytic culture the three chief active principles which are characteristic of the extracts made from the natural sclerotia; viz.*, ergotoxin, histamine, and tyramine; and that they are obtainable to an extent sufficiently large to be of economic significance.

12. Field demonstrations have shown it to be improbable that parasitic culture of *C. purpurea* on a large scale would be desirable, under prevailing conditions in Southern Michigan.

The studies on field culture of ergot, and the pharmacological work with extracts of saprophytic cultures, were carried out under the supervision of Parke, Davis & Co., Detroit, Michigan, to whom the author expresses her sincere appreciation for the privilege of using this material, and for their encouragement during the work on the problem herein discussed.

Grateful acknowledgment is made to Dr. C. H. Kauffman, under whose direction the work has been done, for his unfailing help throughout the years spent in these studies. Due to the illness of Dr. Kauffman, the final criticism of manuscript was kindly given by Prof. H. H. Bartlett and Dr. B. B. Kanouse, but the author assumes full responsibility for any errors that may appear.

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DESCRIPTION OF PLATES

PLATE VII

FIG. *a*. A section through a young pseudosclerotium, with the ordinary mycelial mat showing at each side. The lower, vacuolated area is overlain by denser layers, with loose weft of mycelium over all.

FIG. *b*. Detail of a center section of a typical pseudosclerotium. The vacuolated inner portion of pseudoparenchymatous appearance is overlain by denser layers of mycelial growth.

FIG. *c*. The dried mycelial mat from a culture which has undergone three weeks of forced transpiration. Pseudosclerotia may be seen embedded in all parts of the surface.

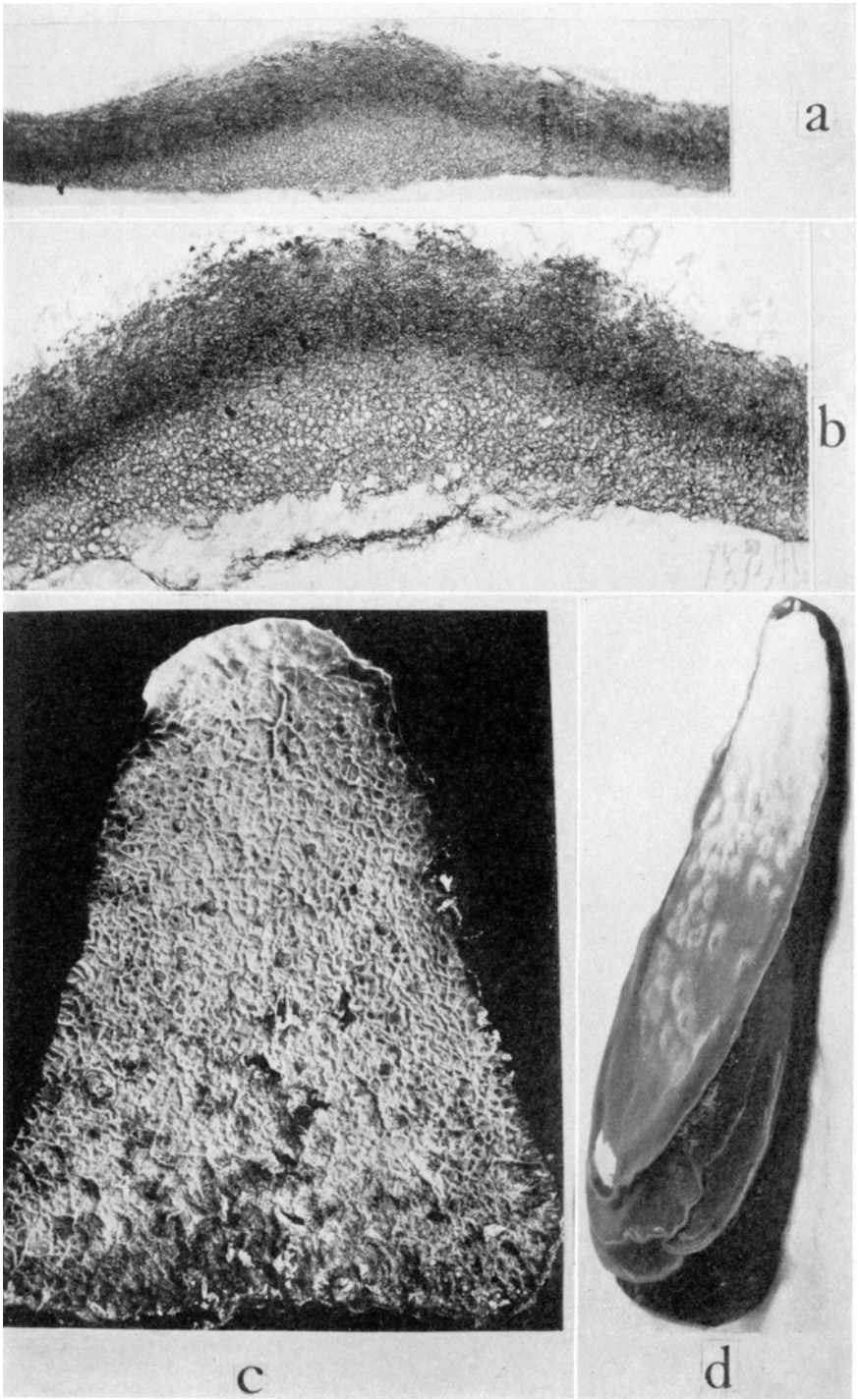
FIG. *d*. Pseudosclerotia forming in an actively growing culture having a surface about 10 × 3.5 cm. Typical mycelial overgrowth is seen starting over the thinner portion of the slant.

PLATE VIII

FIG. *a*. Culture exposed to continuous flow of oxygen about three weeks, showing heavy mat 2.5 to 3.0 mm. thick.

FIG. *b*. Rear view of culture shown in (*a*). The deeply plicate mat shows, at the right, the early stage of formation of pseudosclerotia.

FIG. *c*. The apparatus used for oxygen experiments showing, from the left, the supply tanks of oxygen and carbon dioxid, the washing tubes, and the calcium chlorid containers for drying the gas.



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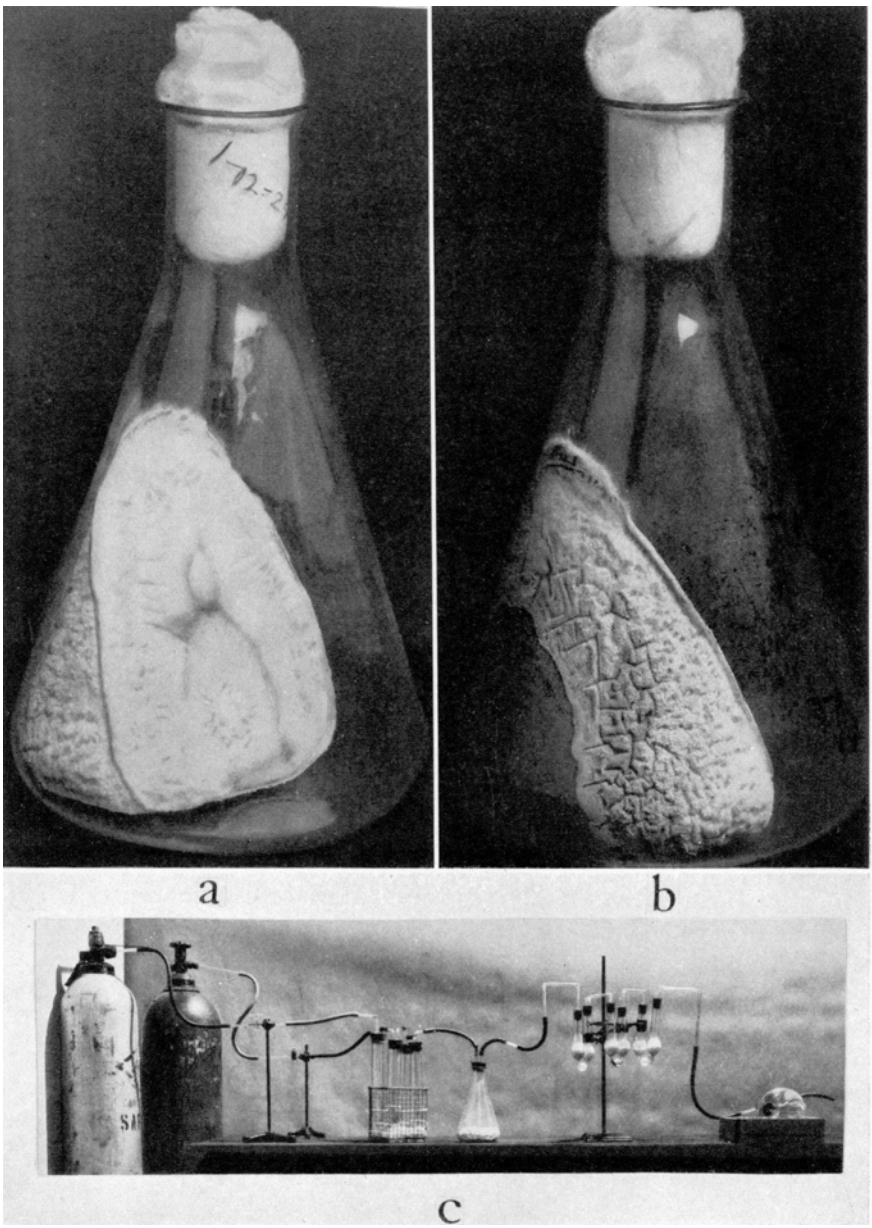

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