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On a Form of Botrytis cinerea, with Colourless Sclerotia

William B. Brierley

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III.—*On a Form of* Botrytis cinerea, *with Colourless Sclerotia.*

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Communicated by Dr. E. J. Russell, *F.R.S.*

[Plate 5.]

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1. Introduction.

Botrytis cinerea is perhaps the commonest and best known fungus, and has been a centre of mycological research since the time of de Bary. Few, if any other fungi, have been studied so thoroughly by so many able investigators, or are the subject of so extensive a literature; and one need only draw attention to the researches of de Bary (6), Pirotta (61), Marshall Ward (80), Kissling (45), Nordhausen (58), R. E. Smith (70), Farneti (27), Beauverie and Guilliermond (9, 10), Istvanffi (33), Reidemeister (65), and the more recent researches from the laboratory of Prof. V. H. Blackman (13, 16, 17, 84). There is a body of experimentally ascertained and exact knowledge concerning the bionomics of this fungus, which can be exceeded by that of few other micro-organisms.

For the particular purposes in view in my investigations, it has been imperative

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that all experimental work be carried out with pedigree cultures. It was very early discovered that the ordinary "pure-culture" of mycological and bacteriological laboratories may be and very often is a poly-genotypic population, and that very considerable genotypic differences may usually be found in apparently homogeneous populations exhibiting only one single type, around which the individuals fluctuate. In order to eliminate this vitiating factor, single-spore cultures were prepared either by BURRI's (19) Indian-ink method, plate isolation, or, more usually, by a combination of the plate and dilution-drop method. These cultures served as the initial source of experimental material. In all, over seventy such pedigree stocks have been prepared from original sub-strata, representing about sixty different species of host plant, derived from all parts of the country. From these stocks some fifteen thousand cultures have been made. During the course of the investigation, many of these have been subjected to the most diverse environmental conditions, and all have been maintained under the closest scrutiny. With the exception of the single culture to be described, no change that could be interpreted as a permanent heritable alteration or mutation has been observed.

2. LIFE CYCLE OF *Botrytis cinerea*.

In nature, the fungus usually appears as a delicate smoke-grey velvety pile, covering diseased plant tissues or organic *debris*, or, on more resistant sub-strata, in the form of isolated grey pustules. This superficial growth consists of the branched conidiophores bearing spores. On germination, which under favourable conditions occurs in a few hours, each of the latter gives rise to a mycelium, which in two or three days produces a further crop of conidiophores and spores. When old, the hyphæ and spores may produce microconidia, which germinate directly giving rise to mycelium. If at any stage of development conditions unfavourable to sporogeny intervene, the mycelium produces sclerotia, and these, under more favourable conditions, give rise to tufts of conidiophores bearing spores. Under certain adverse conditions, the hyphæ may produce chlamydospores, or themselves break up into oïdia, and these, on germination, give rise to normal mycelium.

It was the opinion of DE BARY (5, 6), VIALA (79), ZOPF (89), and other early investigators, an opinion based purely on superficial resemblances and the frequent contiguity of growth of the organisms, that *Botrytis cinerea* is only the conidial phase of a Discomycetous fungus, which was referred to either *Sclerotinia Fuckeliana* or *S. libertiana*. In the absence of any proof of this connection, and despite their own negative experience, this opinion was accepted by MARSHALL WARD (80), KISSLING (45), and others, and so became an integral part of mycological literature. In 1905, ISTVANFFI (32) published a voluminous memoir, purporting to bring forward proof of the genetic relationship of *Botrytis cinerea* and *Sclerotinia Fuckeliana*. In spite of the prior and much more incisive work of R. E. SMITH (70), this evidence has been generally accepted. It is, however, of

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doubtful value, for a critical examination of the investigation makes clear that ISTVANFFI has confused two distinct fungi, which possess certain superficial resemblances, often grow together on the same host, and are not easily separable in culture.

Recently, SEAVER and HORNE (68) claim to have established the relationship of an unnamed species of *Botrytis* with a new species of *Sclerotinia*, which they have termed *S. Geranii*. The evidence is very brief, and awaits confirmation, and, in view of the fact that many "pure-cultures" of fungi are undoubtedly mixed populations, that *Botrytis cinerea* and various species of *Sclerotinia* and other genera harmonise perfectly in their growth when developing intermixed, both on natural hosts and on many artificial media, and that, unless specially searched for, the very minute microconidia of both forms are easily overlooked, and may act as a contaminating factor, one may perhaps hesitate to accept unreservedly this evidence. Furthermore, in my own study, some fifteen thousand cultures of various strains of *Botrytis cinerea* and nearly related "species" have been closely observed under the most varied environmental conditions, and much experimental work has been specially directed towards the elucidation of the genetic relationships of the fungus. I have, however, found no evidence indicating that *Botrytis cinerea* is in any way a developmental phase within the life-cycle of *Sclerotinia Fuckeliana* or genetically related to any other species of this genus. Repeatedly, strains of *Botrytis cinerea* and various species of *Sclerotinia* have been grown side by side by inoculating alternate quadrants of a plate culture, and, under all conditions, have remained separate. My experience merely confirms that of LIND (47), R. E. SMITH (70), PELTIER (59), PETHYBRIDGE (60), and others, who have paid special attention to this aspect of the problem.

Botrytis cinerea as a discrete entity is an asexual fungus, and the critical importance of a *Sclerotinial* relationship lies in the possibility which this introduces of a sexual process. If the organism be sexual, any single individual may possibly be heterozygous, and there is then no inherent improbability that segregation may occur, resulting in the appearance of apparently new forms which might mistakenly be interpreted as mutants.

In many *Mucorineæ*, *Chytridineæ*, *Saccharomyces* and possibly other *Ascomycetes* the sexual process is allogamous and factorial segregation is not impossible. In the great majority of fungi, however, in which sexuality occurs, and here is included so far as is known the entire group *Discomycetineæ*, which contains the genus *Sclerotinia*, the process is autogamous. Now it is well known, and has recently been mathematically demonstrated by JENNINGS (35), that even in an originally heterozygous organism, self-fertilisation if continued generation after generation leads rapidly to a condition in which the offspring are homozygous. In such case JOHANNSEN (36, 37) has shown that the mere isolation of the progeny from a single reproductive member produces a pure line.

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Even, therefore, in the remote contingency that *Botrytis cinerea* is merely a developmental phase of *Sclerotinia Fuckeliana* and that this possesses a sexual process—which has yet to be demonstrated for any species of this genus—it would appear that the complications resulting from heterozygosis are absent from our problem. For critical purposes *Botrytis cinerea* is, on all evidential criteria, an asexual homozygotic organism in which the isolation of a single spore strain necessarily implies the isolation of a "pure-line." A genotypic change in a pure line is a mutation.

3. Origin of Colourless Form. General.

On July 10, 1917, a diseased specimen of *Crassula perforata* from the succulent house of the Royal Botanic Gardens, Kew, was examined, and the causal pathogen identified as *Botrytis cinerea*. The growth was normal in every respect, and from it six cultures, (A. 1–6), were prepared. These appeared to be free from contamination by other organisms, and on July 16, six further cultures, (B. 1–6), were taken from (A. 6). On July 22 all twelve were carefully examined and found to be free from contamination. A series of dilution drops were then prepared from (B. 6), many of these drops containing but a single spore. The latter were transferred to a thin plate of potato-agar and their development scrutinised carefully during two days. A colony whose origin from a single spore had been ascertained with certainty was then picked up on a platinum wire and planted in a tube slant. The spores are comparatively large, the most common size on the host plant being $7·5\ \mu \times 9·5\ \mu$; they are thus easily seen and isolated. On germination they become still more obvious, and the obtaining of a single-spore strain is thus a simple procedure. On August 10 twelve cultures (D. 1–12), were prepared from the pedigree stock (C. 1), and on August 23 six further cultures, (E. 1–6) were made from (D. 12). So far the work had merely been a part of the ordinary laboratory routine, the cultures which were all on tube slants of potato-agar being used for various experimental purposes.

About a week later cultures (E. 1-6) were examined and some three or four days later re-examined. The six cultures had all been placed together in a wire basket and left on the laboratory bench. It was noted that in tube (E. 6) one of the sclerotia had not turned black like the others but had remained colourless. In certain strains of *Botrytis cinerea* these bodies do not form the black pigment characteristic of the genus until they are mature, and although this particular sclerotium differed from its fellows in apparently following this mode of behaviour, it was merely regarded as a somewhat youthful individual, perhaps a little unusual, but not particularly noteworthy. On September 10, however, when tubes (E. 1–6) were again examined and the aberrant sclerotium in (E. 6), now quite mature, was found to be still lacking any sign of pigmentation, it was decided to isolate this sclerotium and breed from it. In tube (E. 6) there were in addition to the colourless sclerotium 24 normal black sclerotia and a black sclerotial crust around the bottom of the tube, where the medium had contracted away from the glass.

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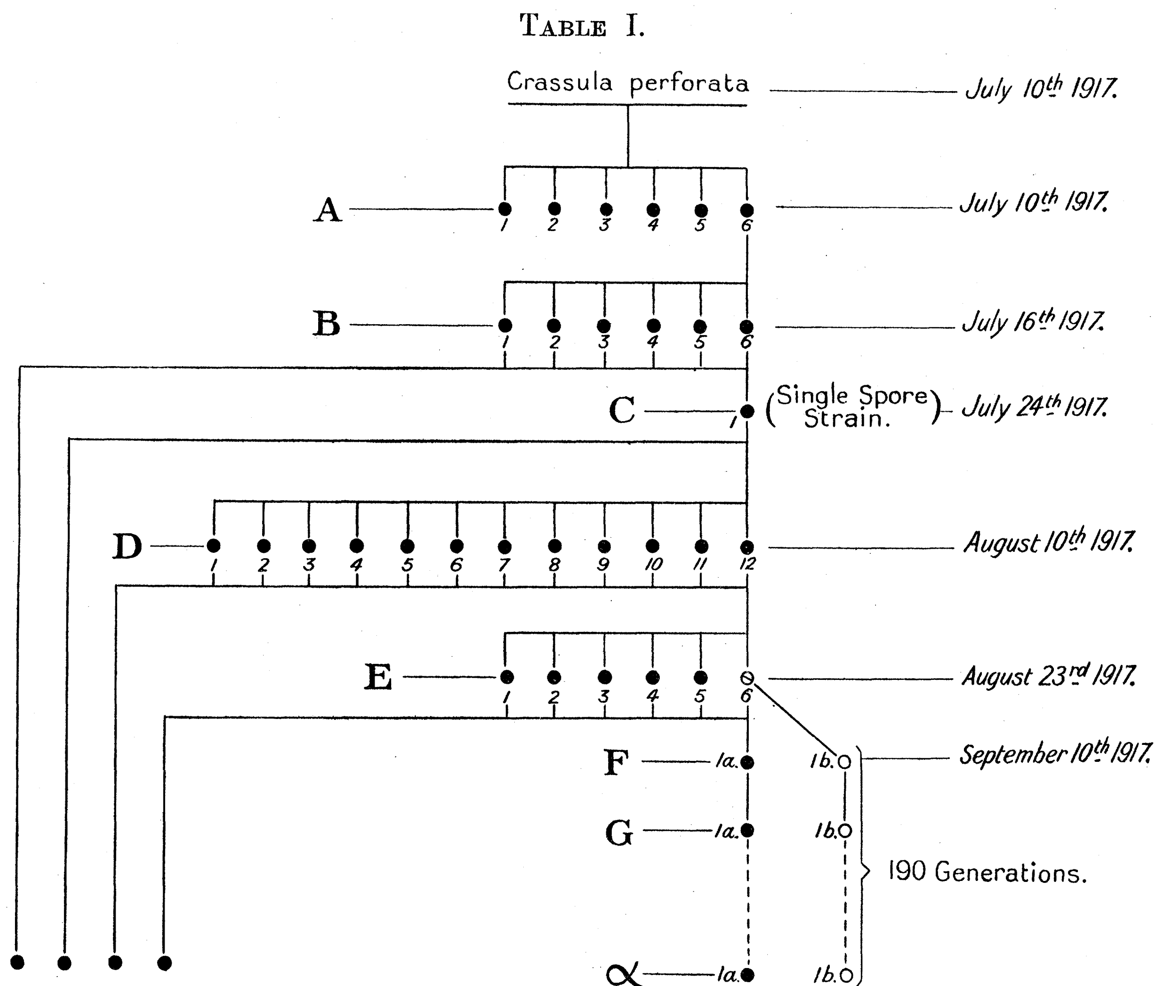
The colourless sclerotium was removed and thoroughly washed in sterile distilled water to remove adherent spores. It was then broken open and a fragment from the centre taken out, placed on a tube-slant of potato-agar (F.I. *b*) and incubated at 22° C. A vigorous growth ensued and a week later sclerotia began to be formed. In the tube there were ultimately formed 17 sclerotia and all were colourless.

TABLE I.



In a normal sclerotium the pigmentation is confined to the outer one to three layers of cells (text-fig. 1) and as it is from these that the conidiophores arise on germination, it was considered not impossible that these cells and their derivatives only might carry the power of colour formation. If, therefore, a fragment of the inner hyaline tissues of such a normal sclerotium were planted on a nutrient medium it seemed possible that colourless sclerotia similar to those obtained in tube (F.I. *b*) might develop. Accordingly, a black sclerotium from (E. 6) was treated thus—exactly as the aberrant sclerotium had been treated. The subsequent culture (F.I. *a*) contained uniformly black sclerotia.

There were now in existence two distinct strains (F.I. *a*) and (F.I. *b*) of the fungus

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both derived from a single-spore stock (C. 1). Of these the parental form (F.I. *a*) possessed the highly characteristic black sclerotia; whilst the new strain (F.I. *b*) which had arisen from this apparently by a single direct saltation, possessed colourless sclerotia. The history of the two forms is shown in a diagrammatic manner in Table I. The distinguishing lettering (A. 1–6) and so forth, and the arrangement of descent in this Table have been adopted for the sake of clearness in the account. In practice (D. 1–12) were merely 12 similar cultures taken from (C. 1), and (E. 6) was taken from one of these cultures chosen at random. In the same way six cultures were made on July 16 and from one of these chosen at random (C. 1) was prepared.

It seemed possible that an albinistic tendency might be a "weakness" inherent in this particular race of *Botrytis cinerea*, and that if the preceding generations were further subcultured colourless sclerotia might again appear. Unfortunately the original diseased plant and the first six cultures made from it had been destroyed, but all subsequent cultures had been retained. As no particular care had been taken of these some had become contaminated. In all cases, however, it was possible to subculture purely from them and this was done extensively for many generations under the most diverse conditions of light, temperature, food supply and so forth, all the resulting cultures being most carefully examined for any indication of a lack of sclerotial pigmentation. All were perfectly normal.

The parental strain and its colourless derivative have each, up to the time of writing, passed through over two hundred direct tube generations with very many lateral subcultures, and although these have been placed under diverse environmental conditions each strain has remained absolutely constant.

Since this colourless strain arose, many thousands of cultures and fresh specimens of *Botrytis cinerea* have passed under my observation, but in no single instance have I found any similar absence of sclerotial pigmentation. Prof. H. H. Whetzel, of Cornell University, who for some years has been engaged upon a monograph of the genus Botrytis and has received and compared material from many parts of the world, informs me, after having examined a culture of the colourless strain, that he has "never seen anything quite like it." In the voluminous literature devoted to the fungus there is no record of a form with colourless sclerotia.

Having regard, therefore, to our knowledge of the fungus *Botrytis cinerea* and to the particular conditions under which the aberrant strain originated, it would appear difficult on present criteria to interpret it as other than a true mutation.

4. Comparison of Parental Strain and its Colourless Derivative.

The obvious difference between the original and the derived forms lies in the colour of the sclerotia. Mutations in the fungi *Aspergillus niger* and *Penicillium glaucum* have been described at length by Arcichovskij (2), Waterman (83) and Schiemann (69). In these cases, however, although the obvious change was in spore

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colouration, there were other less visible changes in several independent respects, certain morphological characters and physiological activities of the derived fungi being different from those of the original forms. In addition to these facts, the results of recent researches on the linkage of characters and the multiple allelomorph interpretation of quantitative inheritance made it appear probable that in the present case the colour change might be only one of many related changes less obviously visible.

Moreover, whether one regards the black sclerotial pigment as a mere excreted product or as an integral and actively functioning substance in the metabolic activities of the organism, the pigment itself is the result of a long and elaborate series of causally dependent processes, and a sudden and permanent loss of the power to excrete or form such matter can only be the visible expression of deep-seated physiological changes in the developing organism. But a living organism is an extremely complex and delicate equilibration of rhythmic metabolic processes in a colloid substratum, and it is difficult to conceive of any sudden derangement of these activities which has not reverberations in many directions.

It seemed, therefore, very desirable to make some comparison of the parental strain and its colourless derivative to ascertain the nature of any changes other than pigmentation which might be present. This comparison proceeded along several lines, the main directions of which are indicated briefly below.

A. *Physiological Activities.*

If, as evidence would seem to show, the physiological reaction of a particular organism is constant only in an unvarying environment, it follows that identity of response to like stimuli implies identity of constitution, and, conversely, that a change in physiological constitution will be reflected in a changed reaction to unaltered conditions. Only if this is true may a comparison of the physiological activities of two organisms be instituted; and being true, it will afford the most delicate test of individual or genotypic identity, for the technique is quantitative and the nicest differences may be measured.

(a) *Germination of Spores.*—The effect of the character of the nutrient medium upon the germination of fungus spores is often very marked, and not infrequently this relation may be used to differentiate two or more genotypes otherwise difficult to distinguish. The percentage germination within certain periods of time when the spores were immersed in distilled water, 15 per cent. gelatine in water, and COONS' solution (21) were compared in the two experimental forms and in two strains used as a control. The spores were taken from three-day cultures on potato-agar incubated at 22° C., and the hanging drops in which the germinations were tested were maintained in the cool incubator at 18° C. The results are shown below, the numbers representing percentage germinations.

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Medium.	Parental Strain.			Colourless Strain.			Control Strain A.			Control Strain B.		
	6 hours.	12 hours.	24 hours.	6 hours.	12 hours.	24 hours.	6 hours.	12 hours.	24 hours.	6 hours.	12 hours.	24 hours.
Distilled water	10	27	55	12	25	53	33	51	68	25	55	93
15 per cent. gelatine	43	67	93	41	66	90	47	78	90	47	72	90
Coons' solution	63	83	97	61	80	96	63	90	98	61	72	98

The lethal temperature of the spores was tested by making spore suspensions in 15 per cent. gelatine in distilled water and heating corresponding tubes of parental, derived, and control strains together in a water bath for ten minutes, the temperature rising by increments of five degrees. The experimental strains both gave growth in tubes heated to 45° C., but not in those heated to 50° C., whilst in the control tubes growth was inhibited by temperatures of 45° C. and 55° C. respectively.

(b) *Growth on Nutrient Media.*—One of the most critical and easily determined measures of the identity of two fungi is their growth and behaviour upon various standardised nutrient media under controlled conditions. The general characters of the colony, the colour reactions of hyphæ and medium, and the many other detailed phenomena which may be observed are valuable diagnostic characters, and sharply reflect the physiological differences separating two genotypes. At the end of his paper on "Cultural Studies of Species of *Penicillium*," THOM (77) has drawn up a scheme of comparative cultural data, which, in the present absence of any more detailed formulation, could with valuable results be adopted by mycologists as a basis of systematic diagnosis. In the comparative examination of the experimental strains this technique was followed, and the results compared with those of two control strains. No essential difference could be detected between the former, but these stood in marked contrast with the latter. For laboratory purposes other than this particular investigation, the parental strain and its colourless derivative were largely used in experiment, so that the cultural comparison of the two forms was amplified in very many ways. In all cases where they were grown under parallel conditions no essential difference could be detected. The comparative development of the two strains and control cultures was tested further at various temperatures, and with different intensities of light and conditions of aeration, but the results obtained only confirmed those referred to above.

A few qualitative experiments were carried out to test the comparative enzyme production of the fungi, the methods used being adapted from those described by CRABILL and REED (22) in 1915. On starch-agar the experimental strain and one control showed approximately equal amylolytic action under the colonies, while the second control showed a distinct halo indicating the presence of a diffusible extra-

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cellular amylase. These differences were thrown up more clearly when the plates were flooded with iodine solution. On litmus-cream-agar growth of the experimental strains was profuse, and there was strong acid production, the red colour diffusing through the medium. In both control strains the lipolytic action was equally marked but the redness much more sharply defined. All the four strains liquefy gelatine and slowly dissolve fibrin in fibrin-agar. In both experimental strains the lipase production is feeble and only slight browning of the fibrin occurred, this being markedly different from the results in the controls. On casein-agar moderate growth occurred, and ereptic action was prominent, extending in a distinct band around the colonies. The production of erepsin was distinctly greater in both controls than in the experimental strains. Sparse development occurred on asparagin-rosolic-acid-agar with slight amidase production. The red colour in the experimental strains was less diffuse than in the controls.

Cytase formation was tested by growing the organisms in Dox's solution (23) the carbon source being filter paper. Moderate growth occurred, but after a fortnight there was only a barely perceptible reduction with Fehling's solution in the experimental strains and one control strain, whilst the second control showed more distinct cytolytic action. Calcium-carbonate-agar prepared with Dox's solution (23) plus one per cent. of lactose showed a feeble though distinct production of lactic acid, this being more marked in the experimental than in the control strains. In all the experiments no essential differences in the enzymic activities of the normal strain and its aberrant form could be detected.

(c) *Pathogenicity.*—Marked and constant differences in virulence for different hosts are shown by the various strains of *Botrytis cinerea.* In testing the comparative pathogenicity of the experimental strains, the spores, together with a fragment of potato-agar, were planted on corresponding surfaces such as the opposite sides of a fruit, or opposite halves of a leaf. On banana, apple, and tomato fruits, lily and potato leaves, tulip bulbs, and twigs of horse-chestnut infection readily occurred, and vigorous development ensued. On onion-bulb scales, potato tubers, crab-apple, cucumber, and fruits of *Pyrus japonica*, leaves of *Primula sinensis* and wall-flower, infection occurred with very considerable difficulty and only when the tissues were badly bruised, and the growth tended to die out rapidly. Bulbs of snowdrop and leaves of Portuguese laurel and rhododendron could not be infected. In no case was any essential difference in pathogenicity noted between the parental and colourless strains. Markedly different results were obtained with the two control strains, of which one was originally derived from onion bulbs and the other from fruits of *Pyrus japonica*.

The cultural examination of the parental strain and its colourless derivative, although put separately above, was only part of a much larger series of experiments which were being carried out in the laboratory to ascertain the physiological differences of closely similar morphological strains, and the identity of result obtained

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in this comparison very largely takes its critical value from its relation to this wider issue. Briefly, it may be stated that so far as the physiological constitutions of the parental strain and the colourless strain may be judged from their behaviour, the two strains are identical.

B. *Morphological Characters.*

Phenotypic plasticity is shown by most fungi, and is not only evident in those general characters which together constitute the morphological facies of the organism, but in those critical structures, the reproductive bodies, whose constancy has so often been assumed.

(d) *Dimensions of Spores.*—During recent years it has been shown in a general way by many students of the fungi, notably perhaps by STEVENS and HALL (76), and following their investigations by ELLIOTT (26), MOREAU (53), MUTTO and POLLACCI (57), GÄUMANN (30, 31), and others, that the size of fungus spores is a function of the particular organism and the environmental conditions. With regard to the present fungus, *Botrytis cinerea*, under certain standardised conditions, the spore dimensions are a function of the particular pure line and the quality of the nutrient substratum ; and for any particular pure line, different standardised food-media give different but mathematically constant quantities for the modal values of the variation curves of the spores. On a series of standardised media under constant conditions, therefore, the modal values of the spores of any pure line of the fungus may be represented by a curve which is constant and characteristic for that pure line. The critical quantity is not the extremes of variation, but the curve of modal values ; and, other factors not varying, a difference of genotype is immediately reflected in this curve of modal values.

The modal values of the spores of the parental strain and its colourless derivative were compared in this way, the number of measurements for each particular test being 500. The results of three such comparisons are shown on p. 93.

The conidiophores of the fungus are comparatively large and complexly branched structures, and the mechanical difficulties in the way of their minute comparison are such as to render it difficult to eliminate errors of selection. In so far, however, as these structures could be compared with regard to the length, diameter, manner of branching and abstriction of spores no differences could be distinguished between the normal strain and its aberrant form ; and this holds true also for the colour of the spore mass.

(e) *Haptera.*—One of the most interesting structural features of *Botrytis cinerea* are the organs of attachment, which were described by DE BARY (5, 6) under the name "Haft-organen," and are variously termed "haptera" or "appressoria."

Their presence or absence in a culture is determined by environmental conditions, and the controlling factors are different for the several strains of the fungus. The experimental strains behaved similarly in this respect, forming few haptera when

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Medium.	Spore.	—	Parent.	Derivative.
Potato-agar	Length	Minimum	6·0 u	6·0 u
		Mode	9·5 u	9·5 u
		Maximum	12·0 u	12·0 u
	Breadth	Minimum	4·5 u	5·0 u
		Mode	7·5 u	7·5 u
		Maximum	10·0 u	10·0 u
Steamed potato	Length	Minimum	6·5 u	6·5 u
		Mode	10·5 u	10·5 u
		Maximum	16·0 u	16·5 u
	Breadth	Minimum	5·5 u	5·5 u
		Mode	9·0 u	9·0 u
		Maximum	13·5 u	14·5 u
Czapek's medium	Length	Minimum	6·0 u	6·0 u
		Mode	11·5 u	11·5 u
		Maximum	17·0 u	17·0 u
	Breadth	Minimum	5·5 u	5·0 u
		Mode	9·5 u	9·5 u
		Maximum	14·0 u	14·5 u

incubated at 22° C. on CZAPEK's medium lacking carbon, producing them in abundance when M/10 glucose is added to this medium, and suppressing their formation when M/1 glucose is added. A similar course of behaviour is evident when the substratum remains constant and the temperature varies. The particular relations between hapteral formation and controllable external factors are very individual for the several strains of *Botrytis cinerea*, and constitute a diagnostic feature. The identity of behaviour between the normal strain and the colourless strain is therefore noteworthy.

(f) *Sclerotia.*—The type of correlation between hapteral formation and environment which has been indicated above holds true in the formation of sclerotia, and the presence or absence of these in any particular strain may be determined at will. It may briefly be stated that under all circumstances the modes of behaviour of the two strains with respect to this structure were alike.

Owing to the widely held belief regarding the constitutional weakness of albinos, as compared with the normal type, it was considered not improbable that the colourless sclerotia might show a lack of vitality and power to withstand adverse conditions. No indication of such weakness had appeared in the general cultural comparison of the two strains, but it seemed desirable to test the sclerotia themselves, for it is a common assumption that dark coloured or "carbonised" hyphæ are more resistant than hyaline hyphæ. The comparison was made by testing the relative germinative capacity of selected black and colourless sclerotia after subjection to various treatments. Parallel series exposed to extremes of temperature and desiccation for various periods exhibited no differential resistance, nor was this found on subjecting

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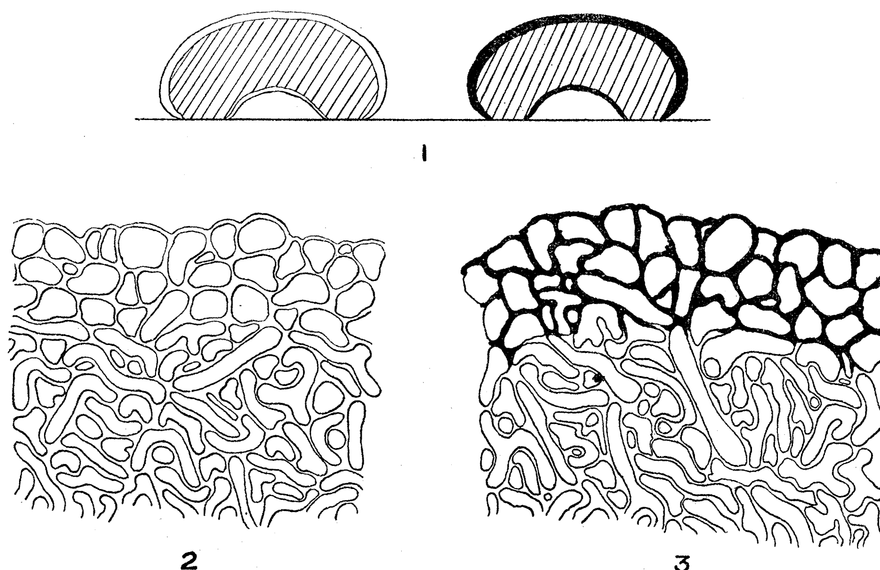
the sclerotia to saturated atmospheres of chloroform and ether, or various dilutions of toxic substances such as mercury bichloride, copper sulphate, carbolic acid, and picric acid. Other strains used as controls gave markedly different results.

In structure the sclerotia vary according to the conditions under which they are formed. In contact with a hard surface they show an outer compact zone two or three cells in thickness which merges into an inner zone consisting of a firm colourless tissue-mass formed by closely interweaving hyphæ (text-fig. 1). On a soft matrix a third central zone of loose spongy hyphæ is usually present. The latter type of sclerotia are more or less spherical in shape whilst the former often fuse



TEXT-FIG. 1.

1. Vertical sections of colourless and normal sclerotia. Diagrammatic.
2. Vertical section of colourless sclerotium. } Swift $\frac{1}{8}$ obj. No. 4 eyepiece.
3. Vertical section of normal sclerotium. }

together into a thin concave crust. The contact surfaces of both forms are colourless, but this is merely due to lack of aeration, for if the sclerotia be removed so that all surfaces have access to air, the previously colourless contact surfaces develop the tissue containing the characteristic pigment. If, however, the sclerotia be cut across so that the inner tissue zones are exposed, these do not develop pigment. The power of colour formation is therefore confined to the peripheral cells. Save for the absence of pigment in these cells, the most minute examination of the structure and form of the sclerotia of the parental strain and its aberrant form failed to reveal any difference.

Like the physiological examination the comparison of the morphological characters of the two forms under discussion has greatly increased value when it is realised that this work was only part of a wider series of experimental researches, which functioned as controls, and the results of which were invaluable in aiding in the

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interpretation of doubtful phenomena. The conclusion drawn from the entire study was that the parental strain and the aberrant derivative differed in respect only to the one character of sclerotial pigmentation. The phenomenon would thus appear to form an exact parallel with mutations by loss of single genes in the higher organisms, and it would stand in contrast with the fungal mutations described by ARCICHOVSKIJ (2), SCHIEMANN (69), and WATERMAN (83), in which there was simultaneous variation in several independent respects.

The explanation of an apparent genotypic alteration on the basis of such a profound change as the sheer "dropping out" of an element from a most delicately balanced reaction-system would appear out of the question. I can only visualise the organism as a unity in itself, in the sense that it consists of a very great number of elements, each an elaborate reaction-system, which bear a specific and causal relationship to one another. The continued harmonious functioning of the total system minus one of its parts is to me unthinkable.

Pure lines of organisms are often separated by characters which are extremely minute and difficult to detect, as for example the obscure serological reactions which differentiate certain races of bacteria, and it would appear probable that were the analysis in the present case carried to a further degree of refinement, many deeply underlying and elusive physiological differences between the two strains would be detected. Such work, however, would constitute a most laborious and intensive study quite beyond the scope of the present paper.

5. Origin of Colourless Form. Special.

A question of some considerable importance is the exact point of origin of the colourless strain. As described it apparently arose in the single colourless sclerotium found in tube (E. 6), and for long no doubt was entertained that this was its actual point of inception. In the absence of further evidence, however, there was no certainty of this, for the colourless strain would only be observed on the production by it of sclerotia, and if sclerotia were not formed the presence of this strain could not even be suspected.

Cultures (D. 1–12) had been made from the pedigree stock (C. 1) in the usual way, by transference of spores on a platinum wire, and a large and indefinite number of these reproductive bodies would thus be implanted in each tube.

Approximately 100 per cent. of these spores would germinate, and the resulting growth would therefore comprise very many intermingled mycelia, some or all of which would produce conidiophores and spores. Similarly, the six tubes (E. 1–6) of August 13, prepared in the same way from one (D. 6) of the previous twelve, would each contain an indefinite number of mycelia, each mycelium corresponding to a single individual. Each tube might, therefore, contain one hundred or more individuals of identical genotypic constitution, growing inextricably mixed together and all or few producing spores and later sclerotia.

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Furthermore, the first crop of spores produced from the mycelia in the tube may fall to the surface of the medium, immediately germinate, and two or three days later again reproduce, when the same process may be repeated. Thus in each *test-tube* generation there may be two or more *lineal* generations, depending partly upon the conditions to which the growth is subjected, and partly upon the available germinating surface of the medium.

From the pedigree stock (C. 1) to the culture of August 13 (E. 6) containing the first colourless sclerotium there were present three direct test-tube generations, which may actually consist of from about six to twenty lineal generations, and at some one point in this series of life cycles the aberrant strain may have arisen. In the series of cultures under consideration there was no evidence that any accessory spore form was produced, nor were the conditions such as would be conducive to their formation. A brief discussion of the two possible points of origin, the spore and the sclerotium, is perhaps desirable.

C. *Spore Formation.*

It will be clear that the colourless strain may have arisen as a variation in a single spore at any point between the second lineal generation in tube (C. 1) and the last lineal generation in tube (E. 6). If, for example, in the former, none of the offspring of this spore, although present in the later cultures, produced sclerotia until a favourable opportunity occurred in tube (E. 6). As, for essential purposes, the colourless form differs from the parental form only in the absence of sclerotial pigmentation, the presence of the former could not possibly be ascertained in a culture of the latter in the absence of the formation of its sclerotia. If, therefore, the aberrant form arose as a single spore variation, it is impossible to locate exactly its point of origin.

Certain facts relevant to the foregoing must, however, be noted. It has been an invariable technique throughout the whole of the research, when transferring inoculum from one tube to another, to take this either from a single conidiophore or, preferably, from a single germinated sclerotium. Now, every conidiophore and each sclerotium arises not only from a single original spore, but actually takes origin in a single mycelial cell. It will be obvious that this very considerably narrows the range wherein the aberrant form may have arisen as a spore saltation.

Furthermore, from its inception the colourless strain has been constantly reproduced by means of spores, and if it arose as a spore variation, the very existence of the new form implies that this particular spore germinated and produced a mycelium giving rise to conidiophores and spores, the latter possessing the new potentiality. But a single mycelium gives rise to an immense number of conidiophores, and the number of spores produced on even a single conidiophore is very great. It is, therefore, almost unthinkable that the new character should not again have appeared in any of the direct and lateral cultures made from tube (E. 6) and from the previous generations.

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For purposes other than this particular piece of investigation it was decided to test the probability of the "throwing up" of the colourless strain in a parental culture, when only a single spore of the former is present. Accordingly, inoculum from (E. 4) was transferred to a clean tube-slant and streaked over the surface, after which there was inserted a single spore of the colourless strain. The circumstances would thus correspond in all essentials to those in tube (E. 6) if the aberrant form had arisen as a variation in a single spore. The results arranged diagrammatically are shown in Table II.

Tube (I) was prepared on March 2 and ten days later, when the sclerotia were mature, it was found that of the 23 sclerotia in the culture 2 were colourless, the aberrant strain thus "throwing up" in the first tube generation. On March 9 cultures (II. 1–6) were taken from (I) and ten days later it was found that 2 of these contained sclerotia of both strains, the remaining 4 containing only black sclerotia. In (II. 1) there were 15 black and 1 colourless sclerotium, and in (II. 2) 27 black and 1 colourless sclerotium. From each of the tubes containing only black sclerotia (II. 3–6) six cultures were made (III. 1–24). Of these 24 cultures 9 contained both parent and colourless form and the remainder black sclerotia only. In the former there were 162 black and 17 colourless sclerotia. From each of the 15 tubes containing only black sclerotia 6 cultures (IV. 1–90) were prepared. Of these, 24 tubes contained both black and colourless sclerotia in the proportion of 528 of the former to 39 of the latter, and the remaining 66 tubes contained only black sclerotia. In the 6 tubes (IV. 79–84), derived from (III. 17), only black sclerotia were present, and it appeared that in this line the colourless strain had "run out." To test if this were so, 6 cultures (V. 1–36) were made from each of these tubes. In at least 1 tube in each set of 6 the colourless strain was again thrown up, there being in all 10 tubes containing both kinds of sclerotia and 26 tubes containing only the black form. The work was not carried further than this; but in the total experiment, which represented 5 tube generations and 157 cultures, the aberrant form appeared in 46 tubes. A parallel control series prepared with similar inoculum from (E. 4) was synchronised with the foregoing, similar in all respects save that the single spore of the colourless strain was omitted. All the sclerotia were black.

It will be clear from this experiment that the presence of a single spore of the aberrant strain in a parental tube may cause the new character to appear in the first generation, and in a large proportion of cultures of succeeding generations. As already stated, the generations (C. 1), (D. 1–12), and (E. 1–6) were all subcultured freely, but the aberrant strain was absent from all descendants. In nearly 200 direct tube generations of the parent strain (F. 1a) the aberrant form has not appeared. This can only be explained by the hypothesis that the colourless strain did not arise as a spore variation, but arose in and was confined to the initial colourless sclerotium, and that the removal of this eliminated the aberrant strain from the parental culture (E. 6). The new strain was thus seen immediately on its inception and isolated.

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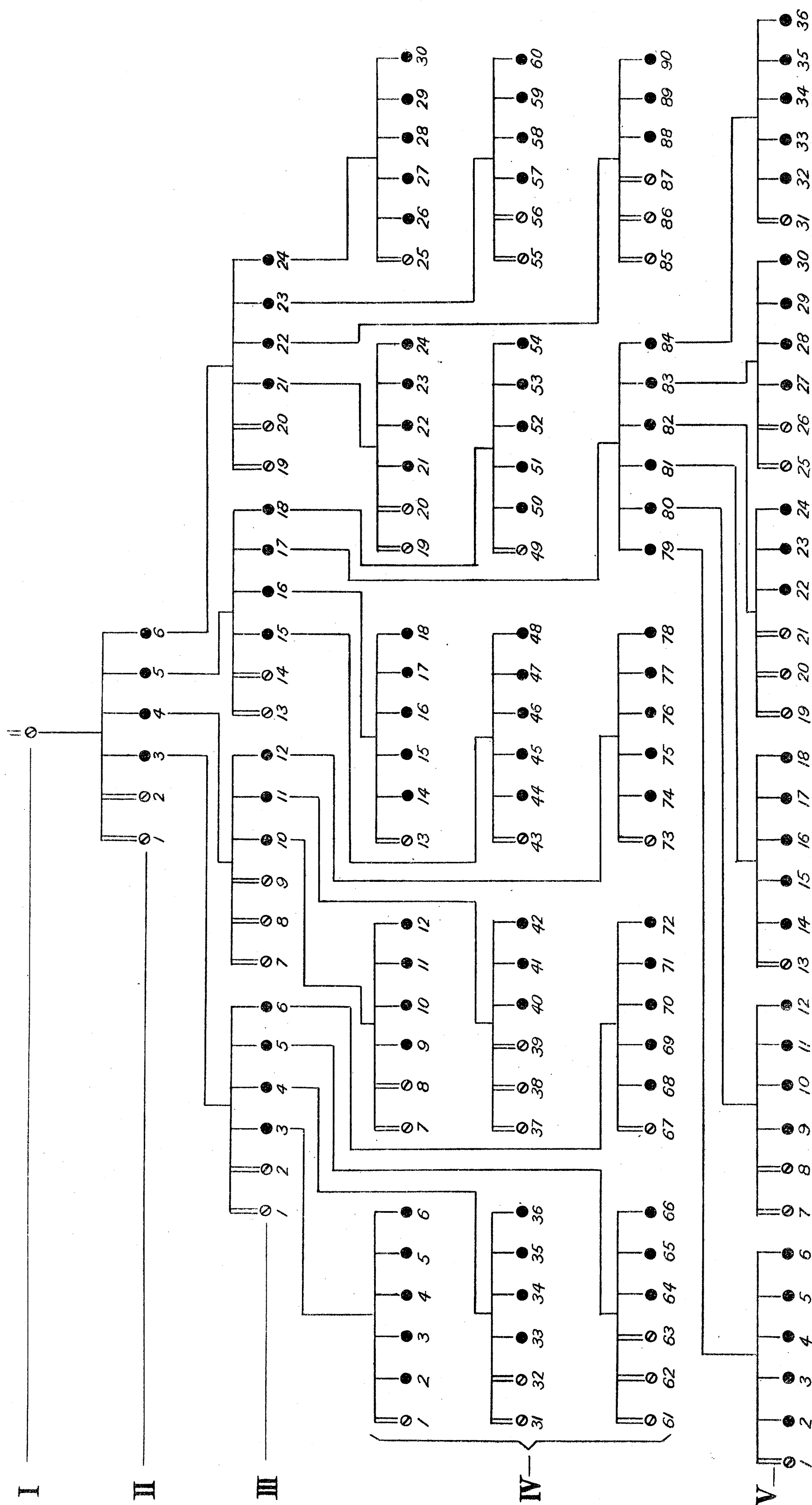
TABLE II.

Inoculum from (E. 4) + one Spore from Colourless Strain.



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D. *Sclerotial Formation.*

It will be evident from text-fig. 1 that the sclerotium consists of a mass of more or less firm tissue, enclosed in a thin layer of cells, in the walls of which the black pigment is located. Now, unless the sclerotium arises in a single hyphal cell, in which the variation may have occurred, the physiological change resulting in loss of colour must have taken place simultaneously in all those peripheral cells which normally would form pigment. In view of the fact that the new character is strictly hereditary, and obtains by the growth of any portion of a single individual, and that the initial colourless sclerotium arose in a tube culture containing a perfectly normal growth, such a deep-seated physiological change occurring simultaneously in a very great number of cells, and to an identical degree in all, is a process extremely difficult to conceive.

Very little is known of the initial developmental stages in the formation of sclerotia, but the latter are usually considered to be a further growth or modification of haptera, structures which are very accurately delineated in the works of MARSHALL WARD (80) and ISTVANFFI (33). In their formation one, or more usually several related hyphæ form short branches on which arise tufts of secondary branches. These are directed vertically to the contact surface, and the clustered hyphæ mat together laterally forming a solid body of a tassel-like shape and structure.

It is of interest to note that DE BARY (5, 6) regarded sclerotia and haptera as discrete bodies, stating that although clusters of haptera "have been mistaken for sclerotia . . . they have no connection." MUNN (56), who has most recently investigated this fungus, writes: "It was observed that in many cases the appressoria are incipient stages of the sclerotia (Plate 3, figs. 23, 24)." Now if MUNN's figs. 23, 24, and 13, which represent stages in the development of sclerotia, be compared with his figs. 25 and 27, which show the development of haptera, the essential difference is plain; and this distinction is obvious in the many delineations of these structures by previous investigators. There are no transition stages. The general laboratory study of the fungus and the relation of hapteral and sclerotial formation to various media and known conditions show that the structures are organs *sui generis*. This was confirmed in a very striking way by the development of these bodies in the aberrant strain. The haptera of the normal strain very rapidly become dark coloured and finally almost black, and this similarity of colouring with the sclerotia is in large part responsible for the confusion. In the case of the colourless strain, however, it is most helpful, for whilst the sclerotia have completely lost their pigment, the haptera have retained it, and thus in the one culture there co-exist the albinistic sclerotia and brown or olive-black haptera. This colour difference is distinctive from the inception of the two structures.

If therefore the sclerotia and haptera are discrete entities, the earliest stages that we know in the development of the former are the clusters of interweaving hyphæ

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depicted by ISTVANFFI, MUNN, and others, and there is no reason why these should not have arisen in the branching of a single mother-cell. On searching selected cultures both by sectioning, and more successfully by mechanical teasing and *intra vitam* staining with neutral-red and methylene-blue, the initial stages were soon found. For the present issue, the main point of interest is that each sclerotium arises symphogenetically from an individual cell of the mycelium.*

On the open surface of a soft matrix the sclerotia usually remain separate, but if developed in a fissure or along a line of contact they often fuse to form a continuous crust. Figs. 50 and 51 on Plate 15 of ISTVANFFI's memoir (33) are to be interpreted in this latter way. If mixed inocula of parental and colourless strains be grown, interesting composite sclerotial crusts may very rarely be obtained. In these there is no intimate mingling of hyphæ, but merely a lateral "grafting," and if such a composite sclerotium be germinated, the conidia and conidiophores, although indistinguishable, each reproduce purely their original parental type.

From the foregoing discussion it will be clear that the physiological change which resulted in the production of the aberrant form must have occurred in the mother-cell which gave rise to the initial colourless sclerotium in tube (E. 6).

6. NATURE OF LOSS OF COLOUR.

A detailed biochemical study of sclerotial pigmentation is beyond the scope of the present paper, but one may perhaps refer to certain simple experiments which throw some light upon the nature of the physiological change which has occasioned the production of the colourless form.

During the last two decades the pigmentation processes in animals and plants have been the subject of much investigation, and although this has followed the two distinct lines of biochemistry and genetics, the results have complemented each other in a quite remarkable way. The general trend of evidence would appear to lead to the view that the pigmentation processes throughout are essentially of an oxidatory nature with associated subsidiary or perhaps preliminary reduction processes.

Albinos have been shown to be divisible into two categories termed "dominant whites" and "recessive whites" respectively. In the latter, the absence of pigment is due to the lack of either the oxidase factor, or the chromogen factor, or of both; whilst in the former, both oxidase and chromogen are present, but their interaction is prevented by a third inhibiting factor. The oxidatory nature of the pigmentation processes is common to so many widely diverse organisms, that one might with some justification expect to find it even in the fungi, and consequently to find also that albinism in such an organism as *Botrytis cinerea* showed common features with the same phenomenon in rabbits or primulas. This fungus, however, is asexual, and the terms "dominant" and "recessive" are inapplicable; but if the pigmentation

* In certain strains of *Botrytis cinerea* the sclerotia originate meristogenetically.

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processes are parallel to those of other organisms, the physiological changes which have resulted in the production of the colourless form may involve either the presence of a new inhibiting factor or the loss of either oxidase or chromogen or both.

The simplest and most direct method of examining the condition of the aberrant form is to test the colourless sclerotia for the presence of oxidase. If this be present and active it follows that the chromogen factor must be absent for otherwise pigmentation would have ensued. If there is no oxidase reaction, then either this factor is lacking or its activity inhibited by a third factor. The technique was based on that devised by KEEBLE and ARMSTRONG (41, 42, 43) in their investigations of flower pigments. Solutions of benzidine, α-naphthol, and p-phenylenediamine gave a marked reaction, whilst with a solution of guaiacum-resin a barely perceptible lavender-blue colour was obtained.

These reactions were unaltered by preliminary treatment of the sclerotia with absolute alcohol or hydrocyanic acid, or by the subsequent addition of hydrogen-peroxide, and in all cases they were produced in a few minutes irrespective of temperature differences ranging from that of the laboratory to incubation at 37° C.

The distribution of the oxidase in the colourless sclerotia was identical in every case, and was noteworthy, being confined to the walls of the peripheral cells which in the parental form contain the normal black pigment. So exact was this agreement that some of the sections of colourless sclerotia treated with benzidine were barely distinguishable from sections of normal sclerotia.

Whether the colourless sclerotia have been formed in light or darkness makes no difference to the intensity of the oxidase reaction, nor is this changed by growth of the fungus at various temperatures or upon nutrient substrata presenting very different physiological conditions.

On WHELDALE's view (85) the chromogens are flavones or flavonal glucosides. The presence of these bodies in tissues may readily be detected by the bright colour given on exposure to ammonia vapour, and the dull-green (or sometimes red-brown) colour with ferric-chloride. When these tests were applied to sections and pulps of the colourless sclerotia a totally negative result was obtained.

NEILSON JONES (39) has shown that even in certain recessive whites both chromogen and oxidase appear to be present, but that owing perhaps to their discrete distribution interaction is prevented. To test the possibility of this explanation of the albinism of the aberrant strain a number of sclerotia were boiled in 5 c.c. of 50 per cent. alcohol to destroy the oxidase, pulped, filtered and the filtrate evaporated to a few drops. Sections of the colourless sclerotia were then immersed in this and incubated at 27° C. No trace of oxidase reaction was observed, confirming the absence of chromogen in the sclerotia.

True brown and black pigments are of the very greatest rarity in the higher plants. MÖBIUS (52) however has shown that they do exist as in the spots on the alæ of Vicia Faba, but even here the colouring matter is dissolved in the cell-sap.

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In thin sections of the normal sclerotia of *Botrytis cinerea* the pigment appears as a true brown-black. Furthermore, as shown in text-fig. 1, it is contained in the substance of the cell-walls and not in the cell-content which is perfectly hyaline. It thus differs very markedly from the usual plant pigments, and would appear to be more closely related to the melanotic pigments, certain of the melano-proteins existing dissolved in the keratin structures.

The mother substance of melanin is generally supposed to be tyrosin, this colourless chromogen being converted into a black pigment by the oxidising enzyme tyrosinase. If, therefore, the black pigment in the normal sclerotia be melanin, the aberrant sclerotia which presumably lack the factor for tyrosin should exhibit a tyrosinase reaction. The tests for oxidase already referred to are only for the detection of phenolase or laccase which BOURQUELOT and BERTRAND (14), ZELLNER (88), PRINGSHEIM (63), KASTLE (40) and others have shown to be almost universally present in fungi.

Tyrosinase is comparatively rare in its distribution in fungi. BERTRAND (11), however, has shown its presence in species of *Russula*, especially *Russula nigricans*, and LUTZ (49) in the stipes and pilei of *Gyromitra gigas* and *Disciotis parlata*; while more recently it has been demonstrated in *Lenzites sæpiaria* by ZELLER (87) and in various species of *Actinomyces* by DRECHSLER (24).

According to BOURQUELOT and BERTRAND (14) the best substrate for tyrosinase is tyrosin. Colourless sclerotia incubated at 18° C., 25° C., and 37° C. for 14 days in a saturated aqueous solution of silk-tyrosin gave a totally negative reaction, nor could this be changed by preliminary treatment with alcohol, ferrous sulphate or hydrocyanic acid or by subsequent treatment with hydrogen peroxide. A pulp of sclerotia, or sections, gave a similar result with this treatment.

CHODAT and STAUB (20) have advocated the use of *p*-cresol with the addition of glycin for the detection of the enzyme tyrosinase. Sclerotia immersed in this and incubated at 37° C. showed after many days a diffuse pale-yellow tint instead of the rich violet-blue reaction characteristic of tyrosinase.

In 1907 ABDERHALDEN and GUGGENHEIM (1) showed that N/100 hydrochloric acid permanently destroyed the activity of tyrosinase, but in the present case an immediate and direct oxidase reaction with benzidine was obtained after preliminary treatment of the colourless sclerotium with N/40 hydrochloric acid, although a strength of N/30 destroyed its activity.

It was found by BERTRAND (11) that tyrosinase could be differentiated from other oxidising enzymes by its lower lethal temperature, its activity being inhibited at 50° C. The oxidase in the colourless sclerotia is inhibited only by a temperature of from 80° C. to 85° C.

Furthermore BACH (3, 4) has shown that the pigment formed by the action of tyrosinase upon tyrosin may be oxidised further to a colourless compound by a dilute acid solution of potassium permanganate. The normal black sclerotia of *Botrytis*

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cinerea remained unaltered when treated in this way. The black pigment is indeed extraordinarily stable, remaining apparently unchanged after boiling in concentrated acids and alkalies. When treated thus the stuff of the tissues disappears whilst the pigment remains as a most delicate gossamer-like cellular film. This stability immediately distinguishes the sclerotial pigment of *Botrytis* from other black fungal pigments such as the Aspergillin of *Aspergillus niger* which is soluble in hot water, or the colouring matter of *Daldinia*, *Hypoxylon* and various other carbonaceous Ascomycetes, which is soluble in alcohol.

The foregoing simple experiments only show (*a*) that the colourless sclerotia possess a direct oxidase system of the laccase type in a region corresponding exactly with that in which the black pigment is present in the sclerotia of the parental strain; and (*b*) that flavones or chromogens are lacking in the colourless sclerotia, or if present, they are inactivated in some way not understood. The experiments do not prove that the pigment in the sclerotia of the parental strain is due to the oxidation of a chromogen or that the aberrant strain has arisen by the loss or inactivation of a factor for chromogen. When, however, the above results are considered along with those of GORTNER (32), KEEBLE and ARMSTRONG (41, 42, 43) and others, which cumulatively form such positive circumstantial evidence, it is somewhat difficult to resist drawing the deduction that the albino strain of *Botrytis cinerea* has indeed arisen from the black parental form by the loss or modification of a factor for chromogen.

7. SIGNIFICANCE OF COLOURLESS FORM.

A. *Discussion of Recorded Mutations.*

The essential facts in the preceding pages may be summarised as follows: The colourless form arose spontaneously without any evident relation to external conditions or stimuli, in a single sclerotium of a culture which on accepted criteria was a pure line. The new form is apparently differentiated from its parent in respect to a single character only, that of colour, and this albinism would appear to be associated with the absence of a factor for chromogen. The change has occurred once only, and has given rise to a form unknown in nature and perfectly constant under all conditions. It would seem possible to place only one interpretation on these facts, that of mutation, and accordingly when I was privileged to exhibit this form to the Linnean Society of London on April 3, 1919, I described it as "an albino mutant of *Botrytis cinerea*."

Previous records of mutation in the fungi fall into two groups. In the first are those changes in genetic constitution which appear to bear a direct and purposive relation to certain conditions which have operated during the development of the organism or that of the preceding generation. As an illustration may be instanced the permanent acquirement of the power to ferment lactose by an organism previously unable to form lactase, this change being brought about by the growth of

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the organism or its progenitor upon a lactose medium. The second category of mutations includes those cases in which the genetic change is apparently quite fortuitous, and this group is divisible into two sub-groups. In the first of these the change is associated with certain conditions which interfere with the normal metabolic reactions of the organism. For example, by treating the organism with toxic substances, or by subjecting it to extremes of temperature or desiccation, various indeterminate morphological or physiological changes are induced, such as alterations in spore dimensions or coloration, or growth upon standardised media. In the second sub-group the changes occur spontaneously under, so far as may be judged, perfectly normal conditions of development, and this category would include the colourless form of *Botrytis cinerea*.

Of the purposive mutations the classical case perhaps is that described by MASSEE (51), in which *Tricothecium roseum* was changed into a virulent parasite by habituating it to a previously immune host. This research to which credence is still largely given was, even for the year 1905, so inexact in all critical details that it may be now be regarded as of merely historical interest. Results worthy of serious consideration are described in the researches of SALMON (66, 67) on the adaptive parasitism of *Erysiphe Graminis* by means of growth upon bridging hosts, or the parallel results obtained with various rust-fungi by MARSHALL WARD (81, 82), FREEMAN (28, 29), JOHNSON (38), POLE EVANS (62), and others. In none of this work, however, were the experiments carried out with single-spore strains of the fungi, under the rigidly controlled conditions imperative in such work. Nor was it realised until the more recent researches of MAINS (50), BROOKS and COOLEY (15), LAURITZEN (46), and others, how vital are the effects of even minute changes in the light, temperature, age, or humidity relations of the host plants. The findings of KIDD and WEST (44) on the importance of physiological predetermination in the life of the plant may also have a very material bearing on questions of relative susceptibility or immunity of host plants to fungal attack, and indicate factors which will be extremely difficult to control. Apart, however, from such collateral evidence as this, the value of bridging species in changing the physiological characters of the fungus is not supported by the work of BIFFEN (12), and has been directly contravened by the researches of REED (64), and of STAKMAN and his associates (71–74). After much intensive study of this subject, the latter conclude "... the writers have not been able to detect any mutation nor to induce perceptible evolutionary changes experimentally." With rare exceptions such conclusions are supported by the invariable experience of all the critical and accurate workers in more recent experimental mycology, and there would appear to be little doubt that when a pure line organism is examined by a careful investigator under rigidly controlled conditions, heritable alterations bearing a purposive relation to educative treatment are absent.

The recorded instances of non-purposive genetic changes in organisms induced by

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exposure to unusual conditions are very few in number. In 1908 ARCICHOVSKIJ (2) described a form of *Aspergillus niger* with yellow-brown spores which arose in a culture of the black form growing in Raulin's fluid to which 0·0001 per cent. of zinc sulphate had been added. Four years later SCHIEMANN (69) obtained various mutants of *Aspergillus niger* by treatment of cultures with potassium bichromate, exposure to high temperatures, and so forth.

In the same year WATERMAN (83) caused *Aspergillus niger* to mutate by treatment with 2 per cent. galactose, rhamnose, or glucose, 1 per cent. boric acid, *p*-oxybenzoic acid, or dichloracrylic acid. A fungus which the author speaks of as *Penicillium glaucum* was also caused to throw mutants by treatment with any of the following substances:—*p*-oxybenzoic acid, salicylic acid, trichloracrylic acid, tetrachlor-propionamid, pentachlor-propionamid, and pyrocatechetic acid (pyrocatechuic ?).

In all the above cases the new forms described differed from the parental fungi in many morphological and physiological characters, the most obvious being that of the colour of the spores. Furthermore, the production of the mutant forms was not a constant and specific reaction to certain definite chemical stimuli, but was merely a fortuitous circumstance resulting from the generally unfavourable conditions to which the organisms had been subjected. These were of such diverse nature that one might expect that almost any substance deleterious to growth or interfering with the metabolic processes in any way would produce similar results. Also not every culture subjected to these conditions gave uniform results over the entire growth, but only occasional cultures, and in these cultures only sporadic individuals, conidiophores or spores. Moreover, as SCHIEMANN (69) points out, mutations were also produced when the fungi were developing under favourable conditions.

Such results as these would have a somewhat indifferent reception if obtained in a physical or chemical laboratory. Their anomalous nature is stated clearly in SCHIEMANN's paper as follows: "Die konstanten Farbmutationen sind beide auf mit $K_2Cr_2O_7$ versetzten Nährböden aufgetreten. Von einer spezifischen Wirkung des $K_2Cr_2O_7$ zu sprechen, ist trotzdem nicht möglich. Schon der Umstand, dass der Farbumschlag unter über 100 Kulturen mit $K_2Cr_2O_7$ nur zweimal stattfand, hier wiederum nicht die ganze Decke betraf, sondern unter vielen hunderten von Köpfchen nur einzelne, spricht dagegen. Ferner hat die *Proteus*-Mutante dieselbe Richtung eingeschlagen; diese aber ist aus Hitzekulturen, einmal mit, einmal ohne Zusatz von $K_2Cr_2O_7$ hervorgegangen. Endlich sind nach braun abändernde Kulturen des *Aspergillus niger* auch sonst gelegentlich beobachtet worden—wenn auch selten—ohne dass das Chromal zur Verwendung kam."

In a discussion of the causes of genetic variation, BATESON (7) has written: "The state of knowledge of this whole subject is . . . most unsatisfactory, chiefly for the reason that in none of the cases which are alleged to show a positive result have two observers been over the same ground, or as yet confirmed each other . . . I do not know a single case which has been established and confirmed in such a way that we

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could with confidence expect to witness the alleged phenomena if we were to repeat the experiment."

In the work of ARCICHOVSKIJ (2), SCHIEMANN (69), and WATERMAN (83), mutant forms were produced so easily and with such a diversity of stimuli and simplicity of technique, that it seemed probable that a repetition of their experiments might produce the necessary confirmatory evidence. Accordingly single-spore cultures of two strains of *Aspergillus niger* and of two strains of *Penicillium Italicum* were prepared. (WATERMAN used "*Penicillium glaucum*," but as THOM (77) points out, this "name as used at present seems to be applied collectively to the common green forms, which under examination are quickly found to be not one but several species.") With these fungi, the experiments of SCHIEMANN (69) and ARCICHOVSKIJ (2), and certain of those of WATERMAN (83) (galactose, glucose, rhamnose, boric acid, and salicylic acid) were carefully repeated. Although modifications in the colour of the spores, general morphological facies, and physiological reactions were observed, these affected the whole growth operated upon and to an equal degree; but the reproductive bodies of the modified fungi, when returned to the original conditions, gave in every case the original result. These changes were phenotypic and not genotypic, and throughout the whole series of experiments not a single mutation occurred.

More recently, experiments have been carried out with another strain of *Aspergillus niger* isolated from the air, and with a strain of *Penicillium* (*roseum ?*) from the soil, both these having been in culture in the laboratory for several months and repeatedly subcultured, although not derived from single spores. The fungi were subjected to rather gross treatment with various dilutions of toxic substances, such as silver nitrate, copper sulphate, mercury bichloride, lead nitrate, potassium acid phosphate, sodium chloride, and so forth, and then platings were made from these tubes. The resulting growths were perfectly normal.

In all work on the induction of genotypic alterations in micro-organisms, it is extremely difficult, even when the most scrupulous attention is given to every detail, to be quite certain that one has under control each of the almost infinite number of factors which may influence the results. Mycologists are only now beginning to learn how extremely complex and difficult to isolate such factors may be, and the researches of, for example, DUGGAR (25) and his collaborators on the physiology of certain fungi grown in culture media, or those of COONS (21) on the factors involved in the growth and pycnidium production of *Plenodomus fuscomaculans*, indicate the complexities underlying apparently the most simple physiological phenomena. Not the least interesting of the many experimental studies throwing light on the bionomics of *Aspergillus niger* are those of JAVILLIER (34) and of STEINBERG (75), in which it is shown how even such an unsuspected factor as the different quantities of zinc present in the glass of flasks, test-tubes, and other apparatus may vitiate comparative cultural studies of this fungus.

If, in the work of ARCICHOVSKIJ (2), SCHIEMANN (69), and WATERMAN (83),

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accidental contamination did not occur, one is almost forced to conclude that some accessory factor of an unrecognised nature must have been operative in their experiments; a factor absent in my own repetition of their work and absent from the studies of every other mycologist who has carried out critical experimental investigations on these fungi.

Furthermore, as has been pointed out, these investigators emphasise the fact that their mutations are not of the nature of specific reactions to definite chemical or physical stimuli, but only the results of a generalised interference with the normal course of life of the organism. Thus SCHIEMANN (69) says: " . . . so zeigt sich als ein Gemeinsames, dass in allen Fällen, wo derartige Farbänderungen auftraten, Störungen der normalen Lebens-verhältnisse vorlagen. Es ist deshalb der Schluss berechtigt, dass die Mutation in den beobachteten Fällen durch einen starken Reiz ausgelöst wurde." But the experience of other investigators demonstrates the extreme genetic constancy of species of these fungi even when placed for many generations under the most unfavourable conditions. Numerous workers have shown that the morphological facies and physiological activities of fungi may within certain limits be changed at will in the single generation, but in no case, with the above exceptions, have these phenotypic modifications had any effect whatever on the genotypic constitution of the organisms.

B. *Alternative Interpretations.*

If the recorded cases of mutation in the fungi need not be accepted—and for my own part I do not feel compelled to accept them—how then is one to regard the colourless strain of *Botrytis cinerea*, which has been described in the present paper? Its fortuitous origin as a single sclerotium in a perfectly normal single-spore strain, whose development since its commencement is known, has already been emphasised. Under no conditions whatever, so far as is known, may the black sclerotial pigment of the parent strain, or the albinism of its derivative, be modified, and although other characters may show plasticity, no instance of a permanent alteration has been observed. Does the phenotypic constancy of the parental strain under constant conditions necessarily imply genotypic purity?

In the preceding account the parental strain has been spoken of as a "pure line," and on all accepted criteria this description is merited, for not only is it a single spore strain of an asexual organism, but it has been reproduced for most of its generations either by spores from single conidiophores or from single germinated sclerotia, both of which take origin in a single cell. In his work on 'Evolution by means of Hybridisation,' LOTSY (48) rightly insists that: "*Certainty of purity is a* conditio sine qua non *to obtain proof of the existence of mutation in living beings, just as chemical purity is a* conditio sine qua non *to obtain proof of the existence of mutability of the elements.*" Is there certainty of purity in the parental strain of *Botrytis cinerea* which gave rise to the colourless form? Further, is the fulfilment of such a

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criterion possible in the fungi (or bacteria), and particularly in those forms for which mutation is claimed?

The only positive contribution to this subject is the work of BURGEFF (18), published in 1914, in which the results of the crossing of various forms of *Phycomyces nitens* are described. This fungus possesses a cœnocytic mycelium, containing numerous scattered nuclei. In the asexual form of reproduction spores are delimited within sporangial heads into which there have passed an indefinite number of nuclei. The multinucleate spores germinate and reproduce the cœnocytic mycelium. If, therefore, the original hyphæ are genetically impure, this condition will be maintained in all succeeding generations, for the sporangiospores merely reproduce the genetic condition of the hyphæ which give rise to the sporangia. Opportunity for genetic contamination occurs at sexual reproduction, for this process is merely a fusion of two multinucleate gametes to form a multinucleate zygospore, which on germination gives rise to a cœnocytic mycelium containing nuclei of both parental strains. There will be an equal chance for both types of nuclei to pass into the sporangia and be included in the multinucleate spores. A single-spore strain may thus be heterocaryotic. If now this form at sexual maturity fuses with a third form, and so on, the genotype of any particular isolation may be extremely complex. As, moreover, the sporangiospores are delimited and the walls of the zygogametes laid down without any apparent regard either to the condition, the number, or the position of the nuclei they separate, there is no absolute surety that two single-spore strains derived from an original single-spore strain will have the same genotype.

In such *Mucorineæ* it is, therefore, totally impossible to comply with the criterion of specific purity which is imperative in this issue. There is an almost equal lack of certainty of specific purity in the genera *Aspergillus* and *Penicillium*. The mycelium of these fungi is multicellular and each cell contains many nuclei. On asexual reproduction a vegetative cell gives rise to a conidiophore, and from this conidia are abstricted. The hyphal mother cell is multinucleate and these nuclei, by division and wandering, finally pass into the reproductive cells, these, in many species of the fungi, containing an indefinite number of nuclei. In other species the conidia generally contain but a single nucleus, but almost any preparation of such conidia shows multinucleate individuals.

A single-spore strain thus reproduces the genotype of its parent, and if the latter contains nuclei of different constitutions, this heterocaryotic condition will be maintained in the progeny.

Now, in the case of *Phycomyces*, genetic contamination occurs in the sexual process, for many of these forms are allogamous. There are, however, no species of *Aspergillus* or *Penicillium* known in which sexuality is necessarily allogamous, although there is no certainty that this type of reproduction may not occur, at all events, at intervals. We still know too little of what takes place in the sexual processes of either of these genera to make their discussion profitable; but given a

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union of two parental mycelia only once in many thousands of generations, the mycelia of the offspring can afford no certainty of specific purity.

Moreover in any culture of a single-spore strain of either fungus it is not usually difficult to find two hyphæ which have come into contact, and the walls separating them have been auto-digested, giving cytoplasmic continuity. In a mixed growth of a number of strains these somatic fusions are also to be found, and although one is not able to state that fusions occur between hyphal cells of different strains, neither may the rare possibility of this be denied. If now from such a contaminated cell in *Aspergillus* or *Penicillium* a conidiophore were to arise, or either or both the sexual organs, the offspring of the reproductive bodies would be genetically impure, and asexual reproduction would only maintain this heterocaryotic or heterozygotic condition.

Thus even accepting the evidence of ARCICHOVSKIJ (2), SCHIEMANN (69), and WATERMAN (83), it by no means follows that the permanent changes they observed are necessarily mutations, for they may be interpreted in terms of genetic contamination, and in no case may these organisms comply with that criterion of "certainty of specific purity which is a *conditio sine qua non* to obtain proof of the existence of mutation." Although in this account very little reference has been made to bacterial mutation, there can be little doubt that the possibility of the rare occurrence of "somatic" fusions in these organisms places them out of consideration as subject organisms for the experimental induction of mutations.

Thus, according to the principle of parsimony, all so-called mutations in these groups of organisms may perhaps more wisely be interpreted in terms of the splitting of an originally impure genetic constitution or of gametic or somatic segregation from heterozygotes.

These considerations applied to the fungus *Botrytis cinerea* throw an entirely new light upon the value to be attached to the colourless strain. *Botrytis cinerea* possesses a multicellular mycelium, each cell of which contains many nuclei. The conidiophores are multinucleate and a small but indefinite number of nuclei pass into each conidium. Throughout the whole of the vegetative and reproductive mycelium the septa are laid down by a diaphragm-like growth from the hyphal walls, irrespective of the number or condition of the nuclei thus separated. Each conidium, therefore, merely reproduces the genetic constitution of the original cell of the mycelium in which its conidiophore arose. Many thousands of such asexual generations would, therefore, not alter the genetic constitution of the organism, and there is no sexual process. The possibility of genetic contamination is brought about by the occurrence of hyphal anastomoses. In the extremely rare chance of a fertile conidiophore arising from a cell contaminated by the nuclei or cytoplasm of a genotypically different individual lies, I believe, the explanation of the colourless form of *Botrytis* described. While this possibility exists, it is more consonant with the principles of scientific methodology to accept this interpretation than to formulate a mutational hypothesis of the origin of the aberrant strain.

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Before concluding, a few words may perhaps be said concerning the *rôle* of the nucleus in the heredity of multinucleate fungi. The conidia of *Aspergillus*, *Penicillium* or *Botrytis* contain a variable number of nuclei, and yet if conidia are plated out the colonies which develop are essentially similar in physiological reactions and morphological properties for each particular species. It follows, therefore, that the exact number of nuclei which chance to be enclosed in each reproductive body has no bearing on the absence or presence of particular characters in the progeny, but is a purely fortuitous occurrence. What is vital, is, as BURGEFF (18) has shown, not the number of nuclei in each cell but the kind of nuclei present. Although the evidence on this point is meagre there would yet seem no reason to doubt that the structure of the nucleus in fungi is in general fundamentally similar to that in other organisms.

Following MORGAN (55) the chromosomes might thus be visualised as linear series of loci, like threaded beads. At each locus exist specific factors, making up a reaction system the elements of which bear a more or less specific relationship to one another. Thus, eight loci, representing the factor for black sclerotial pigment in the chromosomes of as many identical nuclei, would therefore have neither greater nor less visible expression than one locus in the chromosome of a single nucleus, or than 15 loci in the chromosomes of 15 nuclei in a single reproductive body. Increased number of nuclei does not signify a cumulative value for any particular character. But if owing to somatic fusion a hyphal cell containing eight nuclei with loci for black pigment became possessed of one or more contaminating nuclei from a genotypically different individual, and if this particular cell gave rise to a sclerotium, the characters of this sclerotium would be determined by the reacting systems of loci present in the two kinds of nuclei, the visible characters being the expression of the resultant of their interaction. The contaminating nuclei might find themselves in a cytoplasmic environment so foreign to their normal metabolism as to remain inert, or the locus for colour might be counterbalanced or cancelled by the locus for black in any one of the eight original nuclei, in which case the seven remaining nuclei would reproduce the original colour. In both cases the sclerotial characters would be wholly normal. On the other hand the loci in the invading nuclei might function as lethal factors, such as MORGAN (54) has shown to exist in the case of *Drosophila*, and development might be partially or entirely inhibited.

Again, the factors in the one system might react in many ways with those of the other system changing the physiological and morphological characters of the adult form so that apparently spontaneous variation in several independent directions might result, producing "mutants" such as those described by ARCICHOVSKIJ, SCHIEMANN and WATERMAN. The majority of the factors of the contaminating nuclei might remain inert, but one or more might react with certain factors in the original nuclei resulting in the inhibition of one or more of the latter. If, for example, the factor for chromogen were thus inhibited or destroyed the adult would be an albino similar to the new form of *Botrytis cinerea* described. If there are several factors for

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colour, as would appear probable in the present instance, that associated with sclerotial pigmentation only might be inhibited, leaving unaltered the factors governing the pigmentation of spores or haptera.

On the other hand, it is not improbable that the contamination may be purely cytoplasmic, and the progeny of such a cell will therefore still be homocaryotic. The foreign cytoplasm, however, would create a new environment through which the nuclei would function, and this might so influence the development of the organisms as to alter materially the outward expression of one or more characters in the adult form. As the added cytoplasm would now be an integral portion of the organism, this changed expression would be permanent and heritable. It is thus possible for apparent "mutations" to occur in the fungi without any change in nuclear character. So far as the nucleus is concerned, regarding this structure as an entity distinct from the cytoplasm, the change would merely be phenotypic; but for the complete organism the change would be genotypic. It is perhaps in such purely cytoplasmic contaminations that apparent mutations in those organisms having uninucleate cells may be sought.

In the case under discussion, nuclear and cytoplasmic contamination must have occurred prior to the isolation of the single-spore strain, and yet the colourless strain arose only in the third tube-generation subsequent to this. If, however, one assumes a contaminated original strain, it is not inconceivable that in the fortuitous septation of the hyphæ, certain nuclei and cytoplasmic matter were separated, such that in all things save chromogen formation the resultant resembled the parental strain. The expression of this resultant would be an albino strain such as that described. Such a concomitance of conditions might well appear almost inconceivable, but the appearance of an albinistic strain of the fungus is equally almost inconceivable, for in the history of the genus as we know it it has occurred but once.

It would seem to me probable that the same concept which BATESON (8) and LOTSY (48) have formulated for the higher organisms may hold true for both fungi and bacteria, but here the recombination of genes may not only take place by sexual fusions, but also by direct "contamination" of one species by the nuclei and cytoplasm of another in vegetative anastomoses. New reacting systems of chromosomal loci impinge upon each other, and the resultant is the new form. This at first may be heterocaryotic, but if true sexual processes occur later, the offspring become homocaryotic but heterozygous. Such a hypothesis fits the facts and explains them as I think no other present hypothesis does.

It is not the possibility of mutation in the fungi (and bacteria) that is here denied, but rather the compulsion to accept that interpretation of the evidence as it now stands. As the facts present themselves to me, avenues of interpretation other than that of mutation are still widely open.

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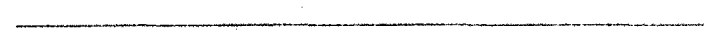
DESCRIPTION OF PLATE.

Fig. 1.—Petri dish culture in which the normal form and its colourless derivative have been grown. Pigmented haptera of the albino strain may be seen at the periphery of the culture in contact with the glass. $\times \frac{2}{3}$.

Fig. 2.—Vertical section of colourless sclerotium. $\times$ 250.

Fig. 3.—Vertical section of parental sclerotium. $\times$ 250.



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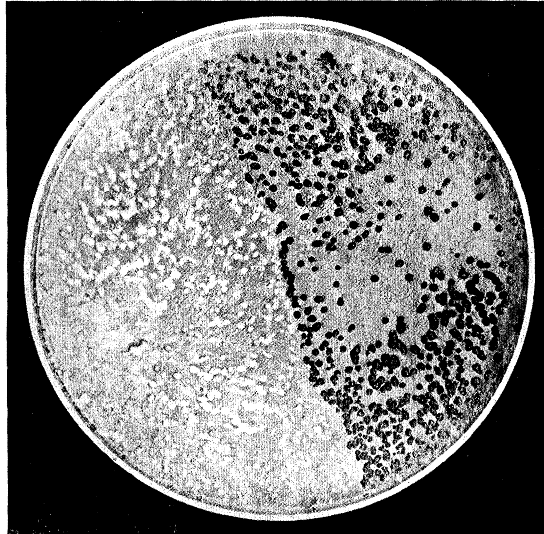

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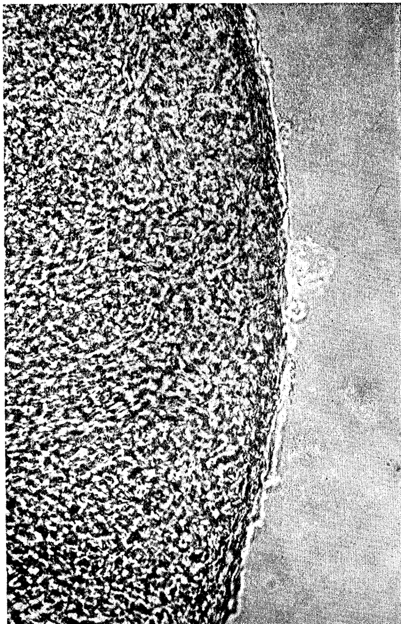

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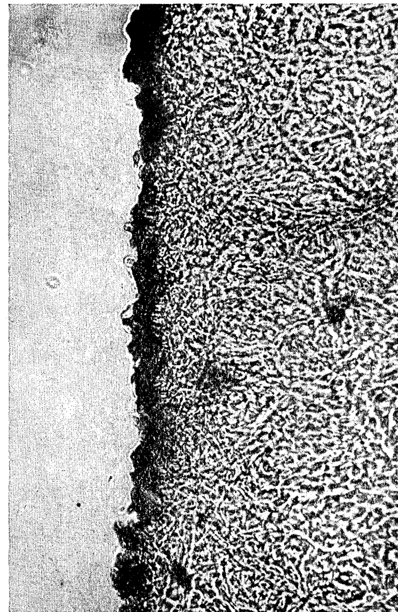

Fig. 3.

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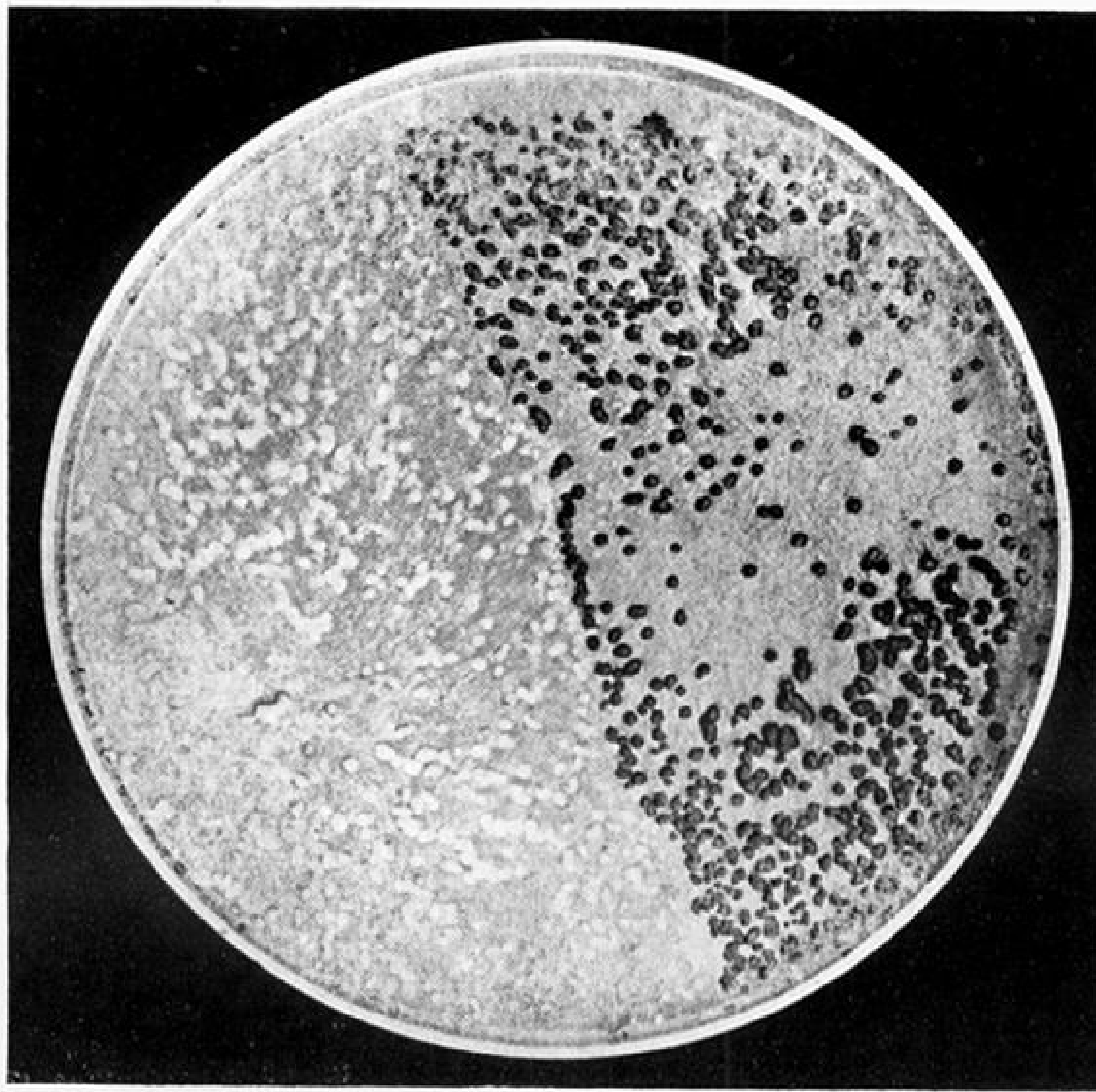

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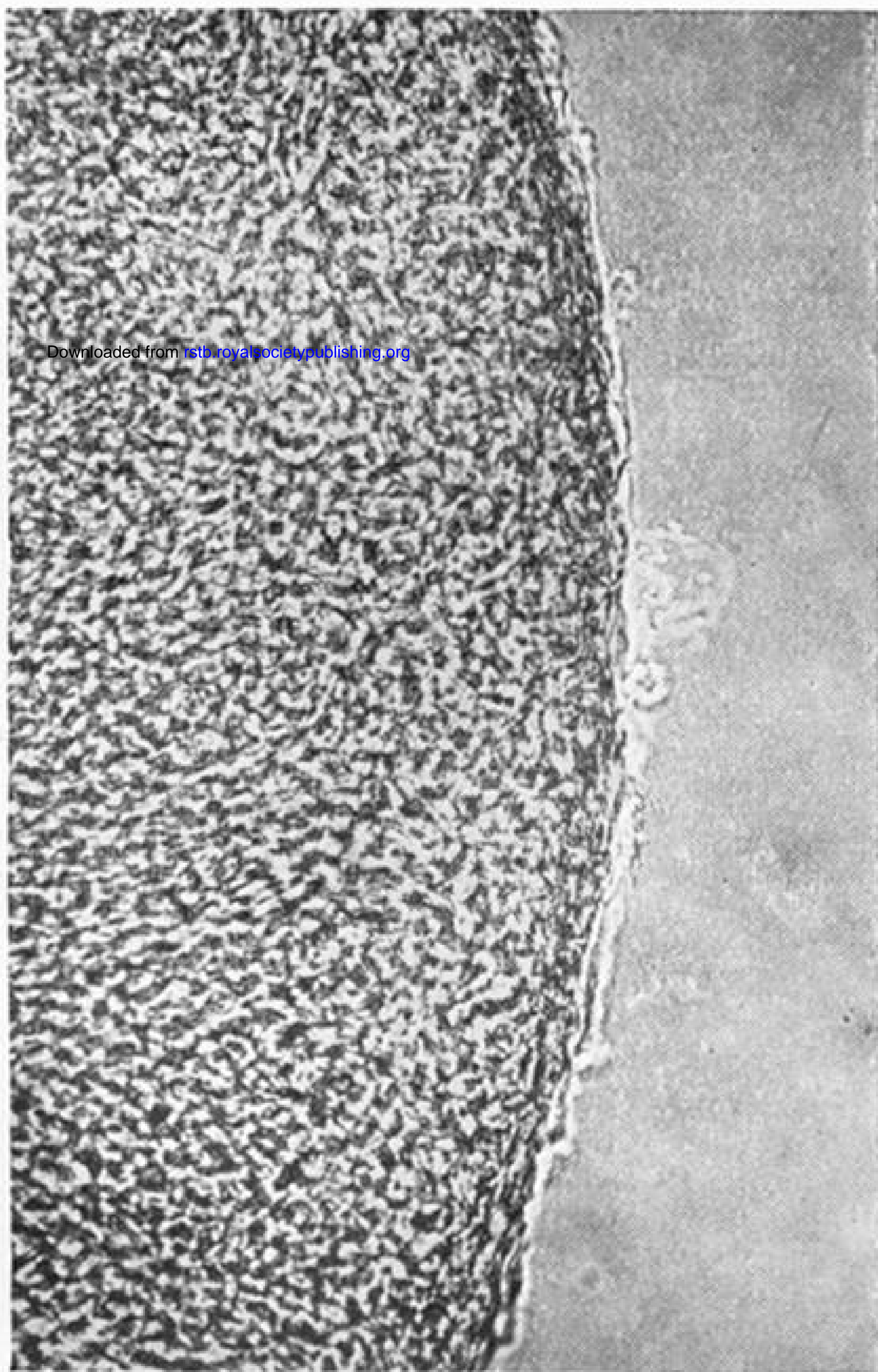

Fig. 2.

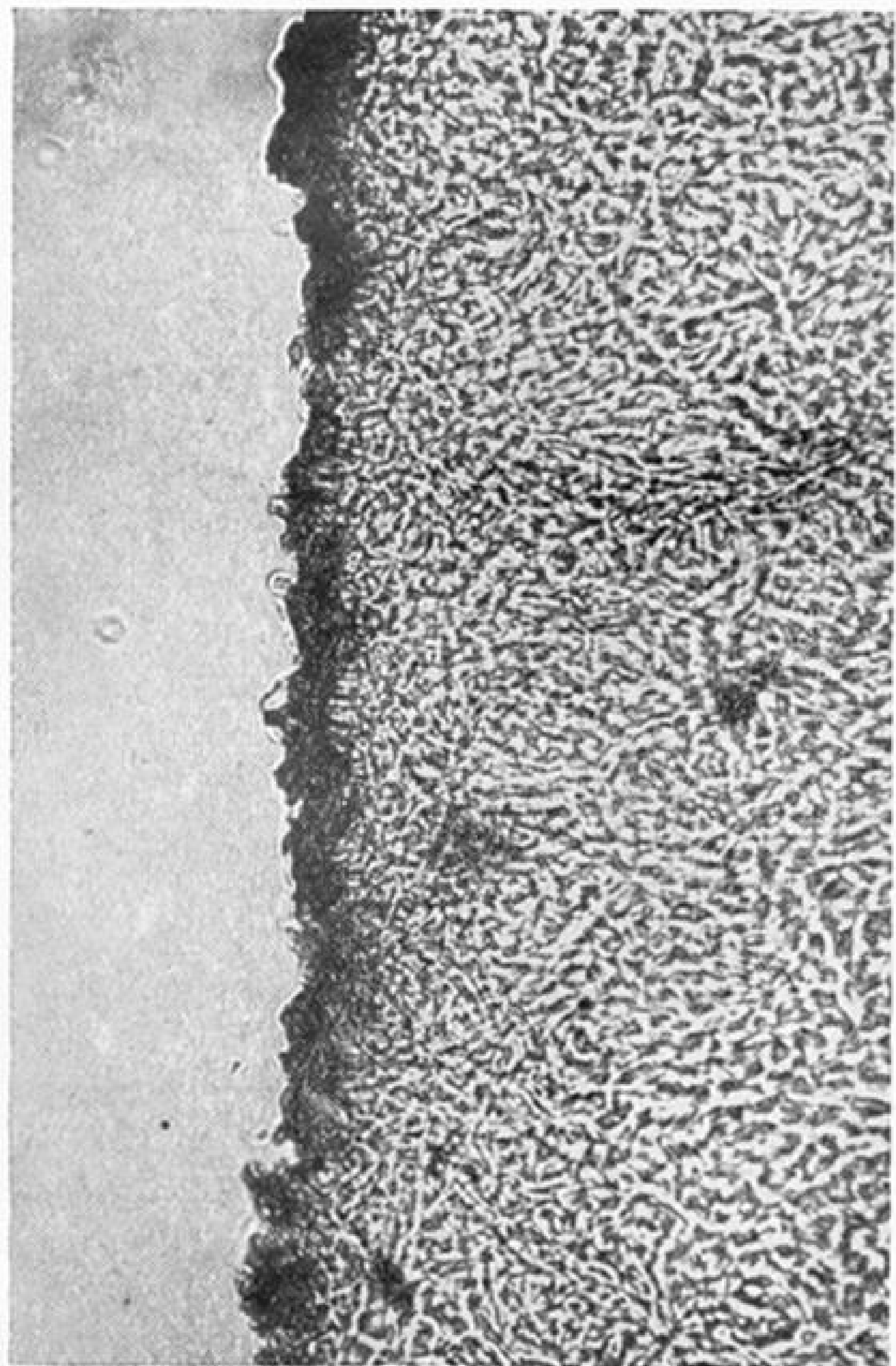

Fig. 3.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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Fig. 1.—Petri dish culture in which the normal form and its colourless derivative have been grown. Pigmented haptera of the albino strain may be seen at the periphery of the culture in contact with the glass. $\times \frac{2}{3}$.

Fig. 2.—Vertical section of colourless sclerotium. $\times$ 250.

Fig. 3.—Vertical section of parental sclerotium. $\times$ 250.