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MORPHOGENESIS AND BIOCHEMICAL PROCESSES IN SCHIZOPHYLLUM COMMUNE FR.

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CONTENTS

ABSTRACT

The investigation concerns a first attempt to unravel biochemical patterns of significance for morphogenesis in *S. commune*. The results derive from two different stocks: one with wild morphology, the other exhibiting an impairment of pileus formation. Some morphological and genetical features of the stocks are discussed.

A special culture technique allowed fair synchronization and control of development via the medium. This technique is based on the fact that differentiation can be prevented by cultivation in a thiamine-free medium.

Measured changes in protein, RNA, DNA, and soluble cell constituents during thiamine-induced primordia formation yielded some information concerning the first steps of differentiation.

Primordial growth was found to depend upon transport of nitrogenous compounds from the mycelium and the presence of a carbon source in the medium. Most of the assimilated glucose is deposited as polysaccharides in the cell walls of the primordia.

Chemical analysis of the cell walls demonstrated that they are mainly composed of two different glucans, called here S- and R-glucan, some properties of which were investigated.

Pileus formation was shown to occur without the uptake of nutrients from the medium and to depend upon the withdrawal of substances from stunted fructifications and stroma in particular. Comparison of the two investigated stocks provided strong support for a causal relationship between degradation of the cell-wall glucans and pileus formation.

An analysis was made of respiration and fermentation during development; some findings probably bearing upon the problem of morphogenesis are discussed.

INTRODUCTION

Morphogenesis encompasses those aspects of the development of an organism that are related to morphological changes. At present it is generally accepted that such processes as cell differentiation and the organization of cells into distinct structures are primarily caused by acquisitions and activities on the molecular level. Moreover, studies on the mechanism of regulation of protein synthesis in bacteria have provided a basis for the construction of hypotheses concerned with mechanisms of cellular differentiation, i.e. the activation of latent gene DNA in time and space and the resulting change in biochemical make-up of the cell (cf. MONOD and JACOB, 1961). However, the fact is that the number of cases in which a change in biochemical events has been related to a change in form is very limited.

The selection of fungi as amenable material for studying the biochemistry of morphogenesis seems justified for several reasons. As compared with higher organisms, the morphological differentiations of fungi are relatively simple and they share with other microorganisms the convenience of rapid and easy cultivation in the laboratory. In a number of thoroughly investigated fungi (cf. WARD, 1959; NICKERSON and BARTNICKI-GARCIA, 1964), two other attributes have proved to be of great value for probing the biochemical mechanisms of form-development. Firstly, in several instances morphogenesis can be conveniently controlled through proper adjustment of certain chemical or physical factors of the external milieu. Once such a factor has been found, it constitutes a reliable tool for tracing biochemical patterns related to the morphogenetic effect. Secondly, a number of fungi have been shown to possess a morphogenetic phase dissociated from the growth phase. It is clear that where such a situation exists, the biochemical events underlying morphogenesis are not obscured by those more especially associated with growth.

Most of the more intensively studied fungi belong to the Myxomycetes and Phycomycetes. By applying modern biochemical techniques, considerable progress has been made in the understanding of the biochemical machinery involved in the development of form in these organisms (see recent reviews by CANTINO and TURIAN, 1959; WARD, 1959; WRIGHT, 1963; NICKERSON and BARTNICKI-GARCIA, 1964).

It is remarkable that higher fungi, such as the Hymenomycetes, have received so little attention in this respect. The production of the fruit-body primordium from the undifferentiated mycelium and the subsequent formative processes that lead to the establishment of the conspicuous carpophores, exemplify an intriguing morphogenetic problem.

In spite of copious references for environmental factors such as light, humidity, temperature, aeration, food supply, etc. which are conducive or inhibitory to sporulation, these studies do not provide any insight into the biochemistry of fruit-body production (HAWKER, 1957; COCHRANE, 1958; for Hymenomycetes, see also LOWAG, 1952;

PLUNKETT, 1956). Nevertheless, the wealth of information emerging from these investigations could provide a good starting point for further biochemical work.

The primary scope of the present work is to report investigations concerned with biochemical aspects of morphogenesis in *Schizophyllum commune*. Fr. The selection of this particular Hymenomycete was suggested by its abundant and reproducible production of fruiting bodies on simple synthetic media. Furthermore, this species has been and still is the subject of intensive genetical research (see Chapter I). On the other hand, there is a general lack of physiological and biochemical data concerning both *Schizophyllum* and other Hymenomycetes. It therefore appeared worth-while to include a report of some additional data whose significance for the problem of morphogenesis is not yet clear. These data may prove useful as a basis for future work in this field.

It was realized that a sound approach to the problem would require at least the following features:

1. The availability of morphological mutant characters, that is to say, mutants which show an impairment of the normal developmental sequence. Comparison of such mutants with the wild type could lead to the recognition of a crucial biochemical event significant for morphogenesis.
2. The technical means to change the external milieu during development, in order to study the exogenous requirements for the different phases of morphogenesis.
3. Synchronized cultures, in order to relate biochemical events to a distinct phase of development.

The first requirement was met by the isolation of a mutant stock (cup mutant) which did not show normal pileus formation: development did not proceed beyond a normally intermediate stage. In Chapter I, the selected stocks are described with respect to their development, morphology, and hyphal structure. A short account of the anomalous segregation of mating factors exhibited by these stocks is also given and compared with the pattern normally encountered in *Schizophyllum commune*.

The methods of cultivation and medium replacement described in Chapter II bear upon the second point listed above. With respect to the third point, the use of a mycelial suspension as the inoculum for surface growth allowed a fair synchronization of development, which could be further improved by the combined use of the replacement-culture technique and the effect of the omission of thiamine from the culture medium. For the sake of convenience, the details of this thiamine effect and the features of thiamineless growth are discussed in a separate Chapter (VII).

The morphological changes in the selected stocks during development having been described in Chapter I, Chapter II goes on to deal with the results of measurements of changes in the amount of nitrogen and carbohydrate contained in mycelium and fructifications during

distinct phases of development and the relationship of these changes to the composition of the medium. One important clue suggested by the results of comparison of the cup mutant with the wild-type stock was a difference in the breakdown of carbohydrates related to the production of the pileus. This turned our attention to the carbohydrate composition of *Schizophyllum* and more particularly to the composition of the cell walls.

Chapter III deals with an analysis of the carbohydrate composition of isolated cell walls by means of chromatographic and X-ray analysis. The major part of the cell walls was found to be composed of two different glucans, denoted here as S- and R-glucan, and to contain in addition a small amount of chitin. These results enabled the design of a fractionation procedure for the cellular carbohydrates that permitted closer inspection of the fate of various carbohydrates in both stocks.

As discussed in Chapter IV, the data obtained strongly suggested an impairment of breakdown of glucans (and chitin) in the cell walls of the cup mutant, indicating this breakdown to be an essential prerequisite for normal pileus formation. It was found that in the wild stock the S-glucan/R-glucan ratio of certain structures rose considerably, the breakdown products of the R-glucan evidently being used for the construction of the cell walls of the pileus. With regard to the biological significance of this process, it was found that the process could not be replaced simply by feeding more glucose (in substitution for the breakdown products of the glucans) to the developing system. Consequently, the hypothesis is put forward that pileus formation is controlled by the existence of a continuous but low concentration of glucose (or other transportable breakdown products of the glucans).

Chapter V describes experiments which strongly suggest that such a mechanism is operative. In fact, by manipulating the external concentration of glucose, it proved possible to inhibit pileus formation in the wild stock and to induce pileus formation in the cup mutant.

Chapter VI contains the results of an analysis of the rate of respiration and fermentation as a function of growth and development. To measure gas exchange in spatially-undisturbed hyphal systems, a special method was employed. In addition to some findings probably more specifically related to the transition from undifferentiated growth to primordia production and to the subsequent period of primordial growth, the results prompted the construction of an hypothesis more generally concerned with the balance of respiration and fermentation in a hyphal system emerging from the substrate into the air.

The last two chapters are concerned with attempts to analyze the first morphogenetic stages; i.e. the formation of primordia upon the undifferentiated mycelium. Chapter VII deals mainly with the problem of thiamineless (undifferentiated) growth and the induction of primordia in such a pre-formed mycelium. Chapter VIII describes quantitative changes in such cell constituents as nucleic acids and proteins

during the transition from undifferentiated to primordia-bearing mycelium under conditions that control the growth of the system as a whole. An important aim of these investigations was to provide a basis for future work on biochemical differentiation at the level of nucleic acids and protein during this early morphogenetic phase.

It will be noticed that results obtained with the cup mutant often antidate those obtained with the wild-type stock, and that the former was used more often for detailed investigations. The reason for this is that at the start of the investigation our interest was concerned primarily with induction of primordia. Because of its abundant primordia production, the cup mutant was chosen for this purpose. During the course of this work, however, our attention was drawn to the process of pileus formation. It was only after some effort that a stock was isolated that combined wild-type morphology of the fruiting bodies with a frequency of primordia production comparable to that of the cup mutant. In those chapters in which the situation in the cup mutant is discussed first, before proceeding to the wild-type stock, this order reflects the actual course of the investigation.

CHAPTER I

GENERAL FEATURES OF THE DEVELOPMENT, MORPHOLOGY, AND SEXUALITY OF SCHIZOPHYLLUM COMMUNE AND SOME SPECIAL NOTES ON THE INVESTIGATED STOCKS

INTRODUCTION

Schizophyllum, a "gilled" mushroom, is commonly classified as belonging to the Agaricales which, together with the Aphyllophorales, is included in the Hymenomycetes. However, this taxonomic position of the genus has been questioned (ESSIG, 1922; HEIM, 1948; SINGER, 1949) and recently arguments concerned with the ontogenesis of the fruit-body and especially of the "gills" have led DONK (1964) to assign it to the Aphyllophorales.

The genus *Schizophyllum* differs from all other gilled fungi in that the "gills" which radiate out from the point of attachment of the fruiting body are cleft at the edges (hence the name *Schizophyllum).* The plant is xerophytic, and under conditions of desiccation the edges of the "gills" curl away from each other and in doing so cover the hymenial surface.

The genus has been divided into several species (LINDER, 1933), of which *Schizophyllum commune* is the most common. Because Linder recognizes the great variability of gross morphology of this cosmopolitan plant, which grows under extremely variable conditions, he emphasizes the importance of microscopic characters in the identification of the species.

Schizophyllum commune Fr. is widely distributed throughout the temperate and tropic zones. It is found mostly on the wood of

deciduous trees, rarely on coniferous wood, and occasionally on the roots and stems of herbaceous plants. It grows saprophytically or weakly parasitically (ESSIG, 1922).

The life cycle and the mode of sexual reproduction of *Schizophyllum* is typical for the Hymenomycetes as a whole.

Basidiospores, four for each basidium as the result of a meiotic division, are produced in profusion on the hymenium at the under surface of the fruiting body. These basidiospores are binucleate at the time of shedding; the two nuclei arise from a mitotic division in the spore following the entrance of a meiotically-produced nucleus from the basidium into the spore (ESSIG, 1922; EHRLICH and MCDONOUGH, 1949).

Fruiting bodies of *Schizophyllum* that have been completely dry for a long time, can be induced to from basidiospores simply by remoistening. The basidiospores, when stored under dry conditions, can retain their viability for many years (AINSWORTH, 1962). Clearly, these properties are related to the xerophytic nature of *Schizophyllum.*

Under appropriate conditions, the basidiospores can germinate and form so-called homokaryotic mycelium, i.e. a septate mycelium composed of mononucleate cells. This mycelium is capable of indefinite vegetative growth.

When two homokaryotic mycelia are brought together, hyphal anastomoses occur. Through the bridges formed, nuclei can migrate and become associated with those of the other mycelium. The subsequent development, however, depends on the mating type of the two mycelia. A full account of the sexuality of Hymenomycetes, and especially of *Schizophyllum,* can be found in articles by WHITEHOUSE (1949), RAPER (1953), and PAPAZIAN (1958); a very brief account of these matters will serve our purpose here.

Schizophyllum commune is a heterothallic, tetrapolar fungus. The first of these terms means that the haploid mycelia of the fungus can be divided into two or more morphologically similar cross-compatible, but self-incompatible, groups. The term tetrapolar, which was first applied by KNIEP (1920), means that sexuality is controlled by two different factors, usually denoted as A and B. In other words: among the progeny of a single fruit-body one can find homokaryons of four different mating types, e.g. A^1B^1, A^1B^2, A^2B^1, and A^2B^2. Normal sexual interaction between these homokaryons occurs only when the mycelia are heterozygous for both factors. Consequently, the established dikaryon contains two dissimilar A and two dissimilar B factors, e.g. in the above-stated case $A^1B^1 + A^2B^2$ or $A^1B^2 + A^2B^1$. In tetrapolar species, thus, only 25 % of the random matings between sibs (i.e. among products of the same fruiting body) are compatible. It has been found, however, that there are many alternative states of the A and B factors in nature (WHITEHOUSE, 1949). In a most extensive study, RAPER *et al.* (1958) estimated the number of different A and B factors in the natural population of *Schizophyllum;* their study indicates about 340 alternate A factors, while the alternate B factors are far less numerous, probably on the order of 60. Practically,

this means that progenies of fruiting bodies collected in different locations can be crossed in all combinations, always giving compatible matings.

Schizophyllum shares this pattern of sexuality, commonly called tetrapolar multiple allelomorph heterothallism, with 55 % of 230 species of Hymenomycetes and Gasteromycetes investigated (WHITEHOUSE, 1949).

In matings between mycelia bearing common A or B factors, special interactions occur which show that the two mating-type loci control different parts of the process of dikaryon formation (PAPAZIAN, 1950; PARAG, 1962). If the mycelia have common A factors and different B factors, a so-called "flat" heterokaryon is set up. Nuclear migration has occurred in both mycelia; the established heterokaryon shows a typical, thin growth with few aerial hyphae, and no clamps or pseudoclamps. These common-A heterokaryons are stable. They exhibit the property of unilateral dikaryotization, acting as donors but not as acceptors in matings with normal mycelia of a mating type compatible with that of either of the "flat" components. In matings between mycelia with common B but different A factors, nuclei of each homokaryon do not generally migrate far into the opposing mycelium and a "barrage" is formed along the region of contact between the mycelia. In this "barrage" region a common-B heterokaryon is formed. This heterokaryon is typified by pseudoclamps. which start to develop like clamp connections (see below) but fail to fuse with the subterminal part of the cell. These pseudoclamps may continue to grow as side branches or they may stop growing; in the latter case they can be distinguished only with difficulty from true clamp-connections (PARAG, 1962). It thus appears that nuclear migration only occurs if the product of interaction is heterozygous for the B factor, while clamps are only formed in a mating involving different A factors. Common-A and common-B heterkaryons never fruit.

If the mycelia are of opposite mating type, i.e. carry both different A and B factors, they are compatible; there follows a sexual interaction with reciprocal "fertilization" of each mate by nuclei from the other. The foreign nucleus in each mycelium now divides rapidly, and its progeny migrate from cell to cell through "pores" in the septae until both parent mycelia have been completely dikaryotized. In other basidiomycetes it has been shown electron microscopically that these pores are not as simply built as in the Ascomycetes, but consist of rather complicated structures which might control the movement of nuclei from one cell to another (GIRBARDT, 1958, 1961; MOORE and MCALEAR, 1962).

The dikaryotic mycelium is also capable of indefinite growth by division of the binucleate cells. When a terminal binucleate cell is ready to divide, the cell forms a short side-branch, the clamp. The two nuclei now divide synchronously. One of the daughter nuclei is formed in the clamp, which now bends over and makes a connection with the subterminal end of the cell. The nucleus in the clamp passes over and becomes associated with one of the products of the other

division. One septum is formed to close the clamp at the point of origin and another septum is formed under the clamp connection, dividing the parent cell into two daughter cells, each containing an identical pair of nuclei. The clamp connection in *Schizophyllum commune* can be used as an invariable diagnostic feature of dikaryotic mycelium; in homokaryotic mycelia clamps are never formed.

According to the terminology used by Raper and coworkers (cf. RAPER and KRONGELB, 1958) a propagated monosporous mycelium will be defined as a strain, and a specific dikaryotic mycelium, formed by the interaction of two strains, a stock.

A genetically adequate dikaryotic mycelium produces fruiting bodies under appropriate environmental conditions. In an extensive study, RAPER and KRONGELB (1958) pointed out that genetic determination is of primary importance in the fruiting process of *Schizophyllum;* proper environmental conditions are of secondary importance only.

The general opinion is that in Hymenomycetes the development of the fruit-body primordium must be traced back, in all likelihood, to a single cell of the dikaryotic mycelium. This opinion rests on both cytological and genetical evidence. As early as 1877, BREFELD could demonstrate cytologically that development of fruit bodies in several *Coprinus* species, *Amanita muscaria,* and other species, starts from a single cell. Another argument stems from the fact that more than two allelomorphs of the mating-type loci have never been recorded for the spores of a single fruit body of *Schizophyllum* or other Hymenomycetes, except for instances in which some of the spores show departure from this rule. This inconsistency was originally attributed to mutation at the mating-type loci, (KNIEP, 1923), later to inter-subunit crossing-over (PAPAZIAN, 1951; RAPER *et al.,* 1960). Thus, it is evident that the progeny arise from basidia, all of which have the same genetic constitution. It is clear that all these identical pairs of nuclei can only arise as daughter nuclei from one cell or a few identical cells.

The general pattern of development of the carpophore has been outlined by ESSIG (1922). The first indication of the fruit-body primordium is a tiny knot of hyphal cells which develops further into a small globose body. This fruit-body primordium becomes provided with a concave pit at the summit in a very early stage. The surface of this cavity is lined with hymenium, indicating a typical gymnocarpic development; i.e. the hymenium is, from its inception, exposed to the air.

Growth at the periphery of the cavity enlarges the sporulating surface, so that the whole structure becomes cup-shaped. In the cup, usually starting from the margin, the radial double "gills" now become established.

The further growth of the young fruiting body depends on an indeterminate peripheral growth of one side of the structure which becomes more and more extended; in this way the typical pileus is produced. At the same time, a positive geotropic reaction of the

hymenial surface can be observed. Because of the unilateral growth of the hymenium, only the "gills" in the growing region become enlarged, so that in a fully-developed fruiting body, the "gills" seem to radiate out from the point of attachment of the carpophore to the substrate.

It should be noted that unilateral growth does not occur when the fruiting bodies are grown in an inverse position, i.e. on the under site of the substratum. In that case, the margin of the pileus expands uniformly, the stipe being attached near the center of the pileus, and on the other side, facing downward, the lamellae radiate out from the centre in a perfectly circular manner (HASSELBRING, 1907; ESSIG, 1922). It thus seems that, regardless of the position of the substrate, the growth of the pileus is directed by the force of gravity in such a way that the hymenium is brought into a more advantageous position for spore discharge.

In the present study the term fructification will henceforth be used irrespective of the stage of development. The term primordium will be reserved for those structures that have not yet developed further than the stage in which the hymenium lines a concave pit with no "gills". The structures beyond this stage will be called fruiting bodies or carpophores.

All the work presented in this paper was done with two descendants of the so-called Kniep stock obtained from the *Centraalbureau voor Schimmelcultures* at Baarn (Holland): *Schizophyllum commune* CBS 103.20. The advantage of this stock as a starting material for the selection of adequate stocks to be used for studies on morphogenesis was early indicated by its high fruiting competence. Moreover, some preliminary crosses with progeny of this stock revealed the presence of aberrant characters which influenced the morphology of the fruiting bodies. This was advantageous because comparison of such genetically-determined aberrations with stocks carrying wild morphology could yield information concerned with the underlying mechanism of morphogenesis in this fungus.

During the initial work on the selection of dikaryons from the Kniep stock (in 1960) we were rather puzzled by the pattern of interactions between monosporous descendants of this dikaryon because it showed no resemblance to the normal pattern outlined above. Although we were not primarily interested in the genetics of the incompatibility factors, some observations were made concerning this topic. Unfortunately, it was not until recently that we became acquainted with a far more extensive study of RAPER and OETTINGER (1962) which deals especially with anomalous segregation of incompatibility factors in the Kniep stock of *Schizophyllum;* all but a very brief account of these matters will therefore be omitted.

A short description of some morphological features of development in the investigated stocks of *Schizophyllum* will also be included in this chapter, since it provides a broader framework for the discussion of the facts to be presented in the following chapters.

METHODS

The basic media routinely employed for propagation of homokaryons and mating were complete and minimal medium (fruiting medium) respectively, as used by Raper and coworkers (RAPER and KRONGELB, 1958; RAPER and MILES, 1958). All cultures were incubated in daylight at room temperature (ca 22°C) unless otherwise indicated.

Single-spore cultures were obtained by allowing a fruiting body to discharge spores in a Petri dish, suspending the spores in water and streaking diluted suspensions over an ager surface with a bent glass rod. After about 24 hours, well-separated germlings were selected and transferred to agar slants. In some instances products of one basidium were isolated with a micromanipulator (Fonbrune), using the method of PAPAZIAN (1950).

Matings were made by placing two mycelial inocula upon the surface of agar medium, not more than 3 mm apart. After dikaryon formation but before fruiting took place, small parts of those dikaryons expected to be of value for further work were removed and transferred to malt agar slants which were incubated at 30°C for 3–5 days and then stored at about 4°C. This method was effective for keeping the dikaryons in a purely vegetative state; fructification was completely prevented. The maintenance of dikaryons proved to be necessary because several instances of instability of the mating factors of the homokaryons were encountered.

For microscopic examination the fungi were grown on agar media inoculated with a mycelial suspension. They were grown either in stationary cultures or in replacement cultures; these methods are described in detail in Chapter VI. Discs (diam. 8 mm) containing agar and fungal structures were fixed in 10 % neutralized formol for about 2 days. Sectioning was done on a freezing microtome. The material was sectioned in rather thick slices (40–60 μ) so that sections would remain entire. The sections were transferred from water to the slides; it was not necessary to use glycerine albumen to fasten the sections.

After trials of several staining methods, the following simple procedure was chosen because it gave quick and satisfactory results. Sections are immersed in a 0.05 % aqueous solution of thionin for 1 min. They are then blotted with filter pater and immediately mounted in an aqueous moutant (Aquamount, Gurr). Final colour develops within one hour. The cytoplasm stains blue, the cell walls take no stain except for an outer layer of certain cell walls (see below) which stains red metachromatically. Fair differentiation is obtained in this way. The contrast between blue- and red-stained structures can be fortified by pre-staining the sections in a solution of 0.05 % aniline blue in 0.5 N acetic acid, rinsing with distilled water, and then proceeding as indicated above. With pre-staining, we found that structures staining blue with thionin were stained more intensively.

RESULTS AND DISCUSSION

1. *Isolation of particular dikaryons and pecularities with respect to incompatibility factors*

In a preliminary experiment, monosporous progeny of one fruiting body of the Kniep stock was mated at random. The resulting dikaryons fell into three classes with respect to fruit-body morphology: a. normal wild-type fruiting bodies, b. cup-shaped fruiting bodies, that did not develop a normal pileus, c. a small number of cultures that developed wild and cup-shaped fruiting bodies in one mating.

A number of the dikaryons that showed cup-shaped fruiting bodies on the minimal medium developed normal pilei on malt agar; others, however, failed to do so. One of the latter dikaryons was chosen for further research. In addition, a wild-type dikaryon was selected. These dikaryons were coded as K.35 (cup-shaped) and K.8 (wild-type). Fruiting bodies of these stocks are shown in Fig. 1 (for an explanation of the "cups" in the photograph of K.8 see section 2 in this chapter).

From 58 crosses among the progeny of a fruiting body of K.8, all compatible matings (12) developed wild-type carpophores exclusively. A sample of 46 random crosses of monosporous progeny of K.35 revealed only cup-shaped carpophores in the resulting dikaryons (16). Although the number of sib matings is small, these results suggest that K.8 and K.35 are homozygous for "wild" and "cup" fruit-body morphology respectively.

The morphology of the cup-chaped fruiting bodies seems much the same as that of a fruiting body indicated as coralloid by Raper and coworkers (RAPER and KRONGELB, 1958; RAPER and OETTINGER, 1962). This character (cor) was found in an old dikaryotic stock collected in 1945 and is also said to be present in the Kniep stock. In the study by Raper and Krongelb, cor was found to be a "single-locus" dominant character, always associated with a mycelial aberration and the production of indigo.

A sample of progeny of K.35 was mated with a monokaryon derived from a fruiting body collected in the field (Teylingerbos, Vogelenzang, Holland). As expected, all matings (73) were compatible, but in no instance did a mating produce cup-shaped fruiting bodies exclusively; they were either wild or mixed, i.e. cup-shaped and wild carpophores from one mating. Similarly, matings between progeny of K.8 and K.35 yielded wild or mixed population of fruiting bodies. It thus appears that the mutant character is not dominant over wild in this case. Moreover, unlike RAPER and KRONGELB (1958), we found no correlation with the production of indigo. On the other hand, cup morphology is associated with high fruiting competence, i.e. early fruiting time and high frequency of fruiting, characters also known to be associated with coralloid. Taken as a whole, we have no evidence as to the genetical identity of cup-

shaped and coralloid fruiting bodies, although they are phenotypically much the same.

The monosporous mycelia derived from the Kniep stock or from the descendants K.35 and K.8 exhibit morphological features quite different from normal monokaryons. Furthermore, sib matings in these stocks do not reveal the characteristic patterns of interactions (flat, barrage, etc.) as generally accepted and also found by us in sib matings of fruiting bodies collected in the field. It was soon recognized that at least part of the abnormal behaviour observed is due to the fact that a large number of monosporous mycelia show unilaterality when crossed with a wild monokaryon. Most of these mycelia show "flat" morphology, typical for a common-A heterokaryon. Fructification occurs only rarely (incidence ± 2 %). Except for a small number of seemingly normal mycelia, the "non-flat" mycelia grow more vigorously with abundant aerial mycelium, bear pseudoclamps at least in most cases, fruit spontaneously (incidence ± 70 %), and exhibit bilateral dikaryotization of a wild monokaryon. These mycelia often sector to give rise to mycelia which then dikaryotize unilaterally.

In the original Kniep stock and K.35, about half of the spores isolated produce "flat" mycelia. In K.8 the frequency of "flats" is much higher, and most of these mycelia show very restricted radial growth on the minimal medium.

In contrast to the wild fruiting bodies collected in the field, it is impossible to isolate complete tetrads from fruiting bodies of either the original Kniep stock or the derived stocks K.35 and K.8. Most basidia bear only two spores, sometimes accompanied by two small ones. One- or three-spored basidia are also present, but less frequently. On the average, only 64 % of randomly isolated spores germinate.

At the time when we observed these extremely anomalous facts, we were unable to suggest any explanation for them. However, it appeared reasonable to connect these facts with the peculiar history of the Kniep stock. In 1920 Kniep deposited two monokaryons in the collection of the *Centraalbureau voor Schimmelcultures*. From his accompanying letter we cite: "Zwei Schizophyllum commune Kulturen mit den Bezeichnungen X und XX. Beide sind Einspormyzelien, die schnallenlos sind und bei Kombination Schnallen ergeben." Strain XX was lost from the collection in 1942. According to a note on the collection card, strain X developed clamps and normal fruiting bodies in 1933. Since then the stock has been propagated by subculturing small pieces of fruiting bodies (personal communication by Miss M. A. A. Schipper of the *Centraalbureau voor Schimmelcultures).*

It thus appears that the Kniep stock arose as the result of spontaneous dikaryosis of a monokaryon. Mutations affecting the incompatibility factors that could account for such a process and for the anomalous morphology of the derived monosporous mycelia have been described recently (PARAG, 1962; RAPER and RAPER, 1964).

RAPER and OETTINGER (1962) also recognized extreme anomalies in their analysis of progeny of the Kniep stock. Monosporous isolates of the Kniep stock were crossed with normal strains marked with biochemical mutations for five generations. Their study revealed that at least a part of the anomalous results obtained was due to irregular events at the time of meiosis, resulting in strains disomic for the A or B factor or for both. Eventual instability of such disomic nuclei during vegetative growth leads to heterokaryosis; disomic A and disomic B lead to common-B and common-A heterokaryons respectively, while a strain disomic for both factors leads to a dikaryon. The common-B is unstable and can lose components by sectoring. These findings are also consistent with some of our own observations, although others, e.g. fruiting in the pseudoclamped mycelia, remain unexplained. However, Raper and Oettinger have also pointed out that disomy can explain only a portion of the anomalies observed; other irregularities must be involved, one of these being indicated as a probable abnormal distribution of nuclei to the spores. The latter assumption is supported by our observation that the number of normal tetrads is small in the Kniep stock and derived stocks. Irregular distribution of products of meiosis to the basidiospores and subsequent irregularities concerned with mating reactions have also been reported some time ago in at least one other basidiomycete (SKOLDO, 1944).

Finally, it should be noted that contrary to the morphological instability of the monosporous mycelia, the derived dikaryons are completely stable in this respect. Stocks K.35 and K.8 have revealed no change in the morphology of mycelium or fruiting bodies during the last four years. During this period the stocks were subcultured vegetatively bimonthly (for about 4 days at 30°C, stored at 4°C).

2. *Morphological features of development in stocks K.35 and K.8*

It should be noted that the specimens taken for microscopic examination (Fig. 2) and used for the photographs shown in Fig. 1 were obtained by growing the fungi from a mycelial suspension spread over the surface of an agar medium. With respect to this cultivation technique, two points are relevant here. Firstly, contrary to the situation when single mycelial plugs are used for inoculation (as in the mating experiments), growth starts from a large number of viable fragments over the whole surface of the medium. As a result, the distinct phases of growth and morphogenesis are synchronized to a certain degree. Secondly, the primordia formed outnumber those formed when single mycelial plugs are used. It is obvious, however, that in "wild" stocks the limited amount of food available cannot support the complete outgrowth of all the primordia present. After the "cup" stage has been reached, competition phenomena become apparent and only a few fruiting bodies develop a large pileus. The supernumerary primordia develop only "cup-shaped" fruiting bodies (arrow Fig. IA) much like those formed in stock K.35 exclusively.

The low magnification photographs in Fig. 2 illustrate some of the

morphological features of development in *S. commune*, as viewed in stock K.35 and K.8 at 25°C on synthetic medium. In both stocks, development is much the same until the cup stage, and these stages are therefore illustrated for K.35 only. These pictures (2A-G) were taken from cultures in which synchronization was improved by means of a replacement technique (see Chapter VI). With this method, the number of developing primordia outnumber those formed in stationary culture and the stroma becomes more heavily developed. Otherwise, the morphological characteristics apply equally well to stationary cultures.

Initially, the hyphae originating from the small inocula grow separately in all directions into the agar and on the wet surface of the substrate (A). Only very few, if any, aerial hyphae are produced in this early stage; a surface view of the culture appears wet and slimy.

The first signs of differentiation soon become apparent when, in localized patches on the surface, the hyphae show a tendency to stick together and to become closely interwoven in dense aggregates (B). In a surface view these aggregates appear as small white tufts against the grey mass of undifferentiated mycelium. Under favourable conditions, implying plentiful availability of nutrients (carbon and nitrogen) as realized in the present cultures, the number of these hyphal aggregates quickly increases. Moreover, the hyphal masses become more and more extended laterally over the surface of the substrate (C). Finally, the whole surface of the culture may be covered with a coherent mass of very closely interwoven hyphae. Henceforth, this structure will be indicated as a stroma (D, E). Macroscopically, the fruit-body initials can be distinguished as small, denser, hemispherical projections embedded in the stroma layer. Microscopically, they can often be traced because the hyphae in the primordia tend to run vertically, whereas the typical stromal hyphae run parallel to the substrate surface.

For the present we are inclined to believe that all primordia arise from the small aggregates of hyphae referred to above and that the stroma is merely a secondary structure. However, the possibility that new initials are formed in the stroma cannot be completely excluded at the moment.

The photographs (Fig. 2B–E) also indicate that during the production of surface structures, submerged growth of undifferentiated mycelium continues to occur. In fact, most of the vegetative mycelium produced is formed after the primordia have been initiated.

The next stage is characterized by growth of the primordia and a further establishment of the stroma. Anticipating evidence which will be discussed in the following chapter, these processes can occur in the absence of an exogenous nitrogen supply, provided that a carbon source is present. As a matter of fact, the specimens in Fig. 2F and G, were held in the absence of exogenous nitrogen; the nitrogen requirement being evidently met by a transport of cytoplasmic constituents from the undifferentiated vegetative mycelium to the developing

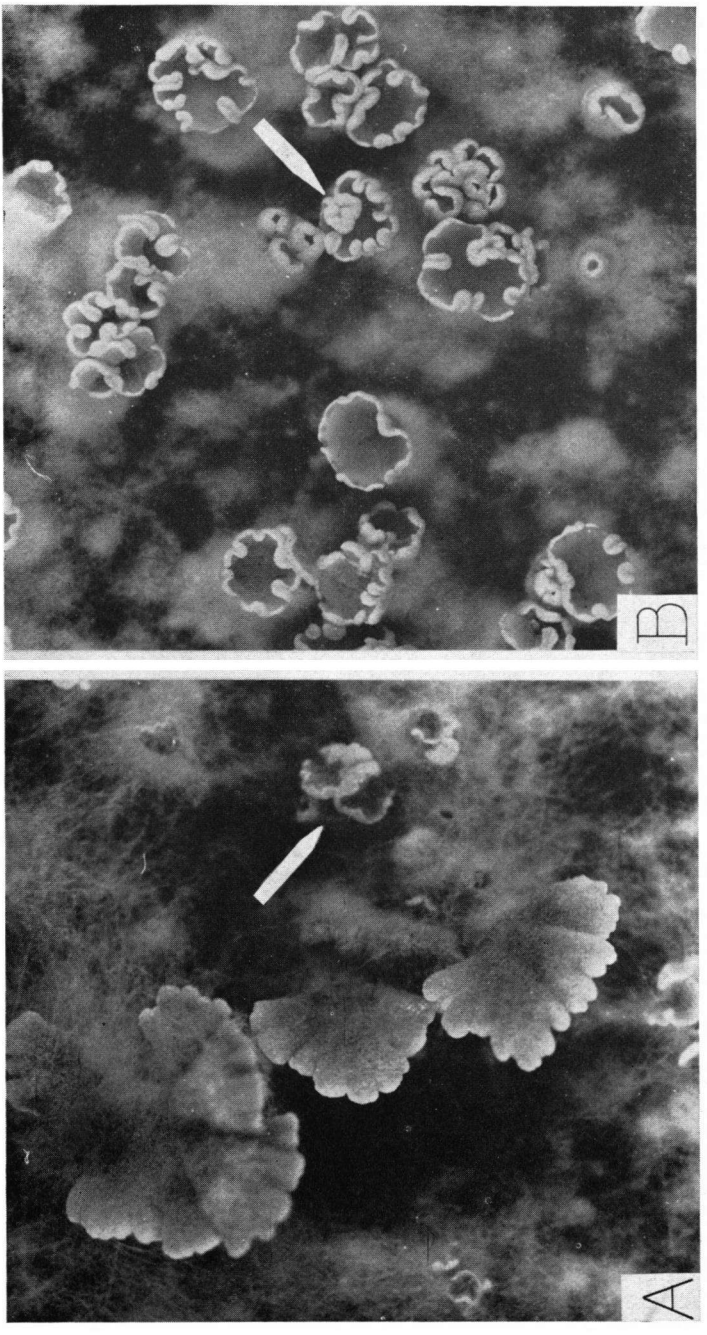
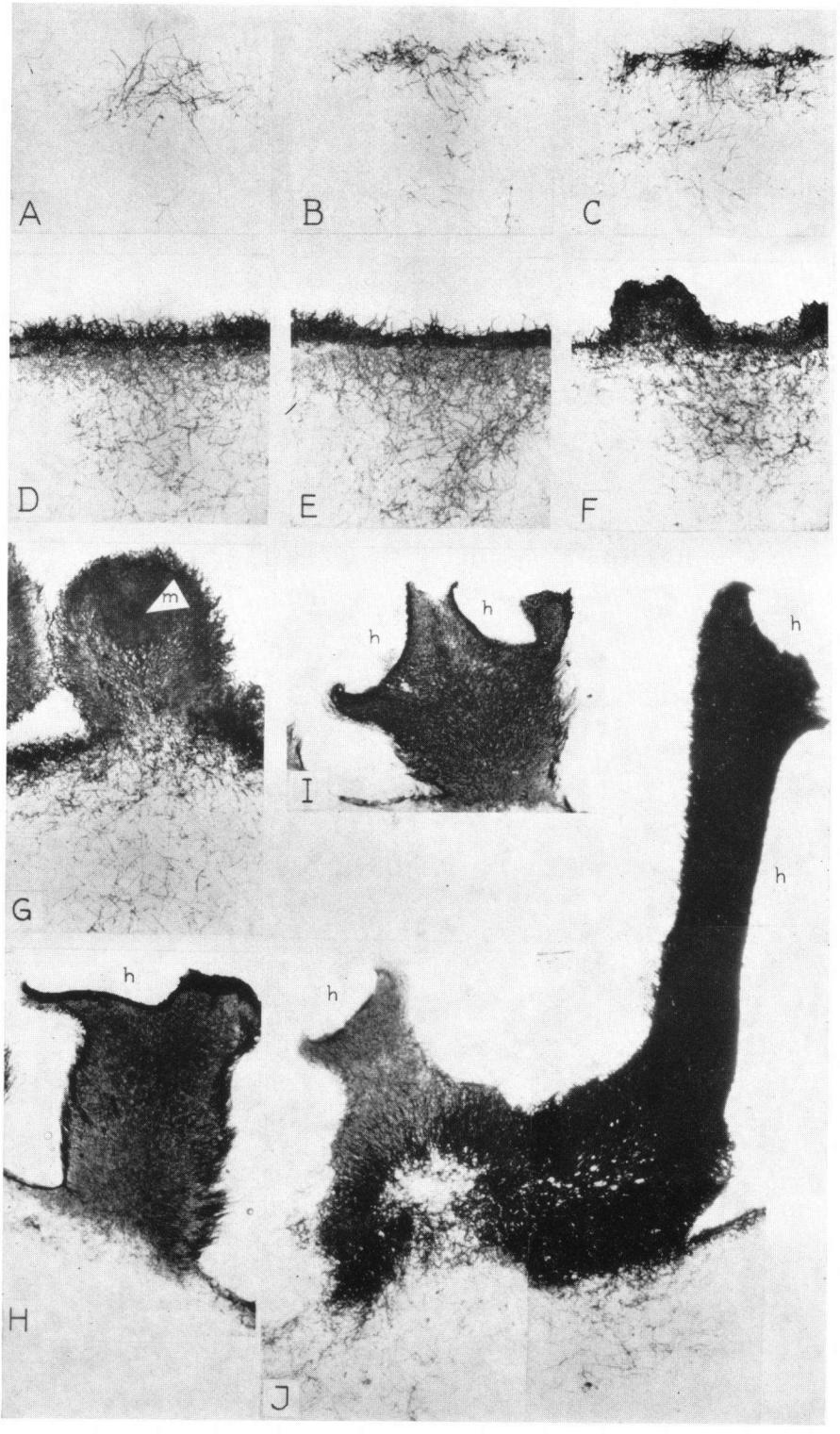


Fig. 1. Fruiting bodies produced by *Schizophyllum commune* stocks derived from the Kniep stock. A: Wild-type stock K.8; B: cup mutant K.35. The fungi were grown on synthetic medium (4 % glucose) for 16 days.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structures. After growth of the primordia (G), the bulk of cytoplasm in the submerged cells appears to be displaced; the cells stain only faintly and are highly vacuolated.

It must be emphasized here that the stroma is not always so strongly developed as in the present case, in which it covers the whole surface of the culture. More often it is only well developed around the base of the primordia, but even in this case strands of stroma seem to connect individual primordia or clusters of primordia with each other.

In an early stage (F), before the primordium has reached its maximum size, the single pore appears at the apex. During primordial growth, no further differentiation seems to occur in this region. However, following the cessation of growth of the primordium, the apical cavity enlarges and "gills" are produced at the margin or on the surface of the original cavity. This simple fruit body is the final developmental stage in stock K.35. In Fig. 2, H and I show median sections through such a fruit body.

Fig. 2J shows a final stage in the "wild-type" stock K.8. Two fruiting bodies are borne on two partly-connected primordia. The lefthand fruiting body has not developed beyond the cup stage. In the righthand fruiting body it can be clearly seen how an essentially one-sided growth of the cup-shaped structure produces the pileus and enlarges the surface of the hymenium. It is interesting to note that the cytoplasmic content of the hyphae of the stunted fruiting body has largely disappeared, suggesting a flow of cytoplasmic material into the developing fruiting body.

With regard to development at 30°C — the preceding description applies to cultures at 25°C — it must be noted that under this condition hyphal aggregates and stroma are formed but no primordia develop. Instead, a woolly aerial mycelium is produced.

The vegetative hyphae which grow into the substrate have thin walls, and these walls remain colourless when sections are stained with thionin. However, the walls of cells constituting the primordia and stroma stain red with thionin. As growth of the primordia proceeds and before cap expansion becomes apparent, the cell walls of the primordial cells (except those in the apical pore region) and of a large number of the stroma cells thicken irregularly. The lumen sometimes almost disappears, but in other parts of the cell the lumen widens and sometimes becomes highly inflated. In these sclerified

Fig. 2. Low magnification micrographs of sections through successive developmental stages of *S. commune* stock K.35 (A–I) and stock K.8 (J). The developmental pattern of K.8 up to the cup stage is essentially the same as that of K.35. To obtain well-synchronized cultures, the specimens used for photographs A–G were grown on agar discs in replacement culture (see Chapter VI). A: Undifferentiated mycelium produced in a thiamine-free medium. B, C, D, and E: Taken at 12-hour intervals after a shift to a complete medium supplemented with thiamine; F and G at 24-hour intervals after a subsequent shift to a medium lacking the nitrogen source. G: Tangential section through a primordium; the dark region at the apex (m) is composed of thin-walled, heavily-stained cells which surround the apical cavity. H–J: 12-day old stationary cultures; h: hymenium.

hyphae only a thin, regular outer layer stains metachromatically red. The sclerifying material does not take any stain. The hyphae that make up the context of the pileus are of one type (monomitic) and originally have thin walls. The walls of these hyphae also stain red. In older carpophores these walls also become gradually thicker, but the wall-thickness remains much more uniform than in the early sclerified hyphae of primordia and stroma.

It is remarkable that only the hyphae contained in multihyphal structures (context of cap, primordium, stroma) show the red colouration of an outer cell-wall layer after staining with thionin. The walls of all other hyphae which grow more individually, such as the hyphae of the vegetative submerged mycelium, the woolly aerial mycelium, and the hairy covering of the fruiting bodies, do not take the thionin stain. This correlation makes it tempting to suggest that the staining characteristics of the cell walls in the multihyphal structures reflect chemical properties of the walls which have something to do with the tendency of these cells to become intimately associated with each other. However, at the moment there is no further evidence to support such a view.

Lastly, we wish to discuss briefly the taxonomic interpretation of the structure and development of the fruiting bodies. The cup mutant K.35 is especially informative in this respect.

Fig. 1 clearly shows that the basic fruiting body is very much the same as that of a cyphellaceous fungus: a hollow cup, lined at the inside with a smooth hymenium and attached to the substrate at the abhymenial side.

The pictures of the cup mutant also clearly demonstrate the ontogenesis of the "split gills". Most of the "gills" are formed by a marginal proliferation of the original cup. This proliferation tends to transform the original simple cup into a lobed structure. The margins of two adjacent lobes do not fuse, and now constitute a "split gill". Two other modes of formation, both based on the same principle however, are apparent from the photographs. Quite often, small secondary cups are formed inside the original cup (arrow, Fig. 1B, compare also Fig. 15). One-sided growth of the pileus (in a wild stock) stretches this structure longitudinally, and the two opposite margins come to form an adventitious "gill". Thus, these "gills" are not connected with the original cup margin. A third mode of formation is realized when two (or more) cups are formed in the same primordium. The adjacent margins then again form a "gill", which now runs transversely through the resulting structure (compare Fig. 15A).

These observations clearly indicate that the ontogenesis of the "gills" of *Schizophyllum* bears no relationship to that of the agaric gills; they are thus in full accordance with the view of Donk (1964), who assigns *Schizophyllum* to the Aphyllophorales (Schizophyllaceae). In this context it is especially noteworthy that specimens of the cup mutant K.35 grown under conditions such that a well-developed

stroma is formed, resemble the genus *Stromatoscypha* Donk (cf. description by REID, 1963) very closely except for the "gills". This genus was also included in the Schizophyllaceae by DONK (1964).

CHAPTER II

CHANGES IN GROSS CELLULAR CONSTITUENTS AND COMPOSITION OF THE MEDIUM ACCOMPANYING DEVELOPMENT

INTRODUCTION

In order to acquire some preliminary information as a basis for further investigation, it was considered necessary to start by studying the growth of the organism in relation to time and development. As will be pointed out under Methods, the cultivation technique made it impossible to use convential dry-weight values as a measure of growth. The total nitrogen value was used instead for this purpose, with the idea that this value would also give a better, although rough, impression of protein synthesis. However, being aware of the fact that in fungi carbohydrates constitute a major part of the cellular mass (especially of cell walls; compare Chapter I), the total carbohydrate value was determined at the same time. It was found possible to express this value in terms of an equivalent amount of glucose because, as will be shown later, at least 90 % of the carbohydrates of *Schizophyllum* are polyglucoses.

METHODS

1. *Culture vessels*

The nature of the present investigation necessitated the use of surface cultures. Several more or less convential techniques for establishing such surface cultures were tried: cultivation on liquid medium, on glass wool or glass-fibre paper impregnated with medium, and on medium solidified with agar. The first three methods suffered from the disadvantage that the growth yield was not very reproducible, the drawback of the last method was that, although the growth yield was quite reproducible, the presence of the agar hindered the analysis and replacement of the nutrient medium. We therefore adopted the following method:

The bottom of a culture vessel consisting of a 1 l pyrex Erlenmeyer flask closed by a glass cap provided with a cotton filter and an aeration tube (Fig. 3A) is covered with 100 g of quartz sand (acid-washed and glowed), and the whole sterilized by dry heat.

After sterilization, the sand is flooded with 30 ml of sterile medium, leaving a 1 mm layer of fluid over the sand. The flasks are inoculated and placed at constant temperature (25°C ± 1°C) under the continuous light of fluorescent tubes (about 1100 LUX). Aeration is

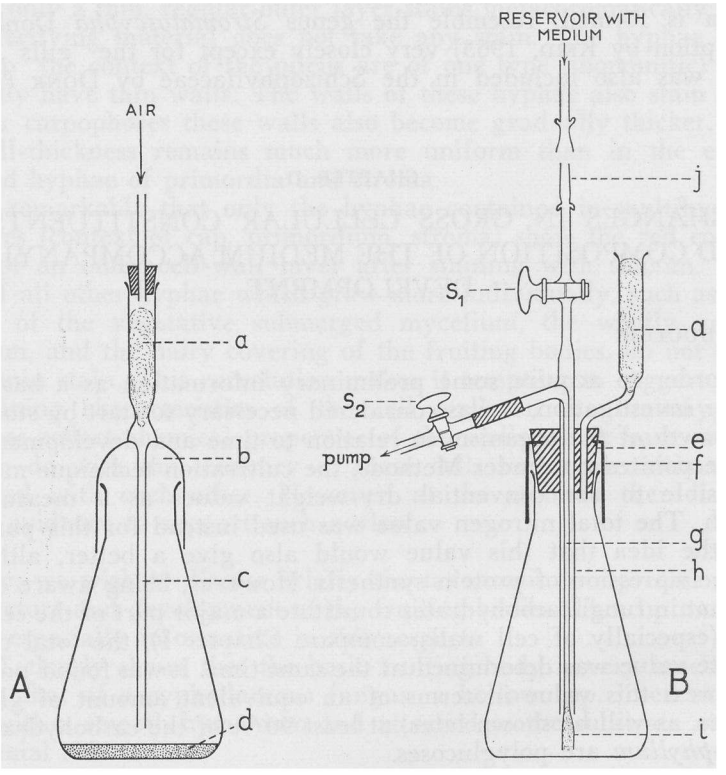


Fig. 3. Culture vessel (A) and replacement apparatus (B). a: cotton filter; b: glass cap; c: aeration tube; d: sand; e: rubber stopper; f: metal sleeve; g: outlet tube; h: inlet tube; i: glass-wool filter.

accomplished by passing a stream of air (about 50 ml/min/flask) via the cotton filters through the flasks, the air having been previously washed with very dilute sulphuric acid and water.

2. *Replacement culture technique*

In order to study the influence of the composition of the medium and to improve the synchronization of development, use was made of replacement cultures. To achieve this, we developed the apparatus shown in Fig. 3B. Essentially, it consists of a combined inlet and outlet tube, the latter with a widening at the base, which, when stoppered with glass wool, serves as a filter. The combined tube can be easily moved up and down through a hole in a rubber stopper fitting into the neck of the culture flask described above. A cotton filter serves to sterilize the air entering the flask during the replacement procedure. The apparatus is placed on an empty Erlenmeyer flask and sterilized in the autoclave. After sterilization, the inlet tube is connected aseptically, by means of ground glass joints, with the siphon of a reservoir containing the replacement medium.

The removal of old medium and the introduction of a new one is carried out as follows. The glass cap of a culture flask containing a pre-formed mycelium is exchanged for the replacement apparatus. The combined tube of the replacement apparatus is lowered until the glass-wool filter in the outlet tube projects into the sand. After making the connection with a water suction pump and opening stop-cock S_2, the old medium is sucked off. S_2 is then closed and S_1 opened, enabling new medium to enter the flask. In order to wash all the old medium from the culture, the washing procedure must be repeated up to 7 times, involving about 200 ml of fresh medium. Furthermore, it is essential that during the washing process the mycelial mat be completely submerged in the new medium. After replacement, the culture flask is closed again with a glass cap and another culture can be processed. The entire procedure lasts about 10 minutes per flask.

The greatest advantage of this method is that the mycelium remains undisturbed.

3. *Culture medium*

The synthetic media are derived from media published by SCHOPFER and BLUMER (1940) and RAPER and SAN ANTONIO (1954); the added trace elements are according to WHITAKER (1951).

The mineral composition per litre of the media is as follows:

KH_2PO_4	0.46 g	$CuSO_4 . 5H_2O$	0.2 mg
K_2HPO_4	1.00 g	$ZnSO_4 . 7H_2O$	2.0 mg
$MgSO_4 . 7H_2O$	0.5 g	$MnSO_4 . 4H_2O$	0.1 mg
$FeCl_3 . 6H_2O$	5.0 mg	$CoCl_2 . 6H_2O$	0.4 mg
H_3BO_3	0.06 mg	$Ca(NO_3)_2 . 4H_2O$	1.2 mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.04 mg		

This medium will henceforth be called basal medium (low phosphate). In some experiments, viz. those involving thiamine-free medium, the phosphate concentration was raised to four times the amount indicated above. This medium will be referred to subsequently as basal medium (high phosphate).

To this mineral medium, glucose and L-asparagine.H_2O were added in concentrations of 2 % and 0.15 % (w/v) respectively, and thiamine.HCl in a concentration of 120 μg per litre. The water was always double glass distilled.

All components were sterilized together at 110°C for 30 minutes. The pH dropped to 6.7 during sterilization. When large quantities had to be handled, the sterilization temperature was raised to 120°C and the time reduced to 25 minutes; in this case the phosphate was sterilized separately and added to the medium aseptically.

When $CaCO_3$ was to be added (conc. 2 % w/v), it was mixed with the sand and sterilized by dry heat.

4. *Inoculum*

The vegetative dikaryotic mycelia of *Schizophyllum* stocks were propagated vegetatively by cultivation on malt agar slants at 30°C for 3–4 days. They were then stored at 4°C. We have observed no differences in growth or morphogenetic characteristics between cultures derived from slants stored in the cold for months and cultures used directly after removal from the 30°C incubator.

Cultivation at 30°C produces a vigorous aerial mycelium which can be scraped off the slant easily and transferred to a tube containing about 2 ml of water and some glass beads (diam. 3 mm). The tube is then shaken manually or in a shaking device (Vortex mixer) until the mycelial clumps are fragmented. The suspension is next diluted with water, after which aliquots (1 ml) are taken for inoculation. Beyond a certain minimum, the density of the suspension does not seem to be critical; the lag phase is somewhat shortened when thick suspensions are used.

5. *Harvest and homogenization*

In experiments related to time, all flasks were inoculated simultaneously. At given intervals the flasks were removed and stored at —22°C. All flasks could thus be handled together in the analytical procedures.

Separation of the mycelial mat from the bulk of the sand was achieved by pouring 100 ml of distilled water into the flask and shaking the contents gently, after which the fluid, containing the floating fungal material could be decanted. This procedure was repeated twice. The decanted fluid with fungal material was then filtered through a piece of glass paper (Whatman GF/A) in a sintered-glass funnel (Jena 3 G/O), the residue washed with water, and the filtrate made up to 1 l for the determination of the residual glucose in the medium (NELSON, 1944).

The fungal material was peeled off the glass-paper filter and homogenized with the adhering sand, 96 % ethanol, and glass beads (diam. 3 mm) in a Mickle disintegrator for 15 minutes. The resulting homogenate was made up to 25 ml with 80 % ethanol; the final concentration of ethanol ranged between 70 and 80 %.

In several cases the fruiting structures were separated from the mycelium. This was accomplished by pouring water over the frozen culture; the fruiting structures could then easily be scraped from the surface and analyzed separately.

6. *Total nitrogen*

Aliquots (0.5–1.0 ml) of the homogenate were pipetted into Kjeldahl flasks. The alcohol was expelled by immersion of the flasks in a boiling water-bath for 3 minutes, after which 2 ml of concentrated H_2SO_4 plus 10 drops of a 10 % solution of $CuSO_4 . 5H_2O$ were added. The contents were mixed until dissolved and then

heated for 15 minutes. After cooling, 10 drops of 30 % H_2O_2 were added and the heating continued for 30 min.

The digest was made alkaline with 10 ml 40 % NaOH and the ammonia distilled into 2.5 ml 0.03 N H_2SO_4. The ammonia was determined colorimetrically with Nessler's reagent (as modified by SNELL and SNELL, 1954) at 430 mμ.

The result obtained with this relatively short and simple destruction method was compared with the much more rigorous procedures of Kjeldahl-Gunning-Dyer and Friedrich (cf. BALLENTINE, 1956); the results were essentially the same.

7. *Total carbohydrate*

From a 1 ml aliquot of the alcoholic homogenate the alcohol was expelled by holding the tubes in a boiling water-bath for 3 minutes. The tubes were then transferred to an ice-bath, 1 ml of cold 72 % (v/v) H_2SO_4 added and, holding the tubes in the ice-bath, the fungal material dissolved by stirring with a rod, after which the contents of the tubes were rapidly diluted with water and made up to volume (25-50 ml). Total carbohydrate was then determined by the anthrone method as modified by FAIRBAIRN (1953). The reagent consists of a 0.1 % solution of anthrone in 72 % H_2SO_4; 5 ml of this reagent is added to 1 ml samples. Maximal colour was obtained, for glucose as well as the *Schizophyllum* sample, by placing the tubes in a boiling water-bath for 9 minutes. After cooling in running water, the fine quartz particles resulting from the homogenization were spun off and the colour measured at maximal absorption (620 mμ).

The absorption spectrum of the chromogen obtained with *Schizophyllum* samples is identical to that obtained with glucose. Further, small quantities of glucose added to the *Schizophyllum* samples can be recovered quantitatively. It is thus unlikely that unknown substances interfere with the determination. As will be shown later, besides glucose, small amounts of xylose and glucosamine are present. However, it was established that these substances do not yield any appreciable colour with the reagent.

RESULTS

1. *Stationary cultures*

Figs. 4 and 5 show the growth curves obtained for the cup mutant K.35 and wild type K.8, using total nitrogen as an index of growth.

After a phase of no apparent growth, in all probability composed of a genuine lag phase and a phase in which growth cannot be measured by the method used, the fungus starts growing. There follows a very brief period in which the hyphae grow in a purely vegetative, submerged manner. This point needs some attention. What is called "vegetative submerged growth" must here be distinguished sharply from "vegetative aerial growth". The former describes a purely undifferentiated type of growth in which no difference can be

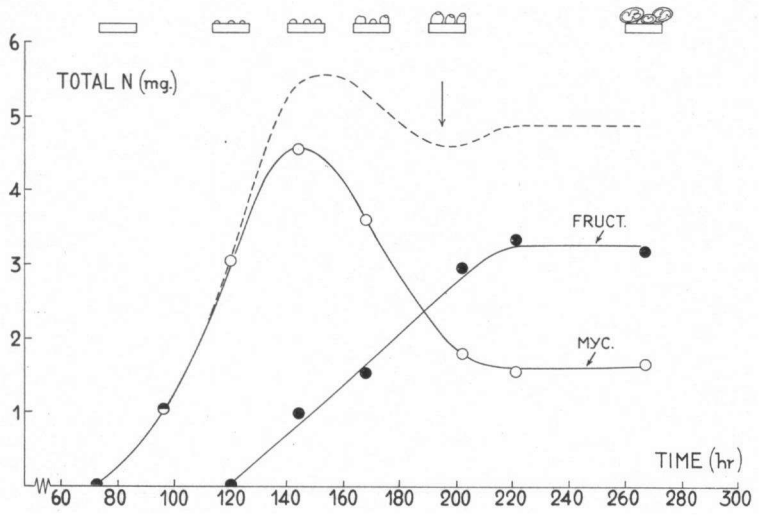


Fig. 4. Changes in total nitrogen of fructifications and mycelium of *S. commune* K.35 (cup mutant) accompanying development. Part of the nitrogen values for the whole organism are obtained by summation of the values for fructifications and mycelium (dashed line). The arrow indicates the moment of exhaustion of the glucose supply in the medium. The schematic drawings at the top represent the various stages of development. Medium: basal medium (low phosphate) plus 2 % glucose, 0.15 % asparagine, and 120 mμg thiamine per ml, pH 6.7, total volume 30 ml. Incubation temperature 25° C. The inoculum was taken from malt agar. Each point represents the average of two cultures.

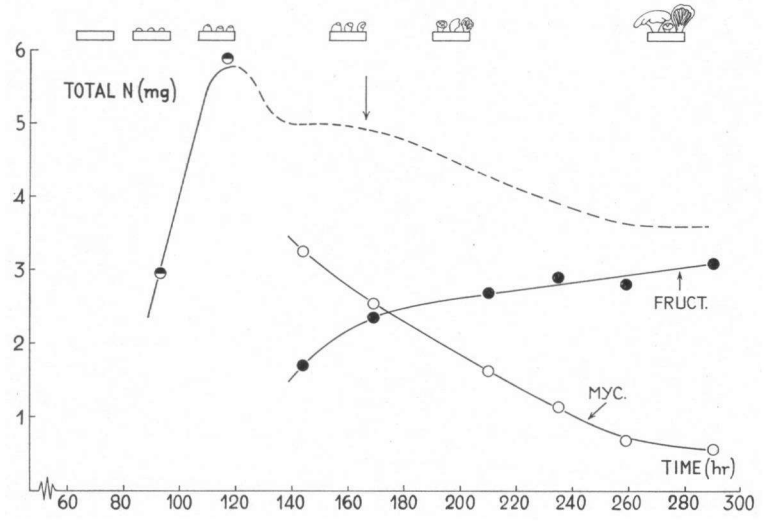


Fig. 5. Changes in total nitrogen of fructifications and mycelium of *S. commune* K.8 (wild type) accompanying development. The arrow indicates the moment of exhaustion of the glucose supply. (See Fig. 4.)

seen between hyphae in different parts of the culture, the hyphae grow just under the surface of the medium, penetrating the substrate ("wet mycelium"). The latter type of growth refers to the formation of differentiated vegetative hyphae which emerge into the air. However, this latter type of mycelium was rarely formed in the experiments reported here. In fact, under the conditions used, the production of aerial hyphae is very scanty and even completely absent in the first growth phase described.

In the stocks used here, which were selected for early fruiting, undifferentiated growth goes on for only a short time (about one day). When the culture has attained a nitrogen content of approximately 1 mg, the first primordia, small aggregates of hyphae, can be seen with the naked eye. The primordia can be distinguished so early because of the nearly total absence of aerial hyphae.

The number and size of the primordia now increase rapidly, coinciding with rapid growth of the culture. Within two days the increase in total nitrogen comes to a halt, at which time the surface bears numerous primordia. At this point of maximal nitrogen yield, the primordia are only small globose bodies, sometimes already provided with a small hole at the top.

In both stocks the nitrogen accounted for in the fungal material (5.5 mg) comprises only a part of the nitrogen supplied (7.0 mg by Kjeldahl analysis after sterilization). The reason for this limitation of nitrogen assimilation is not known; the carbon source is not exhausted at this moment. In this context it is noteworthy that if calcium carbonate is incorporated in the medium, there is not only an increase in growth rate, but also all the nitrogen available is assimilated. It is not very likely that the calcium carbonate acts by functioning as a neutralizing agent in this case, because the pH drops only slightly (minimum value 5.2).

From the curves it follows that, at the moment of maximal nitrogen yield, the greater part of the nitrogen is stored in the mycelium. In the period which follows, and which ends at the moment of exhaustion of the carbon source (see arrows in Figs. 4 and 5), much of the mycelial nitrogen is transported to the primordia, which attain their maximal size. No new primordia are initiated in this phase. In the case of the cup mutant, for instance, the amount of nitrogen disappearing from the mycelium amounts to 60 %.

It is remarkable that, in both stocks, there is an actual loss of nitrogen to the environment in this period. However, this becomes comprehensible in view of the fact that in all likelihood the transport of nitrogenous constituents involves a breakdown and resynthesis of proteins (proteins constitute the bulk of the nitrogenous material, as will be shown later). Since, furthermore, the energy source in the medium becomes exhausted in this period, it may be argued that the dramatic transformations involved cannot proceed with complete efficiency.

Up to the point marked by the exhaustion of glucose from the medium (arrows), the stocks K.35 and K.8 seem to differ only in

detail with respect to total nitrogen and gross morphology. The only apparent difference seems to be that some of the primordia of the wild type K.8 show a tendency to grow unilaterally, resulting in the tilting over of the hymenial surface.

The differences become most conspicuous in the following period. The primordia of K. 35 grow out rather synchronously by expanding the hymenial surface slightly, a small number of "gills" become established, and the fruiting bodies attain their typical cup-shaped appearance. In K.8 however, the outgrowth of the primordia occurs far less synchronously; fruiting bodies of widely differing sizes can be found at the conclusion of development. These fruiting bodies are either typically wild, i.e. a pileus is formed with the lamellated hymenium facing the substrate, or the developing structures are arrested in one of the stages leading from the primordia to fully-developed fruiting bodies.

Coinciding with the changes mentioned above, after the exhaustion of the glucose from the medium there is a profound difference in the pattern of nitrogen metabolism in K.35 and K.8. From Figs. 4 and 5 it can be seen that, for both stocks, there is only a small increase in total nitrogen content of all the fruiting structures together. For K.35 this increase is parallelled by an approximately equal decrease in the mycelium, the total nitrogen contained in the whole organism remaining constant. This situation is probably related to the fact that development comes to a halt in this stage and as yet no autolytic processes are acting on the system. In K.8, however, we are faced with a completely different situation. In spite of the fact that the total amount of nitrogen contained in all fructifications together increases only faintly, the rapid outgrowth of a number of these fructifications can be observed. Because it was taken for granted that there is a relation between growth and nitrogen requirement of the fruiting bodies, there would seem to be some grounds to believe that nitrogenous substances are withdrawn from a number of the fructifications, providing nitrogen for the outgrowth of the developing ones.

At the same time, there is a dramatic disappearance of nitrogen ($\pm$ 75 %) from the mycelium of K.8, which cannot be accounted for by the small increase in nitrogen of all the fruiting structures together.

Perhaps these observations may be interpreted as follows: the developing fructifications withdraw nitrogenous compounds from other fructifications as well as from the mycelium. Due to a limitation of synthetic activity caused by the absence of an energy source in the medium (only endogenous componds can be used as such in this stage), there is an actual loss of nitrogenous intermediates to the medium, or these substances are dissimilated in part and serve as an additional energy source.

Fig. 6 and 7 show the quantitative changes in the total carbohydrate content of mycelium and fructifications of K.35 and K.8, developing along the lines outlined above. The measurements were

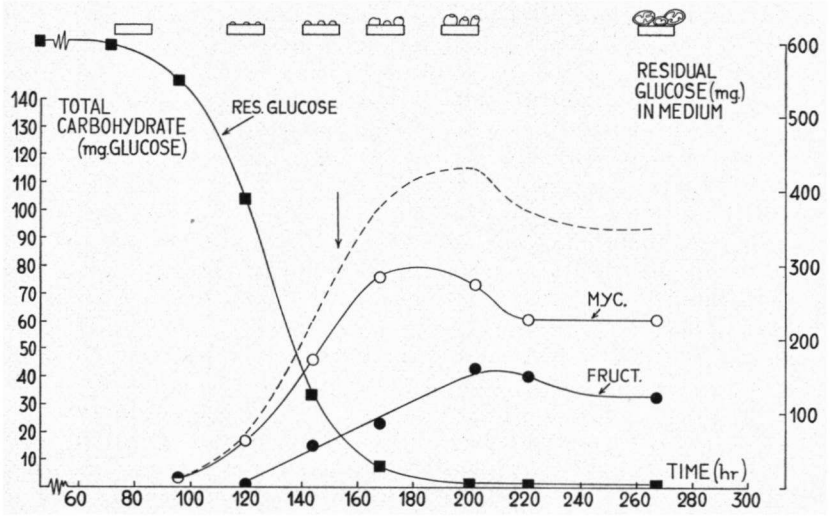


Fig. 6. Changes in total carbohydrate of fructifications and mycelium of *S. commune* K.35 accompanying development. The arrow indicates the moment of maximum nitrogen attainment of the whole organism. (See Fig. 4.)

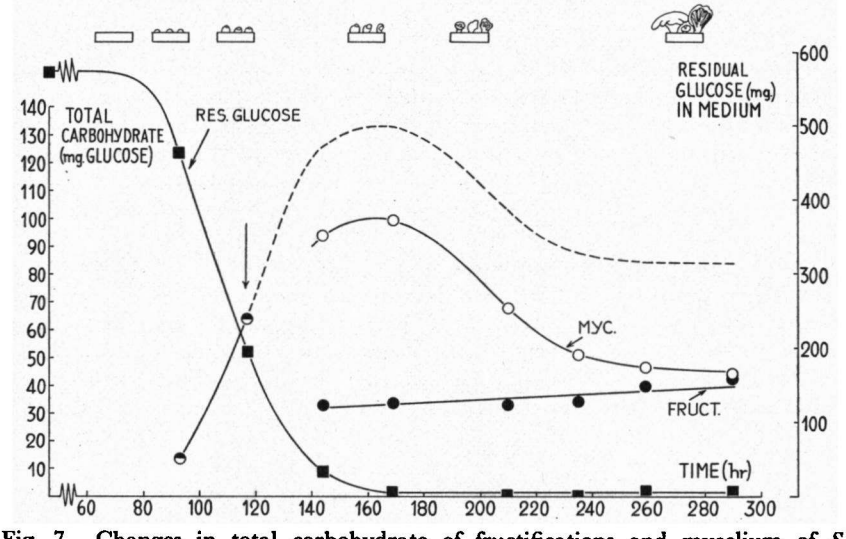


Fig. 7. Changes in total carbohydrate of fructifications and mycelium of *S. commune* K.8 accompanying development. The arrow indicates the moment of maximum nitrogen attainment of the whole organism. (See Fig. 4.)

made on the same cultures as those on which Figs. 4 and 5 are based; the results are therefore strictly comparable.

In the period ending at the moment when the nitrogen content of the whole culture reaches its maximum, only a relatively small fraction of the glucose disappearing from the medium is transformed into cellular carbohydrate, viz. 15 and 17 % for K.35 and K.8 re-

spectively. This phase of primordia production is characterized primarily by the synthesis of proteins and nucleic acids (see Chapter VIII). However, after the net synthesis of nitrogenous material stops, an active accumulation of cellular carbohydrates occurs, correlated with the rapid increase in size of the primordia. In fact, 52 and 35 % of the glucose consumed is assimilated as cellular carbohydrate by K.35 and K.8 respectively.

A striking feature of the accumulation of carbohydrate in the mycelium is that simultaneously there is a considerable loss of nitrogenous material. The most likely explanation that can be put forward here is that during primordial growth, the stroma (which was isolated as a part of the mycelium) also becomes further developed. While a large part of the nitrogenous material from the deeper layers of the mycelium is transported to the growing primordia, the cell walls of a large number of stromal cells thicken, thus contributing to the increment of carbohydrate in the mycelium.

After the carbon source in the medium has given out, clear differences can be noted for the two stocks. Considering first the curves representing the total carbohydrate content of the whole organism, it can be seen that in K.8 about 40 % of the stored carbohydrate disappears, whereas in K.35 this percentage is only 20. In the mycelia the differences are even more pronounced, namely 65 and 25 % respectively.

These facts point to an impairment of the breakdown of endogenous carbohydrate in K.35. This could mean either that the differences are only quantitative or it could imply that K.35 does not have the ability to attack certain types of carbohydrate which K.8 can break down. The shape of the curves shown in Fig. 6 suggests the latter possibility; after an initial rapid disappearance of carbohydrate, the amount remains constant.

Another remarkable phenomenon is the constancy of the total carbohydrate content of all K.8 fructifications together. As has been pointed out previously, the same holds for the total nitrogen content, although to a lesser extent. It is obvious that this again suggests a transport of material, in this case of carbohydrates, from the stunted fructifications to the developing ones.

2. *Replacement cultures*

Some of the features of the developing system and their relation to the composition of the surrounding medium can be studied more adequately by using the replacement culture technique, which enables control of growth and morphogenesis by manipulation of the environment. Another point which emerged during the application of this technique was the possibility of improving the synchronization of the developmental sequence.

This technique was based on the discovery that a thiamine-free medium can support some growth of the organism. This topic will be dealt with fully in Chapter VII; it is sufficient to note here that the mycelium produced in a thiamine-free medium remains com-

pletely vegetative and undifferentiated. The fungus grows submerged; no aerial mycelium or fruit-body primordia are formed. Since the fungus excretes large quantities of acid under these conditions, calcium carbonate is incorporated as a neutralizing agent and high-phosphate medium is used.

Fig. 8 shows the results of a typical replacement experiment with

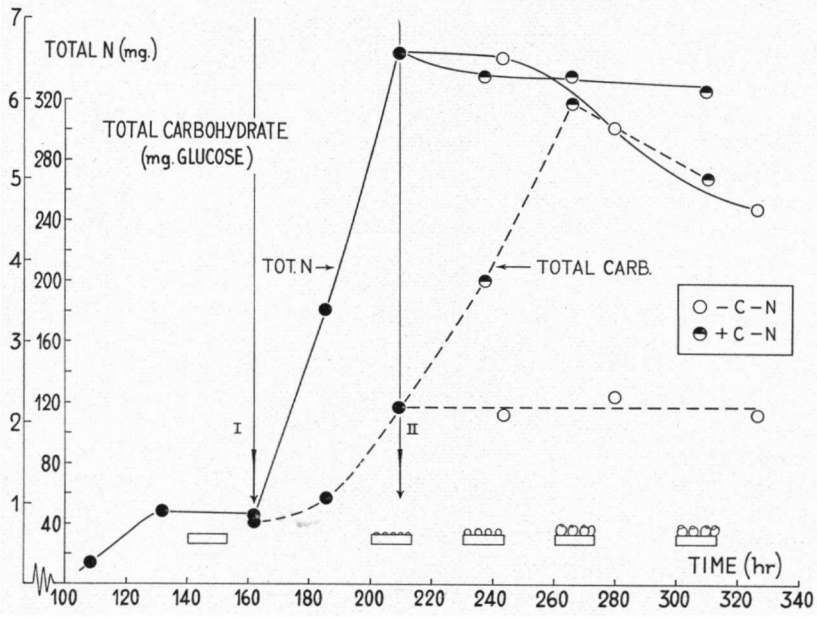


Fig. 8. Changes in total nitrogen and carbohydrate in a replacement experiment with *S. commune* K.35. High-phosphate basal medium was used throughout. Concentrations of glucose, asparagine, and thiamine if added: 2 %, 0.15 %, and 120 mμg/ml respectively. Added $CaCO_3$ remained in the culture flasks during replacement. The curve to the left of arrow I represents growth in thiamine-free medium. At arrow I the medium was replaced by a medium containing glucose, asparagine, and thiamine. At arrow II a new medium was given either with glucose and without asparagine or one lacking both glucose and asparagine. In both cases thiamine was present. Fructifications and mycelium were not separated. The schematic drawings indicate development with the glucose containing medium (+ C–N).

K.35. After 6-7 days growth on the medium lacking thiamine, about 1 mg nitrogen can be found in the mycelium. Removal of the medium and introduction of a medium containing thiamine, carbon, and nitrogen results in a rapid increment of total nitrogen and the formation of primordia. These primordia become visible to the naked eye in the course of the second day after replacement. Net synthesis of nitrogenous cell constituents (at least of proteins) seems to be necessary in this period to induce visible primordia, although the concentration of the nitrogen source can be lowered considerably (see Chapter VII).

Rapid growth of the primordia can be obtained if, two days after

the first replacement, the medium is exchanged again, but now for a medium lacking nitrogen but containing glucose. The subsequent development is highly synchronous; all the primordia grow at the same rate and they also attain the same size and shape. The resulting fruiting bodies are bigger, but even more inhibited in the expansion of the hymenium than in the stationary cultures supplemented with 2 % glucose.

The fructifications outnumber those formed in stationary cultures. This is brought about in the first place by the greater number of initials formed, due to the presence of calcium carbonate. Secondly, the extra glucose evidently permits the outgrowth of more initials.

The data in Fig. 8 show that there is practically no loss of nitrogen from the developing system, in spite of the fact that the medium is not supplemented with nitrogen. At the same time, there is a very conspicuous net synthesis of carbohydrates; the amount present at the moment of replacement increases by about 200 %.

Because this period is characterized by a reorganization of nitrogenous constituents — large quantities of nitrogenous compounds are transported to the growing primordia — these results seem to be at variance with those obtained with stationary cultures in one respect. In stationary cultures there is an actual loss of nitrogen to the environment in this phase but none in this experiment. In the stationary cultures, however, the energy necessary for the processes involved must be derived from a rather limited amount of residual glucose in the medium, whereas in the replacement cultures there is no such a limitation because of the additional glucose. This could offer a reasonable explanation of the discrepancy observed.

It was of interest to study the effect of a medium lacking both a nitrogen and a carbon source on the morphogenesis, total nitrogen content, and total carbohydrate content of the fungus. The results of the relevant experiment showed that there was no primordial growth and, surprisingly, no loss of carbohydrate whatever (Fig. 8). There was, however, a loss of nitrogen in this case. Thus, these findings indicate that growth of the primordia in this stock can only proceed if a carbon source is present in the medium. On the other hand, the results fit in with our assumption that the breakdown of a certain class of endogenous carbohydrates is somehow blocked. The fact that, after intense carbohydrate assimilation, a small amount of carbohydrate disappears, might be attributed to the formation of another small carbohydrate fraction which can be catabolized easily by K.35.

If at the moment indicated by arrow II in Fig. 8 the medium is not replaced, the nitrogen content reaches a value of 7.5–8 mg. The primordia do not grow out under these conditions; the further developmental features are virtually the same as in the case in which the medium was replaced by a carbon- and nitrogen-free medium. If at the moment indicated by arrow II carbon and nitrogen are included in the replacement medium, an asynchronous development results: in addition to the development of fruiting bodies, new primordia arise.

Discussion

The results presented in this chapter enable us to draw a provisional picture of the gross biochemical events underlying morphogenesis in *Schizophyllum commune*. On the basis of these results, the developmental events can be assigned to a number of distinct phases, although it should be noted that this division probably creates a somewhat simplified picture of reality: the data obtained may represent only certain aspects of the developing system.

The first phase represents undifferentiated growth. By this we mean a submerged growth with no aerial mycelium, the hyphae growing into the substratum, as already described. This phase, which is very short in the stocks examined, is followed by a second phase, in which the primordia are initiated.

The system in phase II must be considered as a very complex one in which growth of undifferentiated hyphae and differentiation into and growth of primordia go on simultaneously. As indicated under Results, it is possible to prevent the system from entering phase II by cultivating on a medium lacking thiamine. The problem of uncoupling growth and differentiation in phase II raises many more difficulties, and will be more fully discussed in Chapters VII and VIII.

An important point seems to be that the initiation of primordia takes place in a fully nutritive environment. This seems to contradict the generally stated principle that exhaustion of the substrate is conducive to the initiation of fruiting in fungi. This principle, originally formulated by Klebs (1900), without doubt applies to fructification in Myxomycetes (cf. Bonner, 1959). It has also been mentioned in connection with fruiting in Hymenomycetes, e.g. *Schizophyllum commune* and *Stereum purpureum* (Wakefield, 1909), *Collybia velutipes* (Plunkett, 1953), *C.sasii* (Bille-Hansen, 1953), and *Coprinus lagopus* (Madelin, 1956).

From the literature cited above we cannot judge the exact nutritive conditions at the time at which the primordia are initiated, and thus cannot evaluate the conclusions reached. However, in the stocks of *Schizophyllum* examined in this work, primordia arise very early under fully nutritive conditions. Factors such as exhaustion of the medium become important only at a much later stage, when the pileus is ready to expand (Chapter V).

The third phase to be recognized is the one of primordial growth. The only morphological differentiation that can be observed at the end of this phase is the formation of the small pit at the summit of the primordium. The processes in this stage can proceed without the presence of a nitrogen source in the medium, although the presence of such a source does not seem to be inhibitory. On the other hand, an external carbon source appears to be necessary in this period and a considerable portion of the sugar taken up is transformed into cellular carbohydrate in mycelium and primordia. At the same time, more than half the quantity of nitrogen contained in the mycelium is transported to the growing primordia. It is apparent that dry-weight

determination of primordia and mycelium would have been valueless for revealing the transport phenomenon involved because the weight of the nitrogenous compounds disappearing from the mycelium is approximately equaled by the weight of the carbohydrates synthetized.

Since BULLER's conception (1933) of an acropetal flow from the mycelium into the developing carpophore, several authors have demonstrated that this is true for water and other materials, as judged from an increase in dry weight of the developing fruiting bodies. BONNER *et al.* (1956) and MADELIN (1956) demonstrated this to hold for agarics in the phase of rapid enlargement and maturation; PLUNKETT (1958) demonstrated it for a polypore. In addition, MADELIN (1956) showed that the increase in the dry weight of the fruiting bodies is accompanied by a temporary loss in weight of the mycelium. MADELIN (1960) also obtained cytological evidence: he observed special swollen cells in the mycelium of *Coprinus lagopus*. These cells, containing much glycogen and protein (as detected by staining) were emptied when fruit-bodies were being produced.

Apart from showing that materials other than water can be transported in Hymenomycetes, the studies cited above are not wholly relevant to the phenomenon of nitrogen transport described for *Schizophyllum* in the stage of primordial growth. They are probably more closely connected with what we will henceforth call the fourth stage of development in *Schizophyllum*, viz. the expansion of the hymenium or formation of the pileus. That transport phenomena are also involved in this stage can be deduced from the fact that there is an increase in the carbohydrate and nitrogen content of a number of developing fruiting bodies, while the total amount of carbohydrate and nitrogen in all fructifications together remains nearly constant. Although the results by no means exclude transport from the mycelium to the developing fruiting bodies — there is actually a loss of carbohydrate and nitrogen from the mycelium — they strongly suggest a transport of material from the stunted fructifications to the developing ones. Such a suggestion is in line with the cytological observations described in Chapter I as far as the cytoplasm is concerned. When the pilei expand, the cell content of the hyphae in the stunted fructifications show less staining, indicating a loss of cytoplasmic material.

The concept of competition between fruiting bodies was advanced as early as 1930 by HEIN in connection with the abortion of supernumerary primordia in mushroom beds (LEVINE, 1922). MADELIN (1956), working with *Coprinus lagopus*, obtained experimental evidence for the operation of this mechanism. In consequence of such competition, slight differences in the stage of development of the primordia can subsequently become greatly accentuated when the somewhat more advanced fructifications withdraw material from others. This of course implies an asynchronous development in this phase. We have indeed observed this to be the case in *Schizophyllum* K.8, even in cultures in which the preceding phases were synchronized by means of the replacement culture technique.

As already pointed out, the accomplishment of the fourth phase is completely inhibited in stock K.35. Comparing the two stocks with respect to carbohydrate metabolism, evidence was obtained which suggested that coinciding with the failure of cap expansion of K.35, there is some block in the breakdown of certain cellular carbohydrates.

CHAPTER III

NATURE AND LOCALIZATION OF THE PRINCIPAL POLYSACCHARIDES

INTRODUCTION

In the preceding chapter arguments are given which suggest that the morphological difference between stock K.35 and K.8 of *Schizophyllum* might be related to an impairment of the breakdown of some intracellular carbohydrate in the former. Before this problem could be approached in more detail, it was necessary first to study the nature of the principal carbohydrates synthetized by this fungus.

Preliminary analyses of the carbohydrates of stock K.35 revealed that more than 90 % of the cellular carbohydrates were polysaccharides, as judged from their insolubility in aqueous alcohol. Approximately 60 % of this polysaccharide material was solubilized by dilute KOH (5 %) at room temperature. Further alkaline extraction at 100°C removed only a relatively small portion (about 10 %), leaving a highly resistant polysaccharides that accounted for about 30 % of the polysaccharide material.

Real progress in the effort to design a fractionation procedure was made when it was discovered that the major part of the cold alkali-soluble polysaccharides could be precipitated by acidifying the alkaline extract. This proved that, at most, only a small amount of the polysaccharides contained in the alkaline extract could be attributed to acid-soluble polysaccharides such as glycogen. These solubility characteristics also pointed to the cell walls as the possible localization of the major part of the insoluble polysaccharides, and thus prompted investigation of the carbohydrates of these cell walls freed of cytoplasmic material.

It should be stressed that at this stage our principal purpose is not to give a full account of the composition of the cell walls of *Schizophyllum*. This would require investigation of chemically-intact, clean cell-wall material, preferably isolated by mechanical breakage of the cells followed by exhaustive washing of the ruptured walls (KREGER, 1954). However, because it proved difficult to remove the cytoplasm in this way, especially from the thick-walled hyphae of the primordia, a lipid extraction and an enzymatic cleaning were included in the isolation of the cell walls. Although this introduced the risk of removing specific cell-wall components, we considered this to be of minor importance where only carbohydrates were examined.

Most studies on cell-wall composition and structure in fungi have been done with yeasts (recent summaries in articles by NICKERSON, 1963 and PHAFF, 1963). These studies reveal a very complex structure composed of lipids, polysaccharide-protein complexes, and macromolecules containing hexosamine. Our knowledge of the cell walls of filamentous fungi is very limited, but a few studies applying modern methods have demonstrated a similar complexity in cell-wall composition (KREGER, 1954; ARONSON and MACHLIS, 1959; BARTNICKI-GARCIA and NICKERSON, 1962). As far as we are aware, apart from some observations by KREGER (1954) on *Agaricus campestris* no such studies have been published dealing with Basidiomycetes.

Although this study was initiated primarily to gain information about the nature of the cellular carbohydrates, the reported differences in thickness and staining properties of the cell wall (see Chapter I) could be associated with differences in chemical composition. For this purpose, separate analyses were always carried out for mycelia and primordia. However, the results obtained by means of chromatography and X-ray analysis showed no qualitative differences; only quantitative variations in the amounts of compounds constituting the cell wall were observed. Therefore, only one example of chromatographic and X-ray data will be presented, but it should be kept in mind that virtually the same results are obtained whether the material originated from vegetative mycelium or primordia. However, an important point to be taken into consideration in evaluating these results is that the separation into mycelium and primordia does not constitute a complete separation of different cell types. As has been shown in Chapter I, thick-walled cells and cell walls exhibiting metachromasia when stained with thionin are most abundant in the primordia, but they are also found in the stroma which was included in the mycelial fraction.

The alkali-soluble, acid-insoluble cell-wall component will be shown to be a polyglucose and to have an X-ray diagram corresponding to that of a polysaccharide first isolated from the cell walls of the yeast *Schizosaccharomyces octosporus* (KREGER, 1954). This component will be denoted henceforth as Schizosaccharomyces-type glucan or abbreviated as S-glucan. The resistant, hot alkali-insoluble, non-chitin polysaccharide, which shows an X-ray diagram corresponding to yeast glucan, will be abbreviated as R-glucan.

METHODS

It proved very difficult to get rid of the fine quartz particles resulting from the homogenization of sand-grown cultures in the Mickle disintegrator. These quartz particles caused very strong lines in the X-ray diagrams, obscuring the results. Since normal development of *Schizophyllum* is only possible on a solid surface, the fungus was cultivated on agar. The drawback of this method is that the agar must be removed. This was accomplished by using hot dilute acetic acid. This procedure seems acceptable in view of the insolubility of the

cell-wall polysaccharides under study, but it is clear that it could also have removed soluble components from the cell wall.

All analyses were carried out on material isolated from stock K.35. The fungus was inoculated as a mycelial suspension on low-phosphate medium containing 4 % glucose, 0.15 % asparagine, and solidified with 1 % purified agar (Difco). Cultures were grown for 8 days at 25°C.

1. *Isolation of cell walls*

The fungal material with adhering agar was poured into a two-fold volume of boiling dilute acetic acid (0.5 N). After the boiling point was reached again, heating was continued for 1 minute. The liquid was removed by hot filtration through glass paper (Whatman GF/A) and the residue washed exhaustively first with hot and then with cold dilute acetic acid.

The cells were broken in 0.5 N acetic acid by means of the Servall Omni-mixer run at full speed for 10 minutes. The 400 ml beaker of this apparatus gave especially satisfactory results. The procedure was effective in breaking the cells, except for short ovoid thick-walled cells (chlamydospores?) present in small numbers in the vegetative mycelium. These cells were also refractory to enzymatic cleaning procedures, but in view of their small number no attempt was made to break these cells.

The enzymatic cleaning procedure was adopted from CUMMINS and HARRIS (1956), but a lipid extraction was also included (RALPH *et al.*, 1958).

The homogenate containing the broken cells was centrifuged and the residue washed as follows: twice with water, twice with alcohol, once with alcohol/petroleum ether (1/1), and once with petroleum ether (b.p. 40–60°C). After washing, the material was extracted with petroleum ether in a Soxhlett apparatus for 17 hours and then dried.

The crude cell-wall fraction was suspended in 0.05 M phosphate buffer (pH 7.6) containing trypsin (2 mg/ml) and digested for 4 hours at 37°C. The suspension was centrifuged and after washing the sediment with 0.02 N HCl it was resuspended in 0.02 N HCl containing pepsin (2 mg/ml), and digestion continued for 12 hours. Forty ml of enzyme solution was used for each gram of cell-wall material. Lastly, the material was washed several times with water, resuspended in a small amount of water, and freeze-dried.

2. *Isolation of S-glucan*

About 3 g of cell-wall material was suspended in 100 ml 5 % KOH. The suspension was then incubated at 25°C for 17 hours and centrifuged. The clear supernatant was saved and pooled with the supernatants of two washings of the precipitate with 5 % KOH (50 ml). The alkaline extract was adjusted to pH 4.5 with glacial acetic acid. The copious precipitate that formed was centrifuged off

and washed 2 times with 0.5 N acetic acid and 4 times with water. The residue was taken up in a small amount of water and the paste freeze-dried. The resulting light powder was used for chromatographic and X-ray analysis.

3. *Isolation of R-glucan (contaminated with chitin)*

The precipitate left after alkaline extraction at 25°C was extracted twice with 10 % KOH (1 g original cell-wall material/40 ml) at 100°C for 10 minutes. After centrifugation the residue was taken up in water, acidified with glacial acetic acid to pH 2.8, centrifuged, and resuspended in 0.5 N acetic acid. The suspension was held at 75°C for 30 minutes and then centrifuged, after which the precipitate was washed 4 times with water. Lastly, the precipitate was suspended in water and freeze-dried. Microscopic examination revealed that this preparation consists of cell walls which apparently retain the original shape of the hyphae.

The extraction procedure given above for R-glucan is essentially the same as that used by BELL and NORTHCOTE (1950) and TREVELYAN and HARRISON (1951) for the extraction of yeast glucan from yeasts.

4. *Partial and complete hydrolysis of fractions*

To obtain information about the mode of linkage of monosaccharide units in the alkali-soluble and insoluble polysaccharides, we also tried to effect partial hydrolysis of the polysaccharides, followed by identification of the oligosaccharides formed. A difficulty which arose was the insolubility of the polysaccharides in dilute mineral acids. To effect solution the polysaccharides had to be dissolved first in concentrated mineral acid or concentrated formic acid; dissolution was especially rapid in the latter reagent.

To hydrolyze the samples completely (accepting the risk of considerable destruction of labile components), 50 mg portions were solubilized in 2.5 ml conc. HCl (1.18) and 2.5 ml water added. The tubes were sealed and placed in boiling water for 6 hours. The hydrolysates were filtered through glass paper and the filtrates dried over sodium hydroxyde pellets *in vacuo*. The residues were taken up in water (1 ml) and the drying procedure repeated. Lastly, the residue was taken up in water (0.5 ml).

The samples were hydrolyzed in formic acid by dissolving 100 mg in 5 ml 90 % formic acid and heating in boiling water in sealed tubes for 1 or 12 hours. The contents were then evaporated *in vacuo* over sodium hydroxide, and the residue taken up in 5 ml N HCl. Heating was then continued in sealed tubes for another hour to hydrolyze formyl esters. The hydrolysate was filtered through glass paper, evaporated twice over sodium hydroxide pellets *in vacuo,* and finally dissolved in water (1 ml).

Partial hydrolysis in dilute sulphuric acid was achieved by dissolving the samples (50 mg) in 0.5 ml 72 % (v/v) H_2SO_4. This was

accomplished by stirring the mixtures in an ice-bath for 15 minutes. The contents were then rapidly diluted with 25 ml water, resulting in a normality of 0.53 with respect to acid, and either neutralized directly or after heating at 100°C for 10 of 60 minutes. The acid was neutralized with solid $BaCO_3$ and the insoluble salts removed by centrifugation and filtration. The filtrate was evaporated *in vacuo* and the residue taken up in water (0.5 ml).

Isolated cell walls were also hydrolyzed in a two-step procedure: 500 mg cell-wall material was suspended in 50 ml 0.5 N H_2SO_4 and heated in boiling water for 90 minutes. The insoluble, jelly-like fraction was centrifuged off and washed twice with water. This washing procedure greatly reduced the volume of the precipitate. This precipitate was then washed with alcohol, dried (40 % of original cell-wall material by dry weight), and hydrolyzed with 6 N HCl as described above.

After removal of the insoluble fraction, the combined 0.5 N H_2SO_4 hydrolysate and washing fluids were neutralized with solid $BaCO_3$. The insoluble salts were removed and the clear filtrate heated and adjusted to pH 6.0 with dilute H_2SO_4. The precipitate of $BaSO_4$ was filtered off and the filtrate evaporated to dryness. Part of the residue was taken up in water and chromatographed directly, another part was further hydrolyzed with 6 N HCl.

5. *Paper chromatography*

Whatman paper no. 1 was used throughout. Several solvents were tried; the following systems proved very useful:

Ethyl acetate:acetic acid:water, (9:2:2 v/v, cf. DURSO and MUELLER, 1956). The samples were subjected to continuous descending chromatography for 4–5 days. Excellent separation of oligosaccharides was obtained; glucosamine runs as a distinct spot.

n-Butanol:ethanol:water, (5:1:4 v/v, solvent of HIRST and JONES, 1949). Multiple development by the ascending technique (4 times for 5–6 hours) at 55°C was employed. giving excellent separation of oligosaccharides but glucosamine runs as a streak.

Pyridine:ethyl acetate:acetic acid:water, 5:5:1:3 v/v, FISCHER and DÖRFEL, 1955). Saturation of the tank with the pyridine-ethyl acetate-water mixture was omitted. Rapid descending development (10 hours). Glucosamine runs as a spot.

n-Butanol:ethanol:water, (52:32:16 v/v, PUTMAN, 1957), descending for 18 hours.

Detecting reagents: Reducing substances were detected by a silver nitrate dip as described by STANGE (1959). Another general sugar reagent, which allows differentiation between hexoses and pentoses, was aniline hydrogen phthalate in 95 % acetone (ROBINSON and RATHBUN, 1958). Amino-sugars were detected by the sugar reagents listed above, by ninhydrin (0.2 % ninhydrin in 95 % acetone), and the acetylacetone-dimethylamino-benzaldehyde reagent (PARTRIDGE, 1948). Other reagents employed for the detection of individual com-

pounds were: the ferric chloride-hydroxylamine spray for lactones (ABDEL-AKHER and SMITH, 1951) and the basic lead acetate dip of GEE and MCCREADY (1957) for galacturonic acid.

6. *X-ray diffraction*

X-ray diagrams were made at the Laboratory of Technical Physics, Technological University, Delft, through the kind agency of Dr. D. R. Kreger.

When samples were extracted further, the procedures will be described under Results. In either case, after repeated washing with water the samples were air-dried and subsequently X-rayed. The photographs were made with Cu Kα radiation filtered by a 10μ Ni filter. The specimen-to-film distance was 40 mm and the samples were mounted in 0.5 mm tubes. The diagrams are reproduced in their original size.

RESULTS

1 *Chromatographic analyses*

a. R-glucan (Fig. 9, A and B)

Hydrolysis of the hot alkali-insoluble polysaccharide with 6 N HCl shows the presence of glucose, glucosamine, and some additional low R_f components. These low R_f materials are presumed to be oligosaccharides with glucosamine since they are ninhydrin-positive. This demonstrates the difficulty of obtaining completely hydrolyzed samples of this material.

Hydrolysis in formic acid followed by mineral acid reveals glucose, glucosamine, and a number of oligosaccharides. One of the latter substances has an R_f identical to that of gentiobiose. In a model experiment it was established that gentiobiose is only partially hydrolyzed under these conditions of hydrolysis. The spot intermediate between glucosamine and gentiobiose was not identified but its running distance differs from those of cellobiose, laminaribiose, and maltose. Additional spots with low R_f values and at the origin exhibit strong ninhydrin-positivity and are considered to contain glucosamine.

Chromatography after dissolution in strong H_2SO_4 (72 %) and short hydrolysis (10–60 min.) in dilute H_2SO_4 reveals a somewhat clearer picture because only very small amounts of glucosamine are liberated under these conditions. Moreover, no ninhydrin-positive material is found at the origin. Apart from some spots with very low R_f values (not further identified), two spots were found with an R_f identical to those of gentiobiose and laminaribiose respectively, in the several solvents tried. (As a reference for laminaribiose we used a hydrolysate of laminarine prepared with enzyme from *Rhizopus arrhizus*: this preparation was a gift from Prof. Dr. B. D. J. Meeuse, Seattle, Wash. U.S.A.)

These chromatographic data indicate that the hot alkali-insoluble polysaccharide fraction of *Schizophyllum* cell walls is a glucose-glucosamine polymer. That it is a heteropolymer of glucose and glucosamine seems unlikely in view of the X-ray data (section 2). On the other hand, it may be a copolymer, comprising a polyglucose (R-glucan) and a poly-(acetyl)-glucosamine (chitin) linked together in some unknown way (section 4).

The chromatographic pattern obtained with partial hydrolysates indicates the presence of β-(1→6) linkages (as in gentiobiose) and β-(1→3) linkages (as in laminaribiose) in the R-glucan, although our data provide no information about the quantitative distribution of these bonds.

The presence of these linkages, together with the solubility characteristics, suggests the R-glucan to be nearly identical to yeast glucan, which has been shown to be a polyglucose with 10 to 20 % β-(1→6) linkages, the remainder being of the β-(1→3) type (PEAT *et al.*, 1958a, b).

b. S-glucan (Fig. 9C)

This polysaccharide, which can be precipitated from alkaline solution simply by neutralization, yields only glucose after hydrolysis in 6 N HCl. (The reducing substance with a high R_f value which is also found in 6 N HCl hydrolysates of R-glucan was not identified, but possibly arose as an artifact due to the extreme conditions of hydrolysis.)

However, chromatography of formic acid and dilute H_2SO_4 hydrolysates indicate that xylose is also present. Model experiments with xylose showed that this sugar is completely destroyed during heating with 6 N HCl.

In the several solvents, a spot with an R_f identical to that of gentiobiose can be found when samples are partially hydrolyzed; no maltose, cellobiose, or laminaribiose could be detected, although large quantities of hydrolysate (up to 50 μl) were applied.

These data are insufficient to elucidate the mode of linkage of the glucose residue in the S-glucan unequivocally, but they suggest a predominance of β-(1→6) linkages .

c. Complete cell walls

Heating the cell walls in 0.5 N H_2SO_4 for 90 minutes solubilizes about 60 % of the original cell wall. Chromatography of this solubilized material reveals the presence of glucose, xylose, gentiobiose, and higher oligosaccharides. Further hydrolysis of this material in 6 N HCl results in glucose and a small amount of glucosamine.

The presence of glucosamine in this hydrolysate is of interest. It apparently derives from a small amount of chitin extracted with the hot dilute H_2SO_4, since chitosan, if present, would not appear in the hydrolysate by virtue of the insolubility of chitosan sulphate in neutral solutions. Quantitative determination of glucosamine revealed that the glucosamine content of the cell walls was not significantly

altered if a cold HCl (2 %) treatment (to solubilize chitosan) was included. The X-ray diagrams shown in the following section also give no indication of the presence of chitosan.

Chromatography of a 6 N HCl hydrolysate of the residue left after dilute H_2SO_4 extraction (40 % by dry weight), shows the presence of only glucose and glucosamine.

2. *X-ray analysis*

The solubility characteristics of the S-glucan suggested that this polysaccharide could be identical with an unknown polysaccharide isolated by KREGER (1954) from the cell walls of *Schizosaccharomyces* and some other fungi. This polysaccharide exhibited a typical X-ray diffraction diagram, quite different from that of yeast glucan.

Indeed, the X-ray diagram of the S-glucan of *Schizophyllum* shows a set of rather sharp interferences (Fig. 10c), coinciding with the lines of the *Schizosaccharomyces* polysaccharide. Thus, little doubt remains as to the identity of the two polysaccharides.

The interferences of the precipitated S-glucan are also present in the X-ray diagram of the untreated cell walls (Fig. 10a). The innermost reflection in this diagram will be shown to be due to the presence of yeast glucan.

Heating the cell walls at 100°C for 90 minutes with 0.5 N H_2SO_4 causes almost complete fragmentation of the walls; only a number of thick-walled cells in the primordial preparation retain a recognizable form. The weight of the residue after this treatment is about 40 % of that of the original walls. The X-ray diagram of this residue is essentially the same as that of the untreated walls, but the lines are sharper (Fig. 10b). Thus, no evidence was found for selective hydrolysis of one of the cell-wall polysaccharides, in contrast to the results of KREGER (1954), who found that boiling the walls of *Schizosaccharomyces* in 2 % HCl caused the lines due to the alkali-soluble component to disappear, giving place to hydro-glucan interferences.

The alkali-insoluble residue, which is composed of the so-called R-glucan and the amino-sugar polymer, does not show distinct lines (Fig. 10d). It is interesting to note that this residue is composed of seemingly undisturbed cell walls, retaining their original shape and thickness.

The insolubility of the R-glucan and the chromatographic data seemed to suggest that it was identical with yeast glucan. To obtain further evidence from X-ray diagrams, the samples were treated as indicated by HOUWINK and KREGER (1953). These authors showed that a sharp X-ray diagram of yeast glucan can be obtained after boiling the walls of yeasts with 2 % HCl. This treatment, which dissolved about 50 % of the glucan, made the remaining glucan alkali soluble (hydro-glucan).

Fig. 11b shows the X-ray diagram of cell walls of *Schizophyllum* treated first with alkali to dissolve the S-glucan and then boiled with 2 % HCl for 1 hour. The pattern of interferences obtained is most suggestive for the presence of both yeast hydro-glucan and chitin.

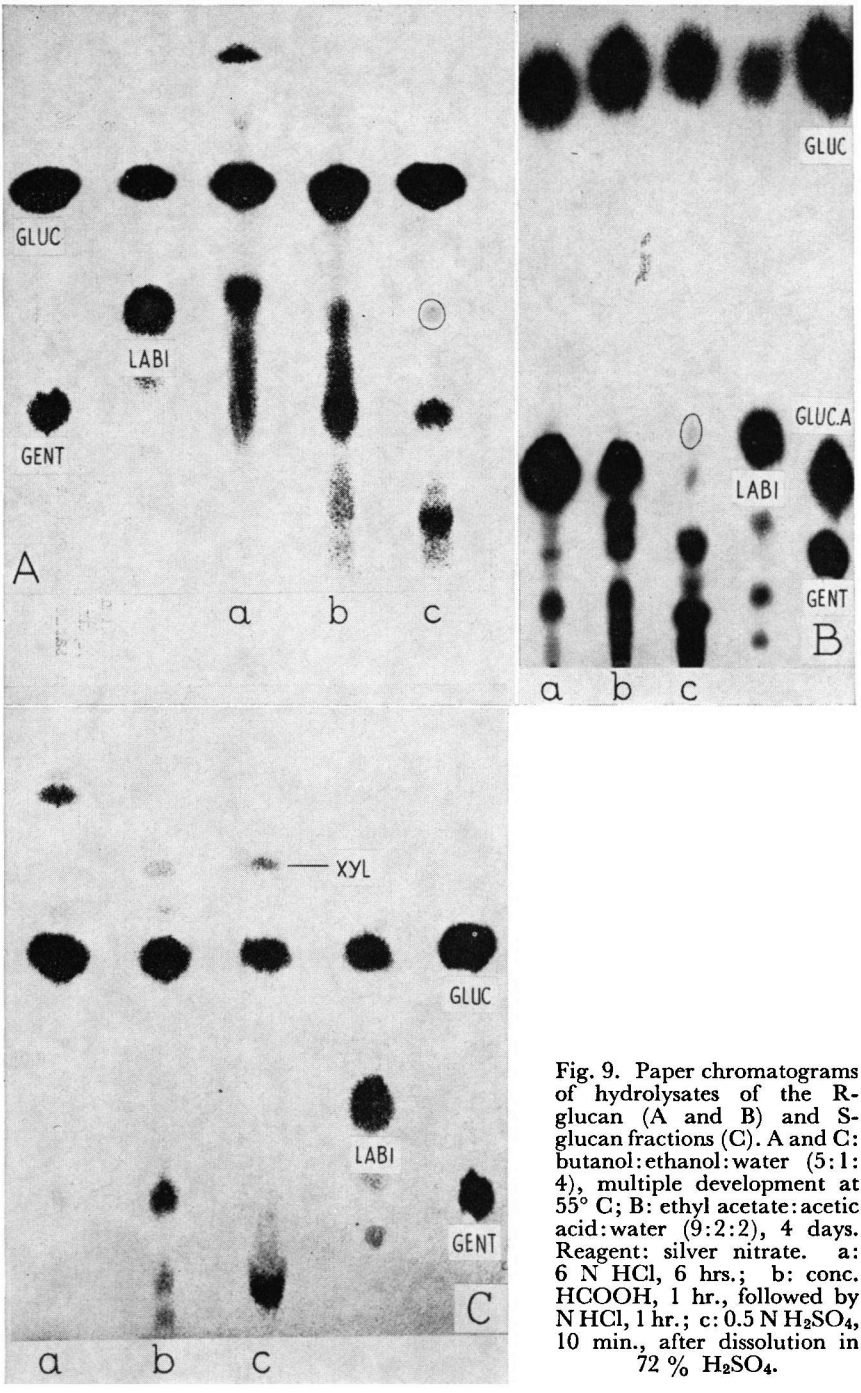
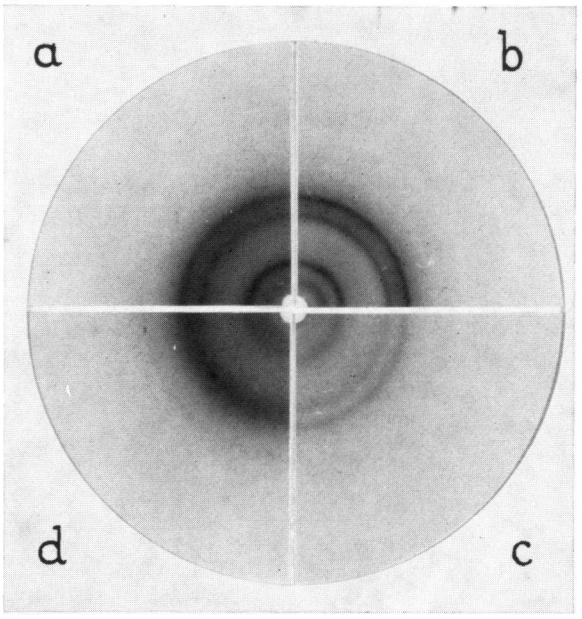
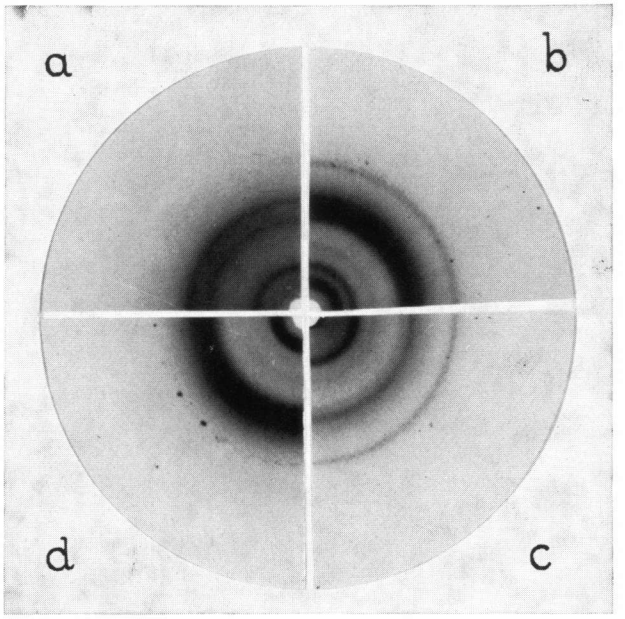


Fig. 9. Paper chromatograms of hydrolysates of the R-glucan (A and B) and S-glucan fractions (C). A and C: butanol:ethanol:water (5:1:4), multiple development at 55° C; B: ethyl acetate:acetic acid:water (9:2:2), 4 days. Reagent: silver nitrate. a: 6 N HCl, 6 hrs.; b: conc. HCOOH, 1 hr., followed by N HCl, 1 hr.; c: 0.5 N H_2SO_4, 10 min., after dissolution in 72 % H_2SO_4.





Figs. 10 and 11. Quadrants of X-ray powder diagrams of cell walls of *S. commune* K.35 and extracted products.

Fig. 10. a: Untreated cell walls; b: after 90 min. treatment with 0.5 N H_2SO_4 at 100° C; c: precipitate in acidified 5 % KOH extracts (S-glucan); d: residue left after extraction with 5 % KOH followed by a treatment with hot 10 % KOH.

Fig. 11. Untreated cell walls; b: after extraction of S-glucan with 5 % KOH for 17 hrs at 25° C, followed by boiling with 2 % HCl for 1 hr.; c: the same as b but hydroglucan dissolved with 3 % NaOH (chitin); d: the same as b but chitin dissolved with cold 30 % HCl (hydroglucan).

To confirm the presence of both substances, the samples were subsequently extracted with 3 % NaOH at 60°C for 30 minutes to dissolve the hydro-glucan, or with 30 % HCl at 4°C for 18 hours to dissolve the chitin. As a result, a pure chitin (Fig. 11c) and hydro-glucan diagram (Fig. 11d) is obtained.

3. *Quantitative composition of the cell walls of S. commune K.35*

In Chapter I we have given a short description of the striking difference in cell-wall thickness of undifferentiated mycelium cells and cells in the primordia and stroma. We also drew attention to the metachromasia of the outer layer of the thick-walled cells of primordia and stroma. Although this staining characteristic was retained by the enzymatically-cleaned cell walls it proved impossible to detect any difference in cell-wall composition that could account for this different staining behaviour.

Table 1 shows that walls from both sources consist essentially of

TABLE 1

Composition of enzymatically-cleaned cell walls of *S. commune* K.35. The organism was grown for 8 days on synthetic medium with 4 % glucose.

	Primordia	Mycelium *
	% dry weight	
glucose ("anhydroglucose")	86.8	81.4
xylose	**	**
chitin ("anhydro-N-acetylglucosamine")	3.1	5.0
non-chitin nitrogen as protein (N × 6.25)	2.6	2.3
ash	0.4	0.5
sum	92.9	89.2

* Stroma and undifferentiated mycelium together.
** Not determined.

polyglucoses, although the glucose content of the primordial cell-wall preparation is somewhat higher. However, the chitin content of these cells is lower. (For the colorimetric determination of glucose and glucosamine see Chapter IV.)

The amount of non-chitin nitrogen is small (about 0.4 %), but in view of the postulated protein-polysaccharide complexes in yeast (NICKERSON, 1963) it must be noted that the enzymatic cleaning procedure could have removed typical cell-wall protein. In fact, amino acids not further identified were found in the 6 N HCl hydrolysates. However, the important point is that no differences in the chromatographic pattern of amino acids were found in the hydrolysates of primordial and mycelial cell walls.

Another point in considering the chitin and protein values is that during preparation of the samples for glucosamine analysis, con-

siderable destruction of glucosamine might have taken place (up to 25 %, cf. TRACEY, 1955). Thus, the chitin values are probably too low and the protein values correspondingly high.

Extraction of the cell walls with 5 % KOH for 17 hours at 25°C removes 53.3 and 52.0 % of the polyglucoses from primordial and mycelial cell walls respectively. In the case of primordial cell walls, 95.5 % of the polyglucoses solubilized by alkali can be precipitated by acidifying the solution (S-glucan), whereas this figure is only 80 % for the extract of the mycelial walls. This could either point to the presence of a different alkali-soluble polysaccharide in the mycelial walls that is not precipitable under acidic conditions, or to a lower degree of polymerization of the S-glucan in the mycelial walls. Although it is known that glycosidic linkages are stable to aqueous alkali at 25°C under nitrogen (WHISTLER and BEMILLER, 1958), the prolonged treatment in normal atmosphere could have caused some fragmentation of the S-glucan, resulting in a small fraction not precipitable by acid.

A further extraction of the alkali-treated residue with hot 10 % KOH (twice for 10 minutes at 100°C) removes only about 14 % of the polyglucoses originally present, leaving a highly insoluble residue with a glucose content (R-glucan) that accounts for approximately 33 % of the glucose originally present in the walls. This residue also contains virtually all the glucosamine (chitin) present in the cell walls.

4. *Correlations between the quantities of the various cell-wall polysaccharides*

The results given above indicate that in addition to S-glucan, the cell walls contain chitin and a different polyglucose (R-glucan), at least part of which is identical to yeast glucan. If we assume that no glucosamine was removed from the cell walls during the cleaning procedure, then obviously all the glucosamine of the cell wall is present as chitin. This can be deduced from the fact that the same glucosamine content is found whether or not the cell walls are subjected to alkali and acid extraction. This seems at variance with the results of KORN and NORTHCOTE (1960) obtained in yeast. These authors concluded that only about 10 % of the total glucosamine in the cell wall could be present in the form of chitin. They conjectured that glucosamine might serve as a structural element in the protein-polysaccharide complexes isolated from these organisms. As yet, we have found no evidence that such a situation also applies to *Schizophyllum* cell walls, although it must be admitted that this could be due to the rather drastic cleaning procedure.

However, in this context it seems sufficiently noteworthy to report here that in *Schizophyllum* a rather good correlation can be found between the amount of R-glucan and chitin in the insoluble cell-wall residue. Fig. 12 gives data derived from experiments which will be discussed in detail in Chapter IV. It is sufficient to remark here that

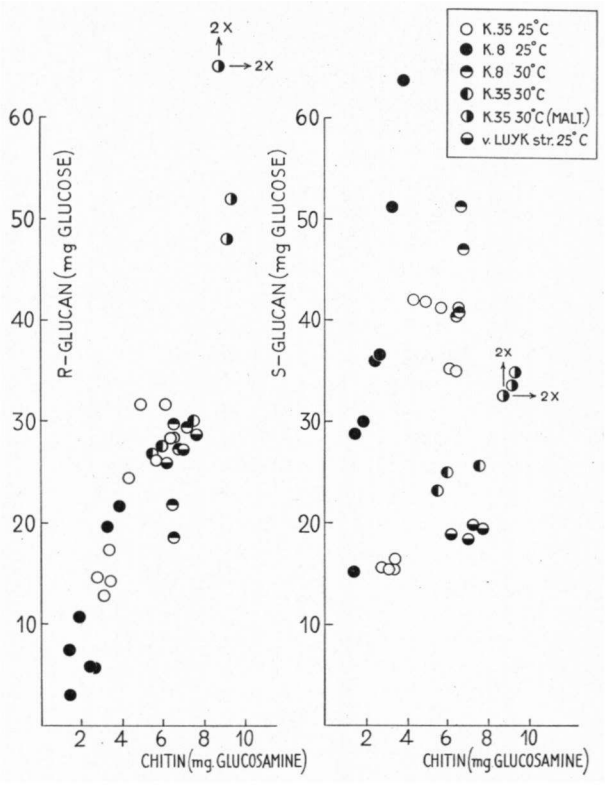


Fig. 12. Correlations between the various cell-wall polysaccharides of different stocks of *S. commune* grown under a variety of conditions.

the points represent data obtained with very variable material. Not only were different stocks used, but the cultivation temperature and age of the culture also varied. Moreover, where fructifications were produced, mycelia and fructifications were analyzed separately. Fig. 12 also shows that no such correlation exists between chitin and S-glucan.

Thus, the X-ray data point to the occurrence of two distinct polysaccharides in the insoluble residue, but the data shown in Fig. 12 suggest that the two polysaccharides are always present in approximately the same proportions. That these two polymers are linked together in a type of copolymer seems to be too far-reaching a conclusion to draw at the moment. However, the fact that the R-glucan and chitin become soluble in alkali and strong mineral acid, respectively, only after the walls have been boiled in dilute acid, seems to lend some support to this idea. The treatment with dilute acid could have cleaved bonds linking the two polysaccharides together, thus rendering the constituent polysaccharides more easily soluble.

Discussion

The results have shown that by far the greatest part of the cellular carbohydrates of *Schizophyllum commune* are deposited in the cell wall. It was found that the main structural components of the cell wall consist of two different polysaccharides, called R- and S-glucan. In addition, a small amount of chitin is present, possibly linked to the R-glucan in some unknown way.

The R-glucan has been shown to be nearly identical to yeast glucan because of its solubility characteristics, linkage of the glucose residues, and X-ray diagram.

The S-glucan has been shown to have an X-ray diagram identical to that of a polysaccharide found by Kreger (1954) in the cell walls of *Schizosaccharomyces octosporus, Pennicillium notatum,* and in small amounts in the cell walls of *Endomyces decipiens* and the stem of the fruit body of *Agaricus campestris.*

It is remarkable that this alkali-soluble, acid-precipitable polysaccharide has not received more attention since its discovery by Kreger. He found that in *Schizosaccharomyces* and *Penicillium* this polysaccharide accounts for about 30 % of the initial cell-wall material. The figures given in the present work for *Schizophyllum* are somewhat higher, viz. 33 and 43 % for the walls of mycelium and primordia respectively. However, in the following chapter it will be shown that these percentages can be much higher and depend on stock, cell type, and stage of development.

As far as we know, no studies are available concerning the composition and structure of a polysaccharide having properties similar to those of S-glucan. Our chromatographic data reveal that it is a polyglucose and indicate the presence of β-(1→6) linkages. No indication of β-(1→3) linkages, as detected in the R-glucan, was found, although the presence of linkages other than β-(1→6) cannot at present be excluded.

Practically all the chemical structural analyses reported in the literature are based on alkali-insoluble glucan. The few cases in which an alkali-soluble cell-wall glucan was investigated provide no evidence that the material was the same as the S-glucan studied here. Bishop *et al.* (1960) studied an alkali-soluble glucan of *Candida albicans* and showed a preponderance of β-(1→6) linkages on the basis of methylation analysis. However, this glucan was soluble in water after precipitation from alkaline solution with ethanol. On the basis of an analysis of a partial hydrolysate of pachyman, the alkali-soluble polysaccharide of *Poria cocos,* Warsi and Whelan (1957) concluded that this polysaccharide contains only β-(1→3) linkages. Duff (1952) studied a crude polysaccharide obtained from the fruiting bodies of *Polyporus betulinus,* part of which was alkali soluble, and suggested that this crude material "consists, in part at least, of a glucan with 1→3 linkages, of which a proportion may be of the α-type".

The origin of xylose in the S-glucan fraction is not clear. It could

be a constituent of the S-glucan proper, but could also originate from a co-precipitated xylan. It is interesting to note that CROOK and JOHNSTON (1962), in a study on the monosaccharides in cell-wall hydrolysates of selected fungi from different taxonomic groups, found xylose only in the cell walls of a Basidiomycete *(Polystictus sanguineus)*.

As to possible qualitative differences in composition which we had expected to find in mycelial and primordial cell walls, the results are inconclusive. New studies concerning the chemical basis of the different staining properties of the walls of cells in primordia and stroma and those of the submerged undifferentiated mycelium are in progress.

CHAPTER IV

THE QUANTITATIVE DISTRIBUTION OF CELLULAR CARBOHYDRATES AND THEIR FATE DURING DEVELOPMENT

INTRODUCTION

The information concerning the nature of the major part of the cellular carbohydrates of *Schizophyllum commune* reported in the preceding chapter enabled us to construct a fractionation scheme based upon the solubility characteristics of the different carbohydrates.

By measuring the amount of carbohydrate in the individual fractions we hoped to check the suggestion put forward in Chapter II, that in *S. commune* K.35 the blocking of the breakdown of certain intracellular carbohydrates is related to the morphological deviation of the mutant.

METHODS

Besides those already described in Chapter II, the following methods were applied.

1. *Fractionation of cellular carbohydrates and their estimation*

Because of the solubility characteristics of S-glucan and R-glucan, it was considered unnecessary to include the enzymatic cleaning of the cell walls in the routine procedure.

The harvest of sand cultures was separated from sand and culture fluid. The fungal material was disintegrated in ethanol in the Mickle disintegrator together with some adhering sand and glass beads. The homogenate was then made up to 25 ml with 80 % ethanol, the final concentration of the alcohol ranging between 70 and 80 % (see Chapter II). The fine quartz particles in the homogenate were found to be very beneficial because they facilitated the sedimentation of the polysaccharides during centrifugation. A flow sheet of the fractionation procedure is shown in Fig. 13.

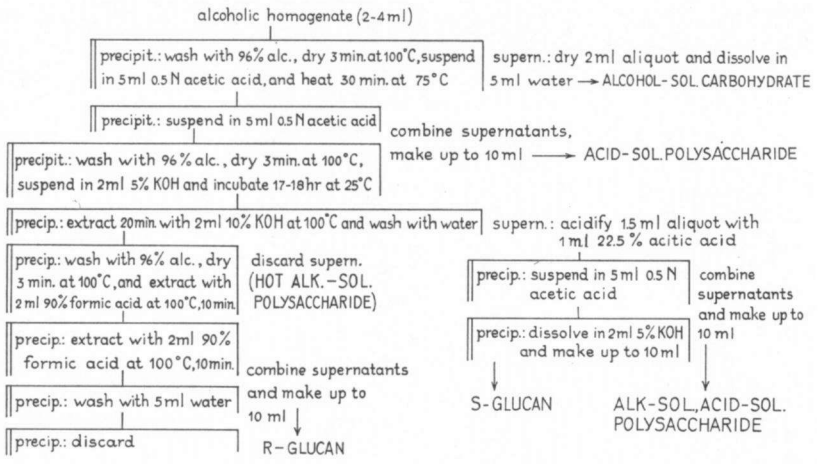


Fig. 13. Flow sheet for the quantitative fractionation of carbohydrates of *S. commune*.

Since chromatographic analyses showed that all carbohydrates in the different fractions were essentially polyglucoses, except for the occurrence of xylose in the S-glucan fraction and hexosamine in the R-glucan fraction, they were estimated with the anthrone reagent (FAIRBAIRN, 1953) using glucose as a standard. It will be recalled that xylose and glucosamine do not react with the anthrone reagent

When the anthrone method was applied to the R-glucan fraction, the formic acid decomposed into carbon dioxide and hydrogen. We got rid of gas bubbles which appeared during colorimetry by shaking the tubes on a Vortex mixer in advance and allowing the gas to escape. Total carbohydrate was estimated by first evaporating the alcohol and then dissolving the polysaccharides in ice-cold 72 % H_2SO_4 (see Chapter II). All values are presented as mg glucose equivalents.

2. *Estimation of chitin*

Purification of the samples and hydrolysis of the chitin was performed according to the procedure used by BLUMENTHAL and ROSEMAN (1957) with a few small modifications.

The alcoholic homogenate (5 ml) was centrifuged and the precipitate dried by immersion of the tubes in a boiling water-bath for 3 minutes. The dried samples were treated with 10 ml 10 % NaOH at 100°C for 30 minutes, centrifuged, and the precipitate washed twice with water. The precipitate was then suspended in 10 ml 2 % HCl and after standing at room temperature for 1 hour, the insoluble material was centrifuged off, washed with water, and treated again with hot 10 % NaOH for 30 minutes. After centrifugation, the precipitate was washed once with water and twice with 96 % alcohol. Drying of the samples in a water-bath for 3 minutes at 100°C was followed by the addition of exactly 5 ml 6 N HCl. The centrifuge

tubes were sealed and heated at 100°C for 6 hours. After cooling, the tubes were opened and centrifuged to remove the insoluble matter. A 4 ml aliquot was taken from the supernatant and dried *in vacuo* over sodium hydroxide pellets. The residue was dissolved in 5 ml water and the samples analyzed for glucosamine according to ROSEMAN and DAFFNER (1956), a procedure which is claimed to be far more specific than the classical ELSON and MORGAN method (1933). In the former, the glucosamine is first N-acetylated with aqueous acetic anhydride and then assayed with Ehrlich's reagent. In our study the chromogen was measured exactly 45 minutes after addition of Ehrlich's reagent at 580 mμ. The absorption spectrum obtained is identical to the one obtained with synthetic glucosamine . HCl. The colour is proportional to concentration up to 350 μg glucosamine.

3. *Estimation of protein*

To 1 ml of the alcoholic homogenate was added 5 ml 6 % trichloroacetic acid (TCA). After standing for 30 minutes at room temperature, the mixture was centrifuged and the precipitate washed with 5 ml 5 % TCA. The protein was dissolved in 5 ml N NaOH by heating at 100°C for 10 minutes and then estimated by the Lowry method (LOWRY *et al.*, 1951), using bovine albumin (Sigma, fraction V) as a standard. The standard was heated with alkali for the same time as the samples. Extinction was measured at 500 or 760 mμ, depending on the concentration of the protein.

In agreement with observations of PANDE *et al.* (1961) on mycelium of *Aspergillus,* the protein values obtained with the Lowry method correlated very well with total nitrogen in our material.

RESULTS

1. *The fate of various carbohydrates in K.35 during carbon starvation in stationary culture*

To check the hypothesis put forward in Chapter II, that certain carbohydrates which can be broken down by K.8 are not subjected to degradation in K.35, we decided to measure the change in carbohydrate content of the several fractions after exhaustion of the carbon supply in the medium. Cultivation was done under exactly the same conditions as mentioned in connection with Fig. 6.

The first analysis was made on material derived from a 7 day old culture. At this time the glucose supply in the medium has given out, the fungus has reached its maximum carbohydrate content, and the fruit-body primordium has reached its maximum size. The morphological appearance is a globose body with a cavity at the top.

In the period which follows, the fungus can only draw upon endogenous compounds. In K.35 this period is characterized by the formation of the typical cup-shaped fruiting bodies, inhibited in the further expansion of the hymenium. After the 12th day, no further morphological changes can be observed in the fruiting bodies; the

second analysis was therefore made on 12 day old material. In this experiment no attempt was made to analyze fructifications and mycelium separately.

The results are shown in Table 2. Total carbohydrate decreases

TABLE 2

Quantitative distribution of carbohydrates in *S. commune* K.35 at two stages of development, under conditions of carbon (and nitrogen) starvation. The italicized values represent percentages of total carbohydrate. Quantities are given as mg per culture (30 ml).

K.35, 25° C	7 days		12 days	
	I	II	I	II
alcohol-sol. carboh.	9.2 *7.8*	9.4 *8.9*	2.8 *2.9*	2.7 *3.1*
acid-sol. polysacch.	13.6 *11.6*	11.1 *10.5*	6.3 *6.4*	6.2 *7.1*
alkali-sol. acid-sol. polysaccharide	12.5 *10.6*	12.4 *11.7*	7.2 *7.4*	6.3 *7.2*
S-glucan	35.1 *29.9*	35.0 *33.1*	41.2 *42.1*	41.2 *46.9*
R-glucan	31.9 *27.2*	28.4 *26.8*	28.4 *29.0*	26.2 *29.8*
not accounted for (incl. hot alk.-sol.)	15.1 *12.9*	9.6 *9.1*	11.9 *12.2*	5.2 *5.9*
total carbohydrate	117.4 *100.0*	105.9 *100.0*	97.8 *100.0*	87.8 *100.0*
S/R ratio	1.10	1.23	1.45	1.57
chitin	6.11	6.40	6.51	5.68
protein	28.8	26.0	25.3	21.3

only slightly (about 17 %) during the 5 days after exhaustion of the external carbon source, which agrees closely with the results presented in Fig. 6. The data in the Table indicate that the disappearing carbohydrate can be fully accounted for by the disappearance of carbohydrate from the alcohol- and acid-soluble fractions, at least a part of which can be identified tentatively as free sugar, glycogen, and "bound glycogen" respectively. At the same time, no such a decrease can be observed for the S- and R-glucan, which constitute the bulk of the cellular carbohydrates. There may even be a small increase in the amount of S-glucan, tending to shift the S-glucan/R-glucan ratio (S/R) to a somewhat higher value. Further, there is no decrease of chitin in this stock during the starvation period.

However, in view of the fact that the S- and R-glucan and chitin

are cell-wall polysaccharides, there seemed to be nothing unusual in the situation observed in K.35. At the time these experiments were performed, we presumed that "reserve carbohydrates" would be contained in the "glycogen" fractions, although the amount stored seemed to be rather small.

It must be stressed here that the term "reserve carbohydrate" is not restricted to carbohydrates that are merely dissimilated under conditions of carbon-starvation. In the wild-type stock morphogenetic events involving the synthesis of new cells (in the developing pilei) take place under these conditions. Thus, the "reserve carbohydrates" must also deliver the building blocks for the synthesis of these new cells.

Since the amount of acid-soluble polysaccharides is relatively small, it could be argued that in K.35 there is an abnormally small flow of glucose into the "glycogen fraction", in this way preventing the accumulation of an adequate pool of building blocks. However, it will be shown that a small "glycogen fraction" is not at all typical for K.35 and that S- and R-glucan, which apparently are not broken down in K.35, can serve as an endogenous carbon supply in the wild stock, notwithstanding the fact that these polysaccharides are intimately associated with the cell wall.

Before going further into this subject, we wish to describe another experiment with K.35, to show the influence of intensive glucose assimilation on the carbohydrate pattern of mycelium and fructifications.

2. *The fate of various carbohydrates in K.35 in replacement culture*

For this study K.35 was cultivated for 6 days on a high-phosphate medium not supplemented with thiamine (calcium carbonate was omitted). Subsequently, the culture medium was replaced by a similar medium containing thiamine and left for another 2 days. One set of cultures was then removed and analyzed for the various carbohydrates, another set received a new medium supplemented with glucose but lacking a nitrogen source. The small primordia initiated on the medium containing nitrogen grew out and attained their maximum size on the 4th day. The primordia were then separated from the mycelium and analyzed separately.

Results are shown in Table 3. At the time of the second replacement, when the culture is covered with tiny primordia, the total amount of carbohydrate is still small (average 39.9 mg). Nevertheless, there is no conspicuous difference in the distribution of carbohydrates among the various fractions as compared with a stationary culture in which intensive polysaccharide synthesis and simultaneous growth of primordia has taken place; the S/R ratio approximately equals that found for a 7th-day stationary culture (Table 2), though the latter contains 3 times as much carbohydrate and much bigger primordia (both cultures contain the same amount of protein). The same holds for the experiment described here: total carbohydrate shows an increase of 275 %, whereas the S/R ratio tends to increase only

TABLE 3

Quantitative distribution of carbohydrates in *S. commune* K.35 before and after a period of intense carbohydrate assimilation in a replacement culture experiment. At $t=0$ the medium was replaced by a medium containing glucose but without a nitrogen source. After 4 days the cultures were analyzed separately for mycelium and fructifications. The italicized values represent percentages of total carbohydrate. Quantities are given as mg per culture (30 ml).

K.35, 25° C	$t=0$		4 days later					
	I	II	I			II		
	M+F	M+F	M	F	M+F	M	F	M+F
alcohol-sol. carboh.	0.7 *1.7*	0.7 *1.9*	0.9 *2.1*	0.9 *0.8*	1.8 *1.2*	1.0 *1.8*	0.7 *0.8*	1.7 *1.1*
acid-sol. polysacch.	4.5 *10.6*	3.6 *9.7*	8.1 *18.8*	15.1 *14.0*	23.2 *15.4*	12.1 *21.6*	12.1 *13.1*	24.2 *16.3*
alkali-sol. acid-sol. polysaccharide	3.5 *8.3*	4.0 *10.7*	4.2 *9.7*	6.2 *5.7*	10.4 *6.9*	4.3 *7.7*	6.1 *6.6*	10.4 *7.0*
S-glucan	16.4 *38.7*	14.3 *38.3*	15.5 *36.0*	41.7 *38.6*	57.2 *37.9*	15.3 *27.3*	41.9 *45.2*	57.2 *38.5*
R-glucan	14.2 *33.5*	12.8 *34.3*	14.6 *33.9*	31.7 *29.4*	46.3 *30.7*	17.3 *30.8*	24.4 *26.3*	41.7 *28.0*
not accounted for (incl. hot alk.-sol.)	3.1 *7.3*	1.9 *5.1*	–0.2 *–0.5*	12.3 *11.4*	12.1 *8.0*	6.1 *10.9*	7.4 *8.0*	13.5 *9.1*
total carbohydrate	42.4 *100.0*	37.3 *100.0*	43.1 *100.0*	107.9 *100.0*	151.0 *100.0*	56.1 *100.0*	92.6 *100.0*	148.7 *100.0*
S/R ratio	1.16	1.12	1.06	1.32	1.24	0.88	1.72	1.37
chitin	3.40	3.07	2.76	4.93	7.69	3.34	4.31	7.65
protein	28.8	27.3	11.8	16.3	28.1	13.0	—	—

slightly. The results recorded separately for mycelium and fructifications show that this small increase is due to a somewhat higher S/R ratio in the primordia.

The total amount of protein remains constant in spite of the fact that large quantities of protein must be involved in cell turnover. This is consistent with the constancy of total nitrogen under these conditions of a plentiful supply of external energy (cf. Fig. 8). On the other hand, there is an increase in chitin. This was to be expected if chitin and R-glucan are linked together in one functional cell-wall polysaccharide as suggested in the preceding chapter.

3. *The fate of various carbohydrates in K.8 during carbon starvation in stationary culture*

As already stated in Chapter II, the developmental features of the wild stock K.8 diverge from those of K.35 after the point at

which the carbon supply in the medium is exhausted. This development, which is restricted to a relatively small number of fructifications, is characterized by unilateral outgrowth of the margin of the hymenium, thus producing the typical pileus. The results suggested that the growth processes in the developing fruiting bodies must draw heavily upon carbohydrates and nitrogenous constituents of the stunted fructifications and mycelium. This suggestion receives further support from the results of an experiment shown in Table 4.

K.8 was cultivated under exactly the same conditions as given in Fig. 7. After a 7-day growth period, the glucose in the medium has given out and the fungus develops further under nitrogen and carbon starvation. After another 5 days development is complete. At this time, the total amount of carbohydrate is reduced by 35 %.

With respect to the several carbohydrate fractions, it can be seen that at the 7th day free sugar and acid-soluble polysaccharides together do not amount even to 10 % of the total carbohydrate, in spite of the fact that the period of intensive carbon assimilation has ceased shortly before. More than 50 % is contained in S-glucan and 20 % in R-glucan.

In contrast to the situation in K.35, these cell-wall polysaccharides can be broken down. The decrease is most dramatic in the mycelium: viz. about 80 % and 40 % for R- and S-glucan respectively; causing the S/R ratio to shift from 2.8 to 10.6. The fructifications also show a substantial disappearance of R-glucan, resulting in a shift of the S/R ratio from 2.4 to 6.3.

In accordance with earlier results on the disappearance of nitrogen during this phase, the small increase of protein and chitin in all fructifications together can not fully account for the decrease of these substances in the mycelium.

It must be taken into account that during the 5 days of carbon starvation, S- and R-glucan are not only broken down but also partly resynthetized, at least in the developing pilei; the observed changes are only net changes. Similarly, the S/R ratios observed on the 12th day must be looked upon as averaged figures. For example at this time the fructifications include stunted and fully-developed fruiting bodies (with a pileus). It was therefore necessary to estimate the S/R ratio in more distinct structures before a dynamic scheme of cellular reorganization could be constructed.

4. *The relative distribution of S- and R-glucan in the cell walls of distinct morphological structures of K.8 after completion of morphogenesis*

Most probably, the data compiled under fructifications in Table 4 for a 7 day old culture of K.8, can be taken as representing the situation in an individual fructification because development is rather synchronous till this point of time. As has been remarked, however, in older cultures the data denote an average value because stunted fruiting bodies and fruiting bodies with a pileus are both present. In addition, what is called mycelium does not refer to a homogenous

TABLE 4

Quantitative distribution of carbohydrates in *S. commune* K.8 at two stages of development, under conditions of carbon (and nitrogen) starvation. The italicized values represent percentages of total carbohydrate. Quantities are given as mg per culture (30 ml).

K.8, 25° C	7 days						12 days					
	I			II			I			II		
	M	F	M+F	M	F	M+F	M	F	M+F	M	F	M+F
alcohol-sol. carboh.	0.5 *0.5*	0.5 *1.0*	1.0 *0.7*	1.0 *0.9*	0.3 *1.1*	1.3 *0.9*	0.2 *0.5*	0.5 *0.9*	0.7 *0.8*	0.2 *0.4*	0.4 *0.8*	0.6 *0.6*
acid-sol. polysacch.	3.2 *3.4*	1.1 *2.0*	4.3 *2.9*	3.1 *2.7*	0.6 *2.2*	3.7 *2.6*	0.8 *1.9*	1.0 *1.9*	1.7 *1.9*	0.7 *1.4*	0.9 *1.8*	1.6 *1.6*
alkali-sol. acid-sol. polysaccharide	2.5 *2.6*	3.0 *5.6*	5.5 *3.7*	2.7 *2.3*	1.7 *6.1*	4.4 *3.1*	1.6 *4.0*	2.9 *5.7*	4.5 *5.0*	1.4 *2.8*	2.5 *5.0*	3.9 *3.9*
S-glucan	51.2 *53.6*	30.1 *55.8*	81.3 *54.4*	63.7 *55.7*	15.2 *54.5*	78.9 *55.5*	28.9 *73.3*	36.6 *72.4*	65.4 *72.8*	39.8 *81.1*	36.0 *70.7*	75.8 *75.8*
R-glucan	19.6 *20.5*	10.7 *19.9*	30.3 *20.3*	21.6 *18.9*	7.5 *27.0*	29.1 *20.5*	3.0 *7.6*	5.7 *11.2*	8.7 *9.7*	3.5 *7.0*	5.8 *11.5*	9.3 *9.3*
not accounted for (incl. hot alk.-sol.)	18.6 *19.5*	8.5 *15.7*	27.0 *18.1*	22.3 *19.5*	2.6 *9.2*	24.8 *17.4*	5.0 *12.7*	4.0 *7.8*	9.0 *10.0*	3.6 *7.4*	5.2 *10.3*	8.8 *8.8*
total carbohydrate	95.6 *100.0*	53.9 *100.0*	149.5 *100.0*	114.2 *100.0*	27.9 *100.0*	142.1 *100.0*	39.4 *100.0*	50.5 *100.0*	89.9 *100.0*	49.2 *100.0*	50.8 *100.0*	100.0 *100.0*
S/R ratio	2.62	2.80	2.68	2.95	2.02	2.71	9.65	6.44	7.54	11.55	6.17	8.17
chitin	3.27	1.85	5.12	3.87	1.39	5.26	1.43	2.64	4.07	—	2.42	—
protein	13.8	16.0	29.8	17.6	10.6	28.2	5.8	20.5	26.3	4.5	18.6	23.1

class of cells, but is in fact composed of purely undifferentiated (submerged) mycelium and a surface layer of differentiated cells (stroma). Thus, we can provisionally distinguish four types of cell populations: cells of the context of the pileus, which constitute the majority of the fully-developed carpophores; cells of the stunted fruiting bodies; cells of the stroma; and, lastly, the morphologically-undifferentiated cells of the submerged mycelium.

To study the distribution of the two principal cell-wall polysaccharides in these cell types, K.8 was cultivated for 16 days on a low-phosphate agar medium containing 4 % glucose and 0.15 % asparagine as a nitrogen source. The higher glucose concentration was used to obtain the more asynchronous development provided by a high-sugar medium: fewer fruiting bodies develop fully than on a medium with 2 % glucose, but those which do so attain a bigger size. This makes it much easier to discriminate between fully-developed and stunted fruiting bodies.

The fully-developed fruiting bodies (diam. of cap 1.5-6.5 mm) were removed first. Then the stunted ones were scraped from the surface. The stroma forms a solid, coherent mat from which the agar containing the undifferentiated mycelium can be removed.

The different parts of the culture were then subjected to analysis for S- and R-glucan as described under Methods. However, before homogenization, the fractions were boiled (3 min.) and washed with 0.5 N acetic acid to remove the agar.

The results, which are shown in Table 5, reveal a striking dis-

TABLE 5

Relative distribution of S- and R-glucan in distinct morphological structures after completion of morphogenesis in *S. commune* K.8. The organism was grown for 16 days on low-phosphate medium containing 4 % glucose and 1 % agar. Quantities are given as mg per culture (20 ml).

	Exp. I			Exp. II		
	S-glucan	R-glucan	S/R	S-glucan	R-glucan	S/R
fully-developed fruiting bodies .	8.86	3.80	2.33	10.68	3.39	3.15
stunted fruiting bodies	72.80	3.18	22.89	74.55	3.38	22.06
stroma	64.06	4.21	15.22	51.48	2.88	17.88
undifferentiated mycelium . .	1.06	0.19	5.58	0.80	0.15	5.33
all fruiting bodies together . .	81.66	6.98	11.70	85.23	6.77	12.59
whole mycelium	65.12	4.40	14.80	52.28	3.03	17.25
whole organism	146.78	11.38	12.90	137.51	9.80	14.03

crepancy in the S/R ratios of the two types of fruiting bodies: the stunted fruiting bodies attain a S/R ratio 7-10 times higher than those of the fully-developed ones. This evidently results from a dramatic degradation of R-glucan in the fruiting bodies, which do not develop further than the cup stage, followed by utilization of the breakdown

products for the synthesis of "pileus cell walls" in the developing fruiting bodies.

The results indicate that in the stroma, too, there is a preferential breakdown of R-glucan, resulting in a high S/R ratio. The process seems to be less operative in the undifferentiated mycelium, which is not surprising since the cytoplasm of these cells has nearly disappeared by the time the breakdown of cell-wall polysaccharides starts.

The relatively low S/R ratios found in the cell walls of the fully-developed, albeit small (1.5-6.5 mm), fruiting bodies, seem to be typical and to change little if the carpophores are allowed to grow into structures measuring about 40 mm (longest axis of the cap). Such fruiting bodies, which were obtained by cultivation on a thick layer of malt agar for 2 months, show a S/R ratio of 3.0 ± 0.2.

5. *The specificity of the observed changes in carbohydrate pattern*

An important question which emerges here concerns the specificity of the changes in cell-wall polysaccharides as described in the previous sections. Is the ability to degrade R- and S-glucan and the shift in the S/R ratio typical for a system undergoing morphogenesis? The fact that K.35, which is not able to perform the last stage of development (cap expansion), does not exhibit these phenomena, strongly suggests this to be the case. To collect further evidence, we examined three systems in this respect.

K.8 and K.35 were grown at 30°C. At this temperature no fructifications and even no visible primordia are formed, but an abundant aerial mycelium is produced. On the surface of the culture medium, under the aerial mycelium, one can distinguish a coherent layer of interwoven, partly thick-walled cells, closely resembling what we have called the stroma. This layer is especially obvious in K.8, although it is also evident in K.35. After 6 days, at which time the glucose in the medium is exhausted, no further morphogenetic events can be observed.

In order to study the possibility of a more direct effect of elevated temperature, we also examined a dikaryotic stock of *Schizophyllum commune* (stock van Luyk, *Centraalbureau voor Schimmelcultures*, Baarn) at 25°C. This stock never forms fructifications; it produces only an abundant aerial mycelium.

From the results shown in Table 6 it is evident that neither K.35 at 30°C or the van Luyk stock at 25°C shows any decrease of R- and S-polysaccharide during carbon starvation. As a result there is also a remarkable constancy of the S/R ratios, which are rather low and in fact represent the lowest values found.

In K.8 at 30°C there is a small reduction in the amount of these cell-wall polysaccharides after the glucose in the medium has been consumed. In a 6-day period there is a decrease of 29 % and 17 % for R- and S-glucan, respectively causing a small shift of the S/R ratio (1.7 to 2.0). At 25°C however, there was a much more pronounced drop in the amount of R-polysaccharide (68 %; see Table 4) and a shift of the S/R ratio from 2.7 to 7.9.

TABLE 6

Quantitative changes in the distribution of carbohydrates in *S. commune* K.8 and K.35 at 30° C and the van Luyk stock at 25° C following a period of carbon (and nitrogen) starvation. The italicized values represent percentages of total carbohydrate. Quantitaties are given as mg per culture (30 ml).

	K.8, 30° C				K.35, 30° C			Stock van Luyk, 25° C			
	6 days		12 days		6 days		12 days	7 days		12 days	
	I	II	I	II	I	II	I	I	II	I	II
alcohol-sol. carboh.	10.2 *6.8*	13.0 *9.2*	5.4 *5.1*	13.6 *11.6*	3.7 *4.7*	4.3 *5.4*	7.7 *9.1*	1.4 *2.4*	1.3 *2.2*	1.5 *2.3*	1.5 *2.4*
acid-sol. polysaccharide	18.9 *12.6*	19.8 *14.1*	17.6 *16.5*	17.1 *14.5*	5.6 *7.1*	4.4 *5.5*	5.0 *5.9*	1.6 *2.7*	1.7 *2.9*	1.6 *2.5*	1.3 *2.1*
alkali-sol. acid-sol. polysaccharide	19.1 *12.7*	17.6 *12.5*	12.7 *11.9*	13.4 *11.4*	7.8 *9.9*	8.1 *10.2*	4.7 *5.6*	3.4 *5.8*	4.3 *7.4*	2.6 *4.1*	3.0 *4.9*
S-glucan	51.2 *34.2*	47.0 *33.4*	40.4 *37.9*	40.8 *34.7*	23.1 *29.3*	25.0 *31.4*	25.5 *30.2*	18.3 *31.2*	18.7 *32.1*	19.8 *31.0*	19.4 *31.6*
R-glucan	29.8 *19.9*	27.3 *19.4*	21.8 *20.4*	18.6 *15.8*	26.8 *34.0*	27.6 *34.7*	30.1 *35.6*	27.2 *46.4*	25.9 *44.4*	29.3 *45.9*	28.7 *46.8*
not accounted for (incl. hot alk.-sol.)	20.6 *13.8*	16.1 *11.4*	8.8 *8.2*	14.2 *12.0*	11.9 *15.1*	10.1 *12.7*	11.5 *13.6*	6.7 *11.4*	6.4 *11.0*	9.1 *14.2*	7.4 *12.1*
total carbohydrate	149.8 *100.0*	140.9 *100.0*	106.6 *100.0*	117.6 *100.0*	78.9 *100.0*	79.5 *100.0*	84.5 *100.0*	58.6 *100.0*	58.3 *100.0*	63.9 *100.0*	61.3 *100.0*
S/R ratio	1.72	1.72	1.85	2.19	0.86	0.91	0.85	0.67	0.72	0.68	0.68
chitin	6.57	6.80	6.46	6.51	5.49	5.95	7.53	6.98	6.17	7.21	7.69
protein	34.0	31.0	28.0	26.5	20.5	24.8	17.8	25.3	30.0	7.5	8.8

These results indicate the presence of activity *in vivo* concerned with the breakdown of S- and R-glucan in K.8 at both 30°C and 25°C and the absence of this activity in K.35 and the van Luyk stock. However, they also suggest that the preferential breakdown and resynthesis of these cell-wall polysaccharides, which leads to a remarkable increase in the S/R ratio, is typical for the system in which pileus formation takes place.

A remarkable feature of the van Luyk stock seems to be the preferential breakdown of protein. During the 5 days of carbon (and nitrogen) starvation the amount of protein drops by 70%, whereas in the other stocks the protein content tends to decrease only slightly.

Discussion

Endogenous metabolism is commonly defined as the metabolic reactions that occur within the cell when it is deprived of a source of compounds which may serve specifically as exogenous substrates. These reactions fulfill a dual role: they serve as a source of energy and/or they provide substrates for the (re)synthesis of cellular constituents. A number of investigations have made it clear that these reactions not only serve to maintain the *status quo* of the cell but also can support processes which lead to biochemical and morphological differentiation (Mandelstam, 1960). It is conceivable that part of the endogenous material must be catabolized to provide energy, and this fraction of the endogenous material is commonly denoted as "endogenous reserve substance". Our knowledge of these compounds in microorganisms and higher fungi is rather incomplete; suggestions as to the nature of these compounds have frequently been made, but only few quantitative studies are available (cf. Foster, 1949; Dawes and Ribbons, 1964).

In this context it must be emphasized that the processes of endogenous metabolism can only be studied unambiguously in unicellular organisms, and even then care must be taken not to include such processes as cell turnover. Lysis of some of the cells may occur, entailing the release of metabolites which then serve as exogenous substrates for surviving cells (cf. Harrison, 1961).

In *Schizophyllum,* conspicuous morphogenetic events — including the production of new cells — go on in the absence of exogenous nutrients. As has already been described in Chapter II, growth of the primordia can occur in the absence of a nitrogen supply in the medium and formation of the pileus occurs even in the absence of both an exogenous carbon and a nitrogen supply. Thus, the whole morphogenetic system may be regarded as a system in which particular structures are produced in sequence, each structure providing the substrate for the following. The vegetative mycelium supplies the nitrogenous compounds for the growth of the primordia and the expanding pilei draw completely upon the stunted fruiting bodies and the stroma.

The feasibility of such processes is obvious from the fact that

translocation in fungi, including Hymenomycetes, has been demonstrated repeatedly (cf. SCHÜTTE, 1956).

The distinction between reserve and structural constituents has now become rather meaningless in the system studied here. It is hardly conceivable that a loss of say 60 % of the nitrogenous components from the mycelium could be accounted for by a breakdown of only reserve substances; at least part of these compounds must have been contained in structural elements. The same holds for the phenomena described in this chapter. Clearly, S- and R-glucan and chitin constitute the structural elements of the cell wall. Nevertheless, these elements are degraded and partially used for the construction of new cells. As a matter of fact, only small fluctuations have been found in the amounts of alcohol- and acid-soluble carbohydrates, both of which can more easily be considered as typically reserve substances. Thus, we arrive at the conclusion that in *Schizophyllum,* under our conditions, typical reserve substances that support morphogenetic processes are of minor importance. Perhaps one might go so far as to say that the relationship between the sequential structures can be described as parasitic, although it must be borne in mind that the cell turnover involved may follow a special pattern.

The results suggest that the ability to degrade the cell-wall polysaccharides is a prerequisite for pileus formation, because only the stock capable of constructing pilei showed activity affecting R- and S-glucan and chitin. There is also an indication that in the genetically-adequate stock there is a relation between these chemical activities and fruit-body production: it has been demonstrated that both the preferential breakdown of R-glucan and the process of fructification can be inhibited by cultivating at an elevated temperature (30°C).

Although we have found no reports of similar quantitative studies on the cell wall material in fungi, there are a number of cytological observations in the literature which bear upon the subject. It is beyond the scope of this paper to review this literature; an extensive résumé of the older observations is provided by LOWAG (1941). These observations concern the sclerotia and related structures produced by many fungi. Apart from being regarded as structures which serve to help the organism to survive periods of unfavourable conditions, they are thought to constitute a store of food reserves which can support the growth of any arising fruiting bodies. During this process, not only does the cell content in the sclerotium disappear but later the cell walls are also attacked and sometimes completely dissolved. Similar processes have been found in the context of a polypore: continuous spore discharge in a starving fruiting body was accompanied by considerable "corrosion" of the hyphal walls (CORNER, 1932).

CHAPTER V

THE ROLE OF BREAKDOWN OF CELL-WALL POLYSACCHARIDES IN THE CONTROL OF PILEUS FORMATION

INTRODUCTION

In the preceding chapter strong evidence is presented for a relation between breakdown of cell-wall polysaccharides and pileus formation. It was further demonstrated that the K.35 stock which does not enter the stage of pileus expansion, shows no net degradation of R- and S-glucan and chitin.

This is the only biochemical lesion we have as yet been able to detect in K.35. Supplementation of the synthetic medium with yeast extract, malt extract, casein hydrolysate, nucleic acid hydrolysate, or a vitamin mixture has no effect on the morphogenetic deficiency.

Now, because the cell-wall polysaccharides are essentially polyglucoses which are presumably broken down into soluble compounds and then re-used as a substrate for the synthesis of pileus cell-walls, it seems remarkable that exogenous glucose cannot be used for this purpose. This is clearly demonstrated by the fact that repeated addition of glucose (with or without asparagine) in a replacement culture experiment effects only an increase of the size of the primordia and a further inhibition of the expansion of the hymenium; the final area of the hymenium is much smaller than in stationary cultures with 2 % glucose.

An explanation of these results can be offered by assuming that the morphogenetic processes which lead to the construction of the pileus can only proceed if the glucose (or larger breakdown products of the cell-wall polysaccharides) is delivered to the developing system at a slow but continuous rate. It seems acceptable that there would be an essential difference between an easily available carbon supply in the medium and a store of carbohydrates in the form of cell-wall polysaccharides which, according to the observations, are broken down slowly. If this view is correct, we can predict the results of a number of simple physiological experiments:

1. The incorporation of increasing concentrations of glucose in a stationary culture of K.8 will only delay the appearance of pilei; their ultimate formation will not be affected. The effect of increasing concentrations of glucose on the K.35 stock will be restricted to an effect on the growth of primordia.
2. The continuous application of a high sugar concentration to K.8 will inhibit pileus formation.
3. The morphogenetic deficiency of K.35 can be overcome by feeding the mutant a continuous, low concentration of glucose after the primordia have been formed.

These experiments will be described below.

METHODS AND RESULTS

Details of the media employed and the replacement technique are described in Chapter II.

1. *Morphological response of K.8 and K.35 to varying concentrations of glucose in the medium*

K.8 and K.35 were cultivated on agar media (low phosphate) containing 0.15 % asparagine and glucose in concentrations of 2, 4, 6, and 10 %. The inoculum consisted of mycelium fragments. Figs. 14 and 15 show the gross morphology of the carpophores after a 16-day period of cultivation.

The start of pileus formation is indeed delayed as higher glucose concentrations are applied to K.8. For increasing glucose concentrations, the first indication of pileus expansion is observed approximately on the 6th, 7th, 8th, and 10th day. The pilei formed within the wide range of glucose concentrations are quite normal; at higher concentrations the fruiting bodies become larger and especially the trama of the pilei grows thicker. Another effect, not shown in the photographs, is that the number of primordia that go on to form pilei decreases with increasing glucose concentrations; those which do so, however, develop more vigorously.

In K.35 high sugar concentrations bring about a bulky type of growth of the primordia. The formation of the "cup", i.e. the rudimentary expansion of the hymenium surface, becomes more and more inhibited as higher glucose concentrations are applied.

2. *Inhibition of pileus formation in K.8*

K.8 was grown for 6 days in sand cultures with high-phosphate medium not supplemented with thiamine. Calcium carbonate was included to neutralize the acid formed. The medium was then replaced by a complete medium containing thiamine. Two days later the surface bore small primordia. One set of cultures was then given a medium without a nitrogen source but containing glucose (2 %), and incubated for 10 days. After an initial synchronous growth of the primordia, which lasted for about 3 days (in this period the glucose supply in the medium became apparently exhausted), the well-known asynchronous development of pilei followed (Fig. 16A).

Another set of cultures received fresh glucose medium every day, in order to maintain a high sugar concentration. Fig. 16B shows the appearance of the culture on the 10th day. Pileus formation was inhibited completely under these conditions. On the other hand, the exogenous glucose supported the growth of the primordia, which grew unchecked until at least the 8th day. It is likely that further growth was blocked only because all the nitrogenous compounds available in the mycelium had been consumed. It must be noted, however, that the cells in these large primordia are mainly of the thick-walled type that contains relatively small amounts of cytoplasm.

The increase in mass is therefore probably due chiefly to synthesis of cell-wall material.

In the photograph, the cavity at the summit of the primordia — the first indication of the hymenium — is just barely visible. Its time of appearance does not seem to be influenced by the repeated renewal of the glucose medium; only the further morphogenetic events which normally go on in this region of the primordium are inhibited.

Exactly the same structures arise when the experiment is performed with K.35, and there is also a close resemblance to the fructifications of K.35 occurring in high-sugar stationary cultures (cf. Fig. 15D).

We may therefore conclude that K.8 can be forced to behave like the mutant stock K.35 simply by maintaining a high level of glucose in the environment.

3. *Induction of pileus formation in K.35*

The logical next step, viz. to induce pilei in K.35 by providing the system with a continuous supply of sugar in low concentrations, was also successful.

To realize such a situation we made use of the slow diffusion of sugar through an agar medium. The simple apparatus is shown in Fig. 17. The Erlenmeyer flasks and the stems of the funnels were

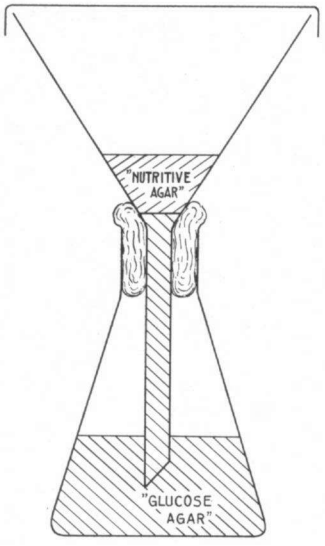
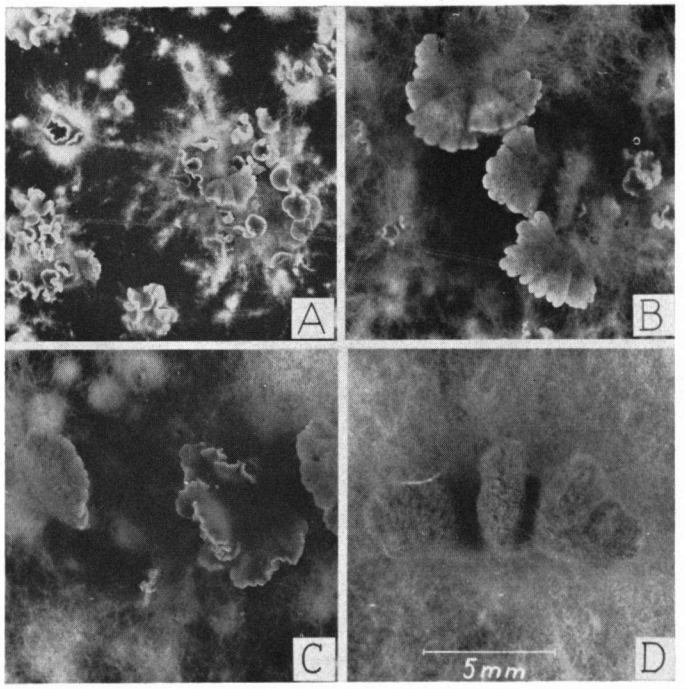
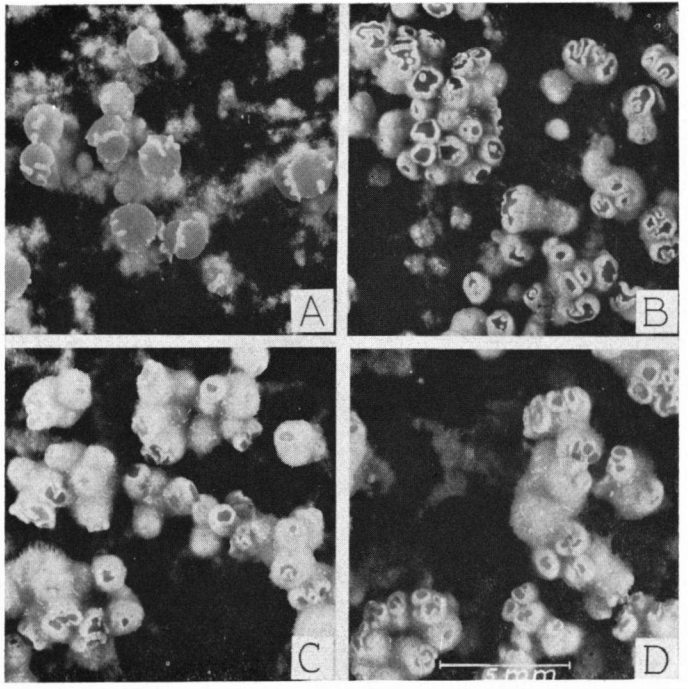


Fig. 17. Device to establish a continuous low concentration of glucose at the site of fungal growth.

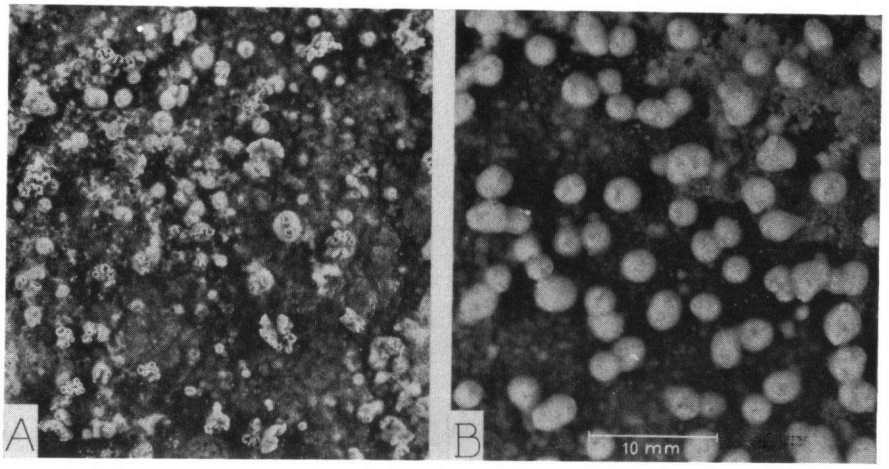
filled with a basal agar medium containing glucose in one of the following concentrations: 2, 4, 10, 20 and 30 %. On top of this medium in the funnel we placed the normal nutritive agar medium containing asparagine (0.15 %) and glucose (2 %) and then inoculated the surface with mycelium fragments. In this way we hoped to establish empirically a steady, low, and probably critical, glucose concentration at the site of growth.





Figs. 14 and 15. Influence of various concentrations of glucose in the medium on the morphology of fruiting bodies of *S. commune* K.8 (Fig. 14) and *S. commune* K.35 (Fig. 15). A: 2 %, B: 4 %, C: 6 %, and D: 10 % glucose. Incubation time 16 days.

Fig. 16. Morphology of *S. commune* K.8 after a continuous supply of glucose in a concentration of 2 % (B); A: control.



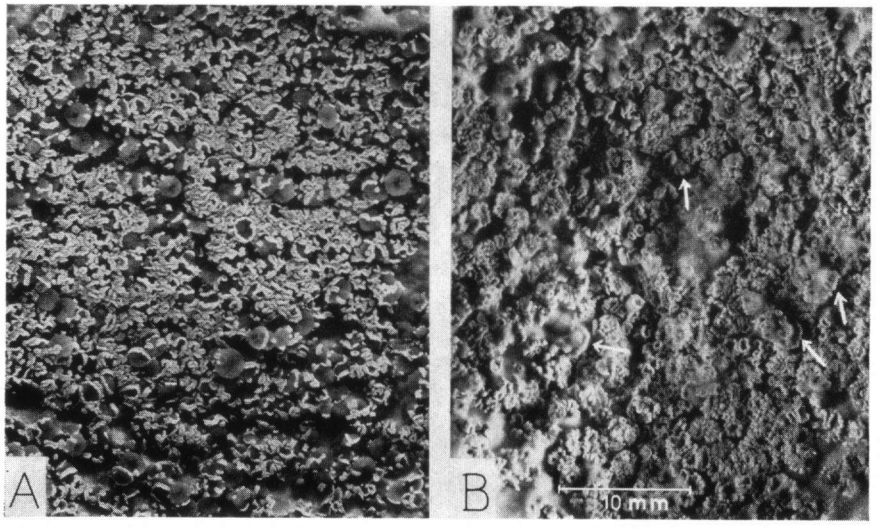


Fig. 18. Morphology of *S. commune* K.35 grown in the apparatus outlined in Fig. 17 for 20 days. Concentration of glucose in the "glucose-agar" 2 % (A) and 20 % (B).

Positive results were indeed obtained, especially with the 20 % glucose concentration; with 10 and 30 % the results were also conclusive, but less evident. The fructifications first developed until they reached the stage typical for K.35 under normal conditions. After the tenth day, however, the production of pilei became evident. The photograph (Fig. 18B) shows the situation after an incubation time of 20 days. Normal, albeit small, pilei can be seen (some are indicated by arrows); others persist in the "cup-stage", but even these show marked proliferation of the hymenial margin.

The fact that the pilei of K.35 evoked by the experimental conditions described here are smaller than those commonly produced by K.8 and other wild stocks is probably due to blocking of their growth by a shortage of non-carbohydrate constituents such as nitrogenous compounds. In K.8 the needs of the developing pilei can be provided for by withdrawal of such compounds from stunted fructifications. Such competition does not seem to operate in the case of K.35.

Discussion

The experimental results clearly indicate that the steady maintenance of a low sugar concentration is required for pileus formation. In a wild stock this requirement can evidently be met by a breakdown of cell-wall polymers, but in the cup mutant (K.35) this mechanism seems to be inoperative for some reason.

It would have been interesting to study the effect of a slowly utilizable carbon source such as cellulose on pileus formation in K.35. Cellulase has been demonstrated in *Schizophyllum commune* (Lyr, 1959). Unfortunately, it appears that the stocks used in the present investigation have lost the ability to produce this enzyme because no growth using cellulose as a sole source of carbon can be observed. Nevertheless, our results might suggest an explanation for the frequently reported fact that the addition of compounds containing cellulose, such as sawdust, to culture media enhances "fruiting" (pileus formation in many wood-destroying fungi (cf. Lowag, 1952).

In stock K.8 (wild type) there seems to be a close relationship between the development of the stroma and the expression of the competition phenomena that ultimately favour the development of large pilei in only a limited number of fructifications. In addition to the proposed role of the stroma in the storage of re-usable cell-wall carbohydrates, it appears to function as a structure that connects individual fructifications with each other, thus providing an expedient for hyphal transport. The observed relationship seems to favour the idea that the transport of cell-wall breakdown products (and other materials used in the construction of pilei) is intra-hyphal and not mediated by prior excretion into the medium.

As to the question of how the concentration of a substance like glucose fits into the cellular machinery that controls pileus formation, we can as yet only speculate. In micro-organisms, the level of glucose can control the synthesis of certain enzymes: high concentrations

of glucose inhibit the synthesis of these enzymes. This phenomenon of catabolite repression (MAGASANIK, 1961; LOOMIS and MAGASANIK, 1964) at least implies the possibility of an effect of glucose on morphogenesis. However, the presence of this phenomenon and its possible significance for morphogenesis in *Schizophyllum commune* can only be conjectured because no direct evidence is available.

Recently it has been demonstrated that chlorophyll synthesis in a particular strain of tobacco crown-gall tissue can be stimulated by cultivation on a medium sustaining a steady low sugar concentration (JASPARS, 1963), but in this case the effect was thought to be caused by the slower growth rate occurring on these low sugar media.

CHAPTER VI

RESPIRATION AND FERMENTATION AS FUNCTIONS OF GROWTH AND DEVELOPMENT

INTRODUCTION

The results pertaining to the breakdown of polysaccharides and the effect of the concentration of thiamine and oxygen, described elsewhere in this paper, suggested that measurement of specific respiration and fermentation as functions of growth and development might yield valuable additional information about the mechanism of development.

Our knowledge of the respiration of *Schizophyllum commune* (and of other wood-decaying Hymenomycetes) is rather scanty. Only a few respiratory studies have been made with *Schizophyllum* (WESSELS, 1959; NIEDERPRUEM and HACKETT, 1961; NIEDERPRUEM, 1964).

METHODS

Sand cultures are not very suitable for respiratory studies involving the Warburg technique. Some preliminary experiments showed that even brief shaking of a sand-grown mycelium (in a Mickle disintegrator, without glass beads), in order to obtain pipettable fragments, resulted in a rapid loss of respiratory activity: 2 and 4 minutes of shaking resulted in a 40 % and 66 % loss of respiratory capacity, respectively, as compared with a shaking time of 1 minute. This loss remained the same whether or not glucose was added as a substrate.

In order to retain the convenient manometric technique for the study of the intact organism, we decided to use discs from agar cultures as was done by NIEDERPRUEM and HACKETT (1961.) Moreover, this method also enables the study of the influence of medium replacements on gas exchange.

1. *Stationary cultures*

Low-phosphate basal medium containing glucose (2 %), asparagine (0.15 %), and thiamine (120 mμg/ml) was solidified with agar (1.0 %

purified agar, Difco). Petri dishes (diam. 14 cm) were provided with 50 ml agar medium, inoculated with mycelial fragments, and incubated at 25°C. At various time intervals, mycelium-agar discs (diam. 8 mm) were removed at random. Part of the agar medium was crushed and used for pH measurements. The discs were transferred directly to Warburg vessels.

2. *Replacement cultures*

Petri dishes (diam. 9 cm) containing 20 ml agar medium of the above-mentioned composition but not supplemented with thiamine, were inoculated and then incubated for 6 days at 25°C. Mycelium-agar discs were cut from the plates and transferred to Petri dishes containing 20 ml liquid medium of the same composition but containing thiamine. Each dish received 20 discs. Care was taken that the discs were not completely submerged: the mycelial surface must be exposed to the air. The discs were held in this solution for 2 days, after which time the surfaces were covered with small primordia. They were then transferred to dishes containing 20 ml of the same solution, but without asparagine. Each dish received 30 discs. After 1 hour the liquid medium was removed and replaced by a fresh one, in order to remove most of the residual asparagine. This medium supported the growth of the primordia, which grew in a perfectly synchronous manner since they were not hindered by a limited supply of glucose.

Transfers to other media, as discussed under Results, were performed in the same way. The developmental features mentioned above apply only to cultures incubated at 25°C. At 30°C a heavy aerial mycelium was already formed after the first transfer.

3. *Measurement of oxygen consumption and carbon dioxide output*

Normal Warburg vessels received 5 discs each. No extra fluid was added. The volume of the discs was estimated by weighing and assuming a specific gravity of 1.0. For the estimation of oxygen uptake, a paper wick and 0.2 ml 20 % KOH were placed in the centre well. The respiratory quotient (RQ) was determined by the direct method, involving a series of vessels which received no alkali. After the measurement of gas exchange in air, these vessels were flushed with pure nitrogen for 15 minutes and the carbon dioxide output determined. The manometers were not shaken in the water-bath, which was held at 25°C (in some experiments at 30°C, depending on the conditions of cultivation). All measurements were done in triplicate and continued for 60 minutes. The time course of gas exchange was a straight line during this period.

The solubility of carbon dioxide was calculated according to the formula $\alpha' = \alpha$ [antilog (pH—pK)] + 1, α' indicating the solubility of carbon dioxide at the given pH and temperature.

Since the amount of fungal material in the different flasks varied somewhat, the gas exchange was first calculated on a protein basis before calculating the RQ.

4. *Estimation of protein*

To relate the gas exchange to the amount of respiring material, it was thought to be most informative to calculate the results on a protein basis. Results based on dry weight (apart from difficulties in estimation) would have little value because of the excessive synthesis of cell-wall polysaccharides without any net synthesis of proteins.

To determine the protein in the discs, we used the following procedure: After completion of the Warburg experiment, the discs were removed from the vessels and transferred to test tubes. To each tube 5 ml 6 % trichloroacetic acid (TCA) was added and the tubes heated in a boiling water-bath for 15 minutes. The contents of the tubes were then homogenized briefly in a Potter-Elvehjem homogenizer and filtered through a small glass-paper filter (Whatman GF/A, diam. 20 mm) fitted to a sintered glass funnel. The residue was washed on the filter with 5 % TCA.

The filter with residue was transferred to a centrifuge tube and extracted with 5 ml N NaOH in a boiling water-bath for 10 minutes. The glass-paper filter and the residue were then centrifuged off and an aliquot of the supernatant taken for estimation of protein by the Lowry method (LOWRY *et al.*, 1951), using bovine albumin (Sigma, fraction V) as a standard.

RESULTS

1. *Gas exchange of K.35 and K.8 in stationary culture*

In a number of preliminary experiments, respiratory quotients (RQ) well above unity were recorded. Since this indicated the presence of active fermentation even under aerobic conditions, it was decided to measure the anaerobic fermentation at the same time. Data on fermentation, uninhibited by oxygen, could be of value in explaining changes in the respiratory quotient. The most complete experiment was done with K.35. Fig. 19 shows the results.

During the transition from undifferentiated hyphae tot primordia-bearing mycelium — a phase in which rapid growth, i.e. protein synthesis, occurs — there is a rapid increase in respiratory rate and fermentative capacity. Respiration continues on a high level until the primordia have reached their maximum size, a moment which has been shown to coincide with the exhaustion of the glucose supply. The respiratory rate then declines rapidly, after which it continues to decrease slowly.

The phase of mycelial growth and primordia formation is marked by respiratory quotients far above unity, indicating the occurrence of decarboxylations relating to anaerobic catabolism. However, the RQ declines rapidly after the primordia have been established, while the respiratory rate remains high for some time. The RQ settles down to mean values near unity which are maintained during the rest of development, although initially a lower value can be attained. This can be explained by assuming that a product of fermentation, e.g. ethanol, is oxidized.

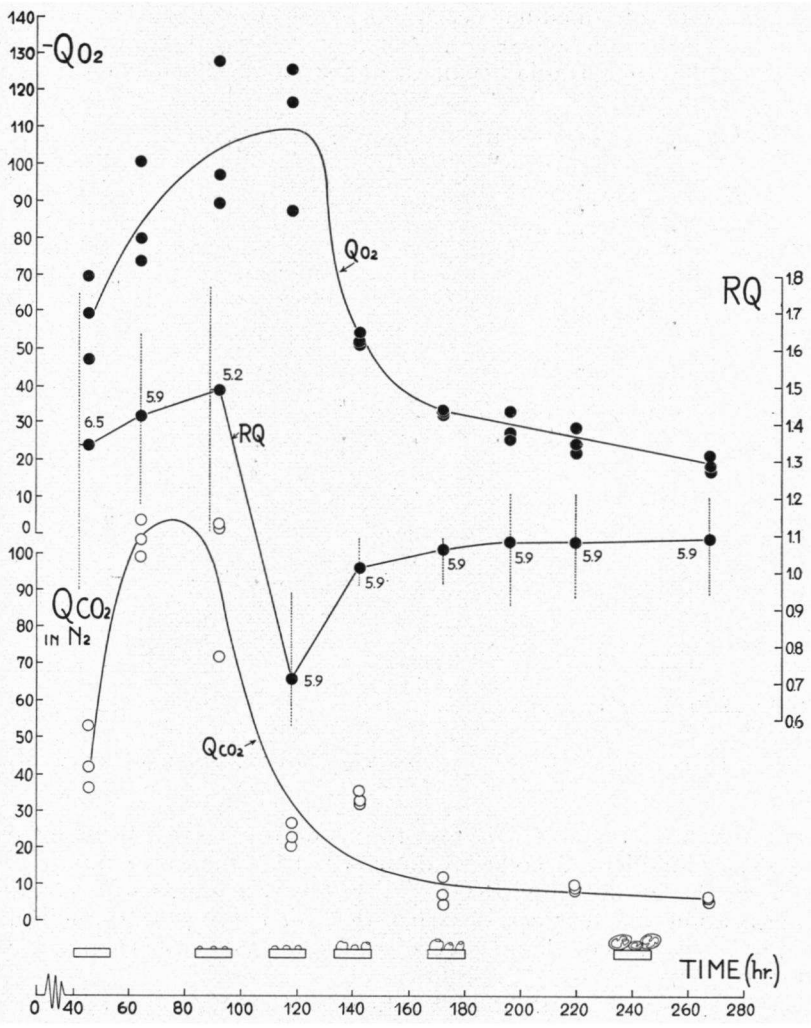


Fig. 19. Respiration, RQ, and fermentative capacity of *S. commune* K.35 during development in stationary culture. Medium: basal medium (low phosphate) plus 2 % glucose, 0.15 % asparagine, 120 mμg thiamine/ml, and 1 % agar. Temperature: 25° C. Dotted lines give the range of RQ values found. The pH value of the medium at the moment of measurement is given near the RQ curve.

It is interesting that as the RQ drops, anaerobic fermentation declines. It is therefore tempting to ascribe the lowering of the RQ simply to a shift towards a type of metabolism with a low fermentative capacity. However, additional evidence to support this view is required because in the period of declining RQ several environmental influences act on the system, e.g. the exhaustion of the nitrogen and carbon supply and the consumption of fermentative products first

excreted into the medium, each of which can have a profound influence on the RQ (see section 2).

Fig. 20 describes similar changes in the gas exchange of K.8. The

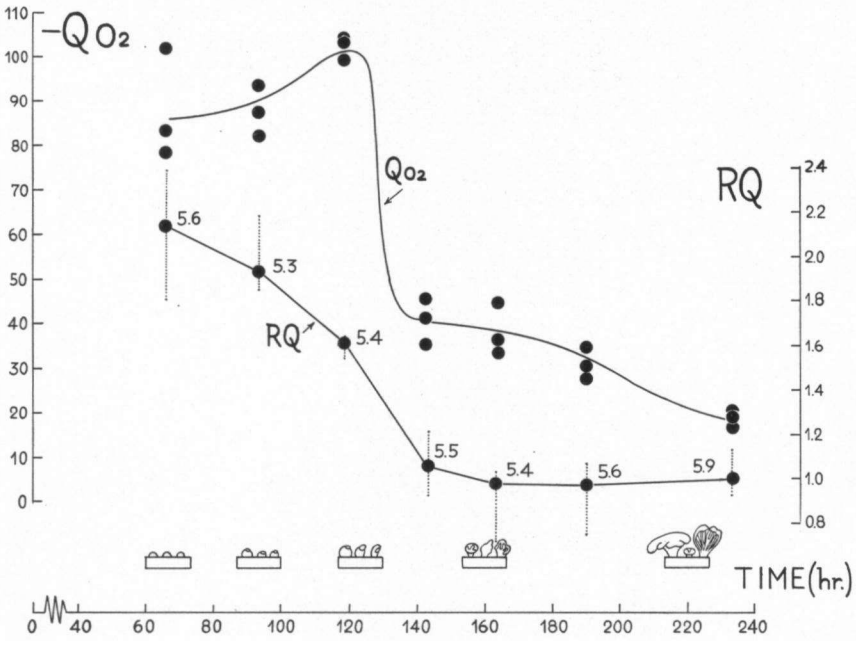


Fig. 20. Respiration and RQ of *S. commune* K.8 during development in stationary culture. (See Fig. 19.)

respiratory rate remains high during primordium growth, while the respiratory quotient decreases. If an ethyl alcohol fermentation is assumed, the initially high RQ (± 2) can be attributed to a balance of respiration and fermentation such that for every glucose molecule completely oxydized, three molecules flow through the fermentative pathway.

Comparison of Figs. 19 and 20 indicates that after the steep decline of the Q_{O_2}, the respiration of K.8 remains at a somewhat higher level during pileus expansion, whereas the respiration of K.35 decreases steadily. This was expected because it has been shown that pileus formation is accompanied by a loss of cell-wall polysaccharides (see Chapter IV). However, the differences are small, notwithstanding the fact that the discs used for the last four measurements shown in Fig. 20 were not collected at random but selected for the presence of pileus-forming fructifications.

2. *Gas exchange of K.35 in replacement culture at 25°C and 30°C*

In order to overcome some of the difficulties with respect to the interpretation of the lowering of the respiratory quotient encountered in stationary cultures, we decided to study the gas exchange in

replacement cultures by means of the technique described above under Methods. It must be borne in mind that these replacements are less complete than those in which sand-liquid cultures were used, as described in Chapter II.

The respiration and anaerobic fermentation of a 5 day old thiamineless mycelium was too low to measure. This was due not only to the small amount of protein produced in the absence of thiamine but also to the fact that both processes were probably severely inhibited by the large amounts of pyruvic acid produced under these conditions (see Chapter VII). When the discs were placed in fresh thiamine-free medium for 24 hours in order to lower the acid concentration (the pH of this medium dropped from 6.7 to 5.7), measurable gas exchange was obtained. Fig. 21 shows these values at the moment of the first transfer.

It can be seen from Fig. 21 (compare with Fig. 23 for changes in protein) that 20 hours after placement of the discs in the complete medium with thiamine (+C+N), a high Q_{O_2} (—130), RQ (1.9), and $Q^{N_2}_{CO_2}$ (+185) are recorded. The first visible primordia become apparent during the next 20 hours of incubation. The RQ remains high, whereas the Q_{O_2} and $Q^{N_2}_{CO_2}$ tend to decrease.

If, after the primordia are established, the discs are transferred to a medium without a nitrogen source but containing glucose (a medium conducive to the growth of the primordia), no further decrease of the respiratory rate occurs but there is even a slight increase during the first three days of the incubation time. The primordia grow rankly during this period. Then the Q_{O_2} falls off rapidly, most probably due to exhaustion of the glucose supply. During the time that the fungus retains a high respiratory rate, the RQ drops steadily until values well beneath unity are reached.

A concomitant of this drop in RQ is a continuous reduction of the fermentative capacity, which suggests a causal relationship between the two phenomena. Thus, it seems that during the growth of the primordia a high respiration rate is retained and fermentative processes are gradually eliminated.

Some of the other data shown in Fig. 21 are worthy of mention. If the mycelium-agar discs bearing small primordia are transferred from the complete medium to a medium lacking both the carbon and nitrogen source (—C—N), practically no growth of the primordia can be observed. Under these conditions both respiration and anaerobic fermentation quickly drop to low values but fermentation seems to be much more affected than respiration by the lowering of the glucose concentration. The rapid adjustment to a respiratory quotient of unity thus seems conceivable. The subsequent rise accompanied by the breakdown of protein (cf. Fig. 23) remains to be explained.

It has been pointed out (Chapter IV) that after intense carbon assimilation only acid-soluble polysaccharides, which constitute only a minor part of the total polysaccharide content, can be broken down by K.35. The time course of the disappearance of carbohydrate

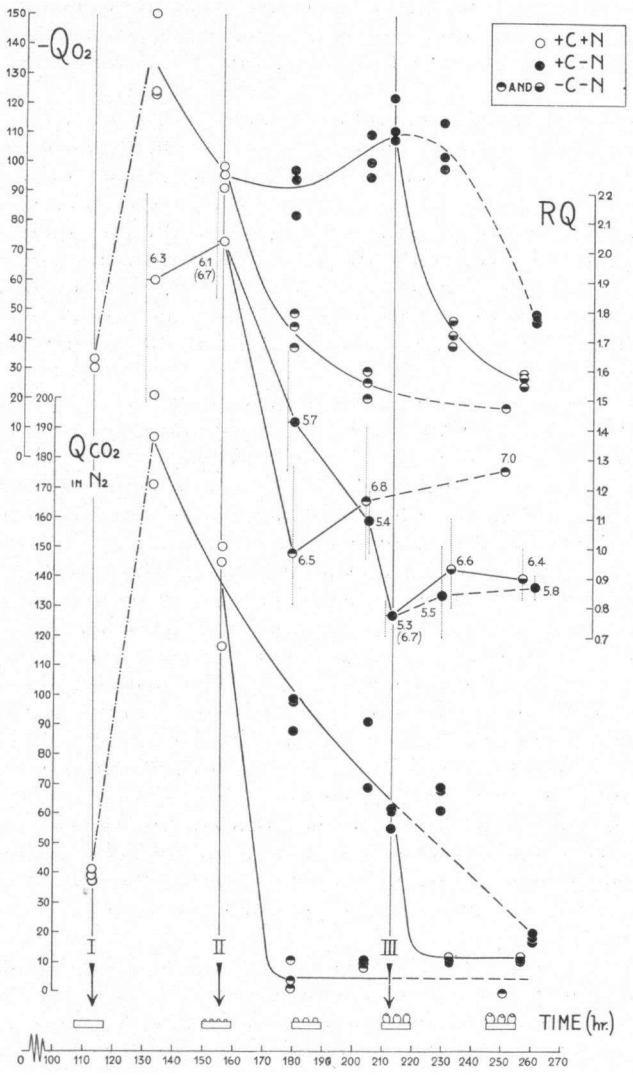


Fig. 21. Respiration, RQ, and fermentative capacity of *S. commune* K.35 in a replacement culture experiment conducted at 25° C. Low-phosphate basal medium was used throughout. Concentration of glucose, asparagine, and thiamine, if added: 2 %, 0.15 %, and 120 mμg/ml respectively. At the moment indicated by arrow I a thiamineless mycelium was transferred to a medium containing glucose, asparagine, and thiamine. Further transfers indicated in the graph. Thiamine was always added to the replacement media. Dashed lines: discs not transferred to new medium; dotted lines: the range of RQ values found. The pH values recorded at the moment of measurement are given near the RQ curve (pH of fresh medium shown in parenthesis). The schematic drawings at the bottom give the developmental sequence if at the moment indicated by arrow II a medium containing glucose but no asparagine (+ C–N) was given.

suggests that this occurred within one day after exhaustion of the glucose supply. Fig. 21 shows that endogenous "reserves" are indeed unable to support a high respiratory rate, as measured one day after transfer to glucose-free medium. However, in this case too, fermentation decreases more quickly than respiration.

As to the question of whether the gradual lowering of the RQ evoked after transfer of the discs to a medium containing glucose (+C—N, transfer II) is brought about by a gradual lowering of the concentration of glucose as a result of its continuous consumption, the following observations are relevant. In a particular experiment an RQ of 1.71 ± 0.14 and 0.81 ± 0.02 was measured 5 and 48 hours respectively after the second transfer. When after 48 hours the discs were put in a new glucose medium for 5 hours in order to restore the original concentration of glucose, the RQ became 1.24 ± 0.10. Repeated renewal of the glucose medium, every 12 hours after the second transfer, resulted in an RQ of 1.13 ± 0.08 after 48 hours. From these observations it can be concluded that the gradual drop of the RQ in a glucose medium can be attributed only partly to a change in the external concentration of glucose; another phenomenon must be involved.

It is now of interest to compare the results obtained at 25°C with changes in gas exchange observed in an experiment conducted at 30°C. Fig. 22 shows the results.

As mentioned before, the primordia, if present, do not grow at this temperature. Only a stroma and an abundant, loose aerial mycelium is produced under these conditions (for a discussion of the relation between stroma and primordia, see Chapter I).

After the first transfer, in which period stroma and aerial mycelium are formed, the only deviation from the picture obtained at 25°C is a higher fermentative capacity. If the discs are now transferred to the glucose medium lacking the nitrogen source, no further morphogenetic events occur; only the stroma acquires a greater toughness, most probably due to thickening of cell walls and perhaps to the synthesis of additional stroma cells. The decline of the respiratory quotient and fermentative capacity seems to proceed along the same lines as at 25°C. However, as distinct from the experiment at 25°C, the glucose present does not prevent a simultaneous reduction of the respiratory rate.

The significance of a number of the data presented in Figs. 21 and 22 can be better appreciated if the ratio of respiratory capacity (assuming full saturation of the respiratory chain by oxygen; to be discussed below) to fermentative capacity is plotted against time (see Fig. 24).

As long as net protein synthesis is possible (compare Fig. 23), the ratio changes only slightly, but after the transfer to a nitrogen-free medium the ratio rises steeply along a linear slope. This slope is nearly identical for the experiments conducted at 25°C and 30°C. In other words, a continuous relative decrease of fermentative capacity does not seem to be characteristic for the process of primordia growth.

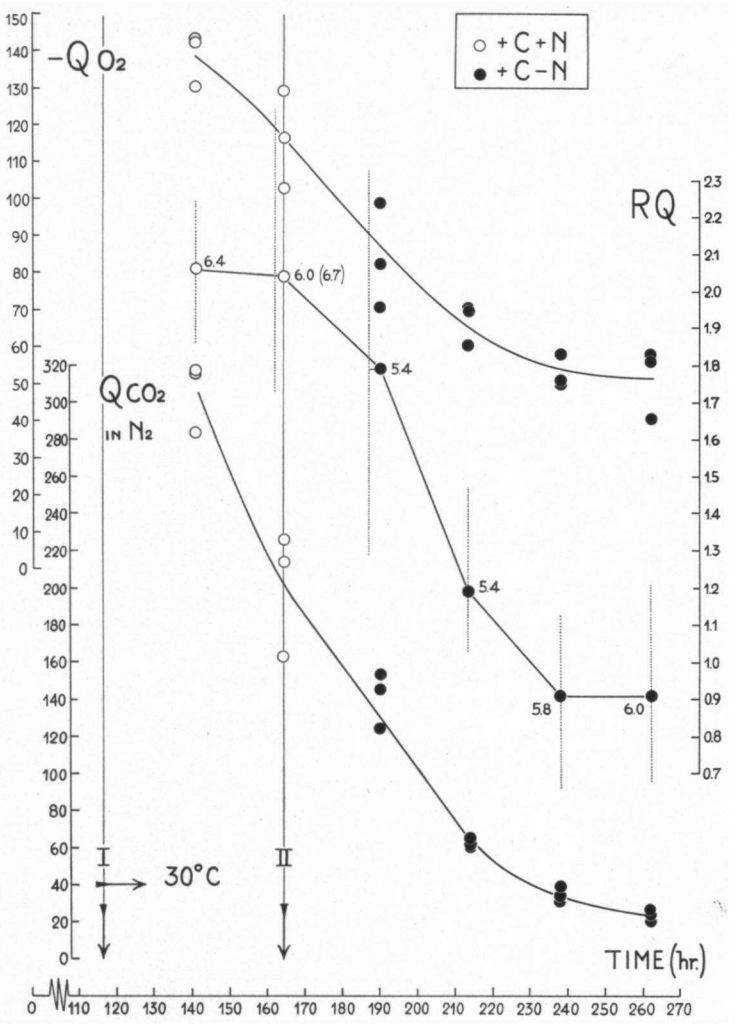


Fig. 22. Respiration, RQ, and fermentative capacity of *S. commune* K.35 in a replacement culture experiment conducted at 30° C. (See Fig. 21.)

Now, if we assume that the respiratory quotient proper of the oxidation of glucose is unity, we can calculate the extra amount of carbon dioxide evolved by aerobic fermentation ($Q_{CO_2}^{air}$). With the available data it is then possible to calculate the Meyerhof quotient (M.Q. = difference between anaerobic and aerobic fermentative carbon dioxide output divided by oxygen uptake). If we accept this quotient as an indirect measure for a Pasteur effect and we assume a normal alcoholic fermentation, then a Pasteur effect is operative if M.Q. $> 1/3$. As can be seen in Fig. 24, after net protein synthesis stops and before the glucose supply becomes exhausted, a fairly constant M.Q. of 0.7–0.8 is obtained for both experiments.

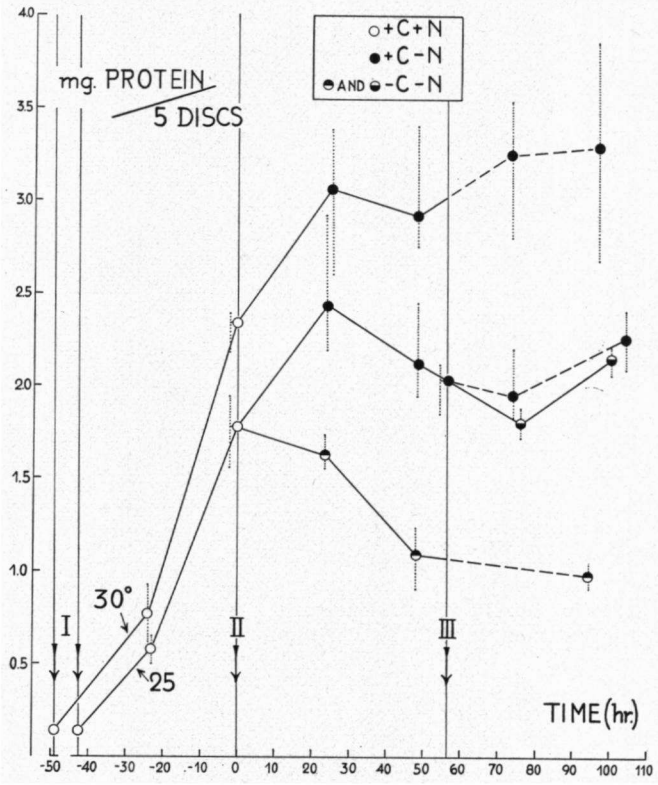


Fig. 23. Protein content of the discs used in the experiments shown in Figs. 21 and 22. For the sake of clarity, the moment of the shift from the medium containing asparagine to the media without a nitrogen supply is indicated as time zero for both experiments. Dotted lines: range of values found for 6 series of 5 discs.

The evaluation of the relationship between the data on gas exchange in Figs. 21 and 22 thus constitutes a strong indication that the lowering of the RQ is principally due to a loss of fermentative capacity, whereas the magnitude of the Pasteur effect remains approximately constant.

The possibility that the mycelium growing in the agar was subjected to semi-anaerobic conditions that allowed high "aerobic fermentation", was ruled out by studying the effect of high oxygen pressure on the respiratory rate and respiratory quotient. An experiment was performed with mycelium-agar discs from a thiamine-free culture incubated in complete medium for 28 hours at 25°C.

Neither an increase in respiratory rate nor any significant decrease of the respiratory quotient was observed (Table 7). It therefore seems that the access of oxygen to the cells was not limited by diffusion, the respiratory pathway being saturated at atmospheric oxygen pressure.

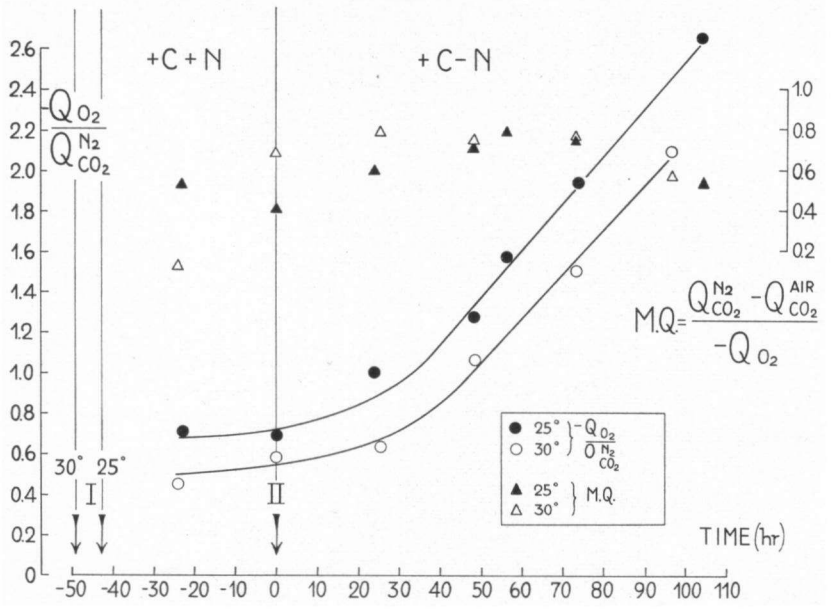


Fig. 24. Ratio of respiration and fermentative capacity and Meyerhof quotient of *S. commune* K.35 shifted at zero time from a medium containing glucose and asparagine to a medium containing glucose but lacking the nitrogen source. Data are calculated from Figs. 21 and 22.

TABLE 7

Effect of high oxygen pressure (100 % O_2) on Q_{O2} and *RQ*. *t* = time after transfer of the discs from thiamine-free to complete medium. Experiments were done in triplicate.

	t (hr.)	$Q_{O_2}^{air}$	$Q_{O_2}^{oxygen}$	RQ^{air}	RQ^{oxygen}
25° C	28.3	137.8 ± 6.2	124.7 ± 11.7	1.68 ± 0.14	1.85 ± 0.23
30° C	28.5	179.5 ± 30.3	167.9 ± 15.5	2.13 ± 0.35	1.80 ± 0.15

DISCUSSION

At first glance, the time course of the respiratory rate of a developing *Schizophyllum* culture (Figs. 19 and 20) greatly resembles similar curves obtained with other fungi (COCHRANE, 1958). However, considering the changes in respiratory rate in relation to environmental changes accompanying morphogenesis as outlined in the early chapters of this study, the curves suggest that the high respiratory rate is maintained for some time after net protein synthesis ceases.

Most studies on respiration during the developmental cycle have

been performed with bacteria, yeasts, and other fungi grown in shaken cultures. It is conceivable that in these systems, after protein synthesis comes to a halt, the metabolic activities, including respiration, gradually decrease. On the other hand, if morphogenetic processes succeeded the growth phase, it would not be surprising to find a high metabolic activity such as respiration in the absence of growth (as measured by the assimilation of nitrogen).

In fact, a fine example of such a situation is provided by the slime molds in which GREGG (1950) has shown that oxygen consumption (on a nitrogen basis), increases from the amoeba stage (the growth phase in this organism) until the stage of multicellular differentiation just prior to stalk formation. It has also been shown that during the differentiation phase there is a striking reorganization of cellular constituents, including the synthesis of cellulose from protein precursors, associated with pronounced changes in the activity of a number of enzymes (WRIGHT and ANDERSON, 1958).

From Fig. 21 it may be concluded that in *Schizophyllum* the phase of primordium growth is also characterized by a high respiratory rate. However, unlike the slime molds, the high respiratory rate and growth of the primordia are only maintained if an exogenous carbohydrate supply is available. It will be recalled that primordium growth depends on the supply of nitrogenous compounds from the mycelium, but involves *de novo* synthesis of cell-wall polysaccharides. In this context it is pertinent that exogenous glucose does not prevent a decline of respiration if primordium growth is inhibited by a higher temperature (30°C).

Concerning the decrease of the aerobic fermentation in the presence of glucose at 25 and 30°C, the results indicate that this decrease is chiefly due to a gradual abolishment of fermentative capacity as judged from carbon dioxide production under nitrogen.

The inhibition of fermentation by respiration seems to be rather low as compared with the M.Q. usually found in yeasts and plant tissues, although the absence of a Pasteur effect has also been reported in these materials (cf. TURNER, 1960). In Fig. 24 it can be seen that even lower M.Q. values are found during growth on the medium containing asparagine. Speculating upon the mechanism of a possible control of aerobic fermentation by protein synthesis, one can assume that the synthesis of amino acids and proteins tends to maintain a high level of ADP and inorganic phosphorus, thus counteracting the effect of respiration upon the rate of glycolysis via the adenylate-phosphate system. Such a mechanism has been shown to be very probable in yeasts (HOLZER *et al.*, 1955), and it has also been suggested that it is responsible for the active aerobic fermentation commonly found in meristems of higher plants (BETZ, 1960).

At present, we have very little information about the possible routes for glucose dissimilation in *Schizophyllum*. The only available data concern the presence of the reactions of the tricarboxylic acid cycle (WESSELS, 1959) and the existence of a cytochrome-dependent electron transport (NIEDERPRUEM and HACKETT, 1961). As long as no

further enzymatic data are available, we can only speculate upon the mechanism of the changes described in this chapter. Nevertheless, such speculations are of interest where they relate biochemical processes to morphogenetic events.

During the transition of undifferentiated vegetative cells to stroma and primordia (25°C) or aerial mycelium (30°C) an important new physiological element is introduced. Whereas the undifferentiated submerged cells are bathed in the nutrient solution, the cells of primordia, stroma, and aerial mycelium are exposed to less favourable conditions because they are far removed from the nutrients. It is clear that glucose, for instance, can reach these cells only via a long path of transport.

For example, during the growth of the primordia in the absence of an external nitrogen supply two concurrent processes probably progressively hamper the access of glucose to the growing — and thus presumably most active — cells of the primordia. In the first place, as has been shown, during primordial growth the greater part of the cytoplasm of the submerged cells is displaced to the primordia and becomes dependent upon translocated sugar, and secondly, such a decrease in the amount of cytoplasm of the submerged cells probably influences the rate of sugar uptake or the transport proper.

Consequently, we are faced with the possibility that the concentration of glucose at the site of the most active parts of the developing organism decreases gradually. It is obvious that similar changes could be involved if no primordia but only a stroma layer and aerial hyphae are produced (30°C).

If we accept that the availability of glucose to the active cells limits the rate of glycolysis, a change in the steady-state concentration of pyruvate is apparent. Studies by Holzer and coworkers (summarized by HOLZER, 1961) with yeast and animal tissue reveal a great difference between the Michaelis constants of pyruvate decarboxylase and pyruvate oxidase. The former enzyme, which catalyzes a practically irreversible reaction leading to fermentation products (e.g. alcohol and carbon dioxide), has a much lower affinity for pyruvate than the latter enzyme, which brings about the irreversible reaction leading to acetyl-CoA and thus accounts for the major part of respiration. Holzer points out that any factor which lowers the steady-state concentration of pyruvate below the saturation point of pyruvate decarboxylase would first influence pyruvate decarboxylation and hence fermentation, whereas a decrease in the oxidation of pyruvate to acetyl-CoA (and further reactions leading to complete oxidation) would not be seen until the steady-state concentration of pyruvate became extremely low.

Such a mechanism could satisfactorily explain the changes in the ratio of respiration and fermentation during development in *Schizophyllum*. In accordance with the operation of such a mechanism is the response of the mycelium-agar discs to transfer to a medium not supplemented with glucose (the second transfer in Fig. 21). As a consequence of this operation, the concentration of glucose in the

environment is considerably lowered and, after one day, respiration is decreased by 50 %. However, it can be seen that at the same time the high anaerobic and aerobic fermentation present at the moment of transfer is practically completely abolished.

Lastly, some comments must be made on the small difference in respiratory rate of stocks K.8 and K.35 after the exhaustion of the glucose supply. The principal reason for this small difference must be the relatively small amounts of cell-wall polysaccharides which are presumably oxidized by K.8. Further, the difference is apparently obscured by a residual respiration that drops only slowly and cannot be accounted for by the disappearance of cell constituents.

This is shown by experiments with K.35. After the exhaustion of the carbon supply and the rapid consumption of a small amount of acid-soluble polysaccharides, no further decrease of cellular carbohydrates can be observed in this stock (see Chapter IV). Likewise, no significant decrease in the protein and lipid content was observed (lipid extracted with petroleum spirit after acid-hydrolysis). One explanation we can put forward here is that a glucose gradient is established owing to the slow diffusion of the glucose through the agar. After the glucose in the immediate surroundings of the hyphae has been used up (a point marked by the sharp decrease in respiratory activity), a slow stream of glucose originating from the deeper agar layers is fed to the cells, thus sustaining a slow respiration.

Another possibility to be taken into consideration is that small quantities of substances previously excreted into the medium (for example water-soluble polysaccharides; cf. WANG and MILES, 1964) are taken up again after exhaustion of the glucose supply. However, in connection with the theory concerning the effect of glucose on pileus formation outlined in the preceding chapter, it must be assumed that the amount of carbohydrates reaching the cells of K.35 in this stage is not large enough to compensate for the incapacity to break down cell-wall polysaccharides.

CHAPTER VII

THIAMINE IN RELATION TO GROWTH AND THE INITIATION OF PRIMORDIA

INTRODUCTION

In the work described in the preceding chapter use was frequently made of the possibility of cultivating *Schizophyllum* in a medium lacking thiamine. Although growth is severely limited, the advantage of this method is that formation of primordia and stroma is completely inhibited.

The decision to use thiamine as the means to control the initiation of primordia was made after the effect of some other environmental factors on this process had been studied.

It will be recalled that cultivation at higher temperatures (30–37°C) restricted fruiting completely and caused the development of an abundant aerial mycelium. The same phenomenon was observed when Petri dishes containing a synthetic agar medium — which normally supported fruit-body formation — were sealed with adhesive tape (in agreement with results of NIEDERPRUEM, 1963). A comparable growth pattern was also obtained when sodium bicarbonate was added to the medium. A bicarbonate concentration of 0.05 M inhibited fructification completely and greatly enhanced the formation of aerial mycelium (unlike all the other experiments, the pH was 8.0 in this case).

The fact that these different environmental conditions all have virtually the same morphogenetic effect suggests a common underlying principle, and it seems plausible that this could be the concentration of carbon dioxide (or bicarbonate ion). In all the cases cited above, carbon dioxide concentrations higher than normally present can be expected.

Carbon dioxide accumulation has been shown to inhibit asexual reproduction in *Choanephora cucurbitarum* (BARNETT and LILLY, 1955) and carpophore development in *Agaricus campestris* (LAMBERT, 1933) and *Collybia velutipes* (PLUNKETT, 1956). Recently, NIEDERPRUEM (1963) has obtained conclusive evidence that at least the effect of sealing cultures of *Schizophyllum commune* is ultimately due to accumulation of respiratory carbon dioxide.

In Chapter I we discussed a possible relationship between stroma and primordia. Now we have found that in several instances a stroma-like layer can be seen under the aerial mycelium of the cultures of *Schizophyllum* referred to above. This suggests that it is not so much the initiation of primordia that is inhibited as the growth of the primordia. If this is true, these mycelia are worthless for studying the first stages of differentiation because differentiation has already occurred to a remarkable extent.

We therefore sought a way to cultivate *Schizophyllum* under conditions permitting only the formation of undifferentiated (non-aerial) mycelium. Two sets of conditions were found to meet this requirement:

Primarily, lowering of the oxygen concentration to 1 % resulted in a decrease of mycelial yield to only 50 % (dry weight of mycelium: agar removed by means of hot water; maximum yield obtained at about 6 % oxygen concentration). However no primordia, stroma, or aerial mycelium were produced at this low oxygen concentration.

Secondly, growth in the absence of or at very low concentrations of thiamine was likewise characterized by the complete absence of primordia, stroma, and aerial mycelium. This seems to be in accordance with results of RAPER and KRONGELB (1958), although these authors refer only to the production of mature fruiting bodies and not to the production of primordia. Because the possibility of experimental control of primordia initiation via a factor in the medium was thought to be more convenient than control via the atmosphere, we undertook a study to explore the former possibility further.

Thiamine deficiency has been generally accepted for *Schizophyllum* and many other wood-rotting Hymenomycetes (ROBBINS, 1938; SCHOPFER and BLUMER, 1940). The basis of this deficiency in*Schizophyllum* seems to be the lack of synthesis of the pyrimidine moiety of this vitamin (ROBBINS and KAVANAGH, 1938), although RAPER and KRONGELB (1958) claim that "fruiting was earlier and better on thiamine than on pyrimidine" (2-methyl-4-animo-5-ethoxymethyl pyrimidine).

METHODS

Stock K.35 was used throughout. The cultivation of the fungus on sand, replacement of medium, and determination of growth yield by measurement of total nitrogen are described in Chapter II. The medium given there will be referred to as basal medium with respect to the mineral components. Further additions are given under Results. Because of good reproducibility of growth yield, experiments were done in duplicate only.

RESULTS

1. *Effect of various thiamine concentrations on growth, production of fructifications, and accompanying changes in the medium*

The results are given in Fig. 25. It can be seen that there is a linear relationship between the concentration of thiamine (up to 20 mμg/ml) and the final yield as measured by total nitrogen determination. Although thiamine in concentrations higher than 20 mμg/ml exerts no influence on total nitrogen yield, there is a distinct influence on the ratio of the amount of nitrogen contained in mycelium and fructifications. These higher thiamine concentrations do not seem to influence the number of fructifications formed; the increase in nitrogen must therefore be attributed to an increase in the nitrogen content of individual fructifications. Parallel to this increase in nitrogen there is also an influence on the final morphogenetic stage reached. Whereas at 20 mμg/ml thiamine the fructifications do not develop beyond the primordial stage, practically normal cup-shaped fruiting bodies are formed at 50 mμg/ml. Comparison of Fig. 25 with the growth curves given in Chapter II shows clearly that thiamine deprivation must have a profound effect on the transport of nitrogenous compounds from the mycelium to the developing fructifications.

At concentrations of thiamine below 6 mμg/ml, no primordia are produced in this stock. Only submerged ("wet") mycelium is produced, albeit in small amounts. At a concentration of 6 mμg/ml, initiation of primordia can be clearly observed; their number increases with increasing thiamine concentration and reaches a maximum at a concentration of 20 mμg/ml.

It could be expected that thiamine deprivation would lead to a decrease of glucose consumption. This was found to be indeed the case in the lower thiamine range, and pH measurements showed

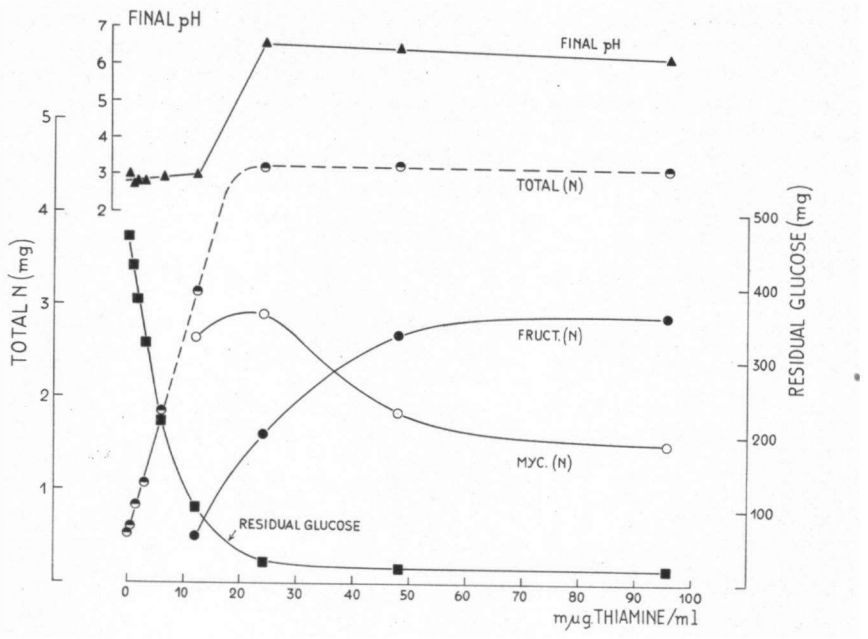


Fig.25. Effect of various thiamine concentrations on nitrogen yield of *S. commune* K.35, final pH, and residual glucose in the medium. Where possible, mycelium and fructifications were analyzed separately. Medium: basal medium (low phosphate) plus 2 % glucose and 0.15 % asparagine. Initial pH 6.7. Glucose initially present per culture (30 ml) determined as 619 mg. Incubation time 11 days.

further that appreciable quantities of acid are produced under these conditions. In the range above 20 mμg/ml total glucose uptake and final pH are not influenced, but it is of course likely that in this range, thiamine would influence either the rate or path of glucose metabolism or both and in this manner would exert an effect on morphogenetic events.

Preliminary experiments showed that repeated transfer of small inocula in the medium not supplemented with thiamine always yielded about the same amount of mycelium (visual estimation). Because this medium was considered essentially thiamine-free, these observations suggested that an active thiamineless growth is possible, and made it seem worth-while to investigate this point further, especially because it implied that the processes leading to the formation of primordia could be uncoupled from the processes of mycelial growth.

2. *Composition of the medium and mycelial growth without thiamine*

If undifferentiated growth is possible without thiamine in the medium, at least some of the effects created by thiamine deficiency could possibly be counteracted by adding certain compounds to the thiamine-free medium. For instance, compounds that neutralize the

acid formed or substitute for metabolites not readily formed in the absence of thiamine could have such an effect.

A number of variations of the medium and their effect on thiamineless growth in *Schizophyllum* are shown in Table 8. Only data with

TABLE 8

Growth of *S. commune* K.35 in media not supplemented with thiamine. Yield expressed as mg N per culture (30 ml). The listed components were added to basal medium containing 36.5 mM PO_4 (high phosphate) and 2 % glucose, unless indicated otherwise. Concentrations of added substances: all nitrogen sources (except trypticase 0.4 %) in amounts on a nitrogen basis equivalent to 0.15 % asparagine; $CaCO_3$, 2 %; $NaHCO_3$, 0.01 M; acetate, succinate, and citrate (sodium salts), 0.05 M. The initial pH was measured before addition of $CaCO_3$.

	Incubation time (days)	Initial pH	Final pH	Yield	
				I	II
NH_4Cl, 9.125 mM PO_4	10	6.7	2.7	0.35	0.59
NH_4Cl	10	6.7	3.9	0.91	0.91
NH_4Cl + $CaCO_3$	10	6.7	5.2	0.95	1.11
NH_4Cl + $CaCO_3$ + acetate	10	6.7	—	1.14	1.34
NH_4Cl + $CaCO_3$ + succinate	10	6.7	—	1.75	—
NH_4Cl + $CaCO_3$ + citrate	10	6.7	5.6	1.98	2.16
NH_4Cl + $CaCO_3$ + citrate	11	5.0	5.5	2.04	2.06
NH_4Cl — $CaCO_3$ + citrate	11	5.0	4.2	1.00	1.10
NH_4Cl + $CaCO_3$ + citrate (— glucose)	11	5.0	—	0.00	0.00
asparagine	12	6.9	3.9	0.81	0.83
asparagine + $CaCO_3$	12	6.9	5.2	0.86	0.92
asparagine + $CaCO_3$ + citrate	12	6.9	5.3	2.19	2.33
asparagine + $NaHCO_3$	12	7.2	3.9	1.02	1.04
asparagine + $NaHCO_3$ + citrate	12	7.2	4.8	1.69	1.71
trypticase (0.2 %) + $CaCO_3$	9	6.7	—	4.29	4.29
trypticase (0.4 %) + $CaCO_3$	9	6.7	—	5.80	6.70
trypticase acid hydrolyzed + $CaCO_3$	9	6.7	—	1.94	2.22
casamino acids + $CaCO_3$	9	6.7	—	2.44	2.50
asparagine + $CaCO_3$ + thiamine	7	6.7	5.9	6.63	6.83

the same incubation time are derived from one experiment, but reproducibility in repeated experiments was very good and nitrogen increment between the 7th and 12th day was very small or entirely absent. It therefore seems justifiable to compare these data.

To prevent severe lowering of the pH, the buffer capacity was increased by quadruplication of the phosphate concentration. Doubling of the yield was indeed obtained. If calcium carbonate was added to this high phosphate medium, only a small further increase was noted, if any.

Acetate, succinate, and citrate stimulate thiamineless growth. Citrate is especially effective, since it increases the yield by 100 %. The optimal concentration was found to be 0.05 M. The presence of glucose is necessary; citrate cannot serve as the sole carbon source at the given pH.

It is of interest that the effect of citrate is displayed only in the presence of calcium carbonate or sodium bicarbonate. Thus, although citrate and (bi)carbonate added separately have no effect, the results

indicate that the combination citrate-(bi)carbonate is very effective. (As a matter of fact, it is important to shake the sand and calcium carbonate vigorously with the liquid in order to obtain satisfactory dispersion of the calcium carbonate particles.)

In none of the cases cited above was the slightest indication of primordia formation observed.

To overcome a possible inhibition of amino-acid synthesis in thiamine-free media as a result of limited availability of tricarboxylic-acid intermediates, the effect of the inclusion of protein hydrolysates in the medium was also studied. From Table 8 it can be seen that substitution of trypticase for asparagine or ammonium in an equivalent concentration (on a nitrogen basis) results in a four-fold increase of the mycelial yield. (Trypticase: pancreatic digest of casein, B.B.L. Baltimore, analyzed 12 % N.) Doubling the concentration of trypticase results in a nitrogen yield of the mycelium comparable to that obtained with asparagine in the presence of thiamine.

Acid hydrolysis of the peptides in the trypticase (as for protein hydrolysis; cf. SNELL, 1957) results in a preparation (7 % N) with only part of the growth-promoting effect of trypticase, although the yield is better than with asparagine as the sole nitrogen source. The same amount of mycelium is obtained with a commercial acid hydrolysate of casein (casamino acids, Difco, vitamin-free, 8 % N). Since tryptophane is destroyed by acid hydrolysis it was tested separately but showed no growth-promoting effect. Possible traces of thiamine present in trypticase seem too small to account for its pronounced effect (see section 3); therefore this suggests a beneficial effect of certain peptides on thiamineless growth. This point was not investigated further.

In contrast to the cultures receiving a simple nitrogen source, cultures receiving protein hydrolysates show an indication of primordia initiation some time after maximum growth is attained. Indeed, in older cultures very small primordia are apparent. However, in this case an effect on primordia initiation of very small amounts of thiamine which could contaminate the protein hydrolysates cannot be excluded. It was shown in section 1 that the concentration of thiamine permitting induction of primordia is much lower than the concentration supporting maximum growth.

3. *Traces of thiamine in medium and inoculum*

It is of course essential to know to what extent growth without added thiamine is influenced by traces of thiamine present in the medium or inoculum. To prepare a medium from which the last traces of thiamine are removed, the following procedure was adopted. The medium, minus the trace elements and Fe, was boiled twice with activated charcoal (pharmaceutical-grade Norit, 5 g/l) for 10 minutes under reflux and then filtered, after which the trace elements and Fe were added (LILLY and BARNETT, 1951).

Table 9 shows the effect of repeated transfer in media with and

TABLE 9

The effect of both the removal of traces of thiamine from the medium and repeated transfer of mycelium on the growth of *S. commune* K.35. Yield expressed as mg N per culture (30 ml). Medium: high-phosphate basal medium plus 2 % glucose, 0.15 % asparagine, 0.05 M sodium citrate, and 2 % $CaCO_3$. The first inoculum was taken from malt agar.

		Incubation time (days)	Yield	
			I	II
medium treated with Norit	first transfer	10	1.75	1.77
	second transfer	10	1.87	1.89
	third transfer	9	1.82	1.90
medium not treated with Norit	first transfer	8	1.89	1.99
	second transfer	10	1.99	2.11

without Norit treatment. The data indicate that no stimulation of growth by traces of thiamine in the inoculum is apparent; repeated transfer in the Norit-treated medium does not diminish the final yield. The fact that the yields are somewhat higher (approx. 10 %) with the untreated medium must be attributed to trace quantities of thiamine as contaminants in the chemicals used. Thus, it seems that an appreciable amount of mycelium can be made in a medium free of thiamine.

These results do not exclude the possibility that growth in thiamine-free media merely reflects a limited capacity for thiamine synthesis stimulated by favourable conditions. However, there are two arguments which make it unlikely that such a mechanism occurs.

Primarily, small amounts of synthetized thiamine, sufficient to support the growth recorded, should also become manifest in the production of primordia. However, not the slightest indication of primordia induction can be observed in the mycelia grown without thiamine in the presence of citrate and carbonate.

Secondly, using an HCl extraction and the microbiological assay of WEEKS and BECK (1960), it was impossible to detect thiamine in a mycelium grown without thiamine and with trypticase as the nitrogen source. On the other hand, appreciable quantities of intracellular thiamine were found in a similar amount of mycelium produced in the presence of thiamine with asparagine as the nitrogen source. With respect to the production of primordia in trypticase media not supplemented with thiamine it must be admitted that, in our hands, the assay method cited above was not sensitive enough to detect quantities sufficient for primordia induction.

4. *The nature of the acid produced during thiamineless growth*

In a preliminary experiment, acids were extracted from the culture fluid by using the ion-exchange method of BRYANT and OVERELL (1953), followed by paper chromatography. This method did not yield clear-cut results, although orthophenylene diamine and semicarbazide

sprays indicated the presence of α-keto acids. In view of the instability of keto acids in this procedure, the acids were converted to 2,4-dinitrophenyl hydrazones in the culture fluid and determined by the method of Friedemann and Haugen (FRIEDEMANN, 1957). The hydrazone formed could be extracted with ethyl acetate and ethyl benzene but not with benzyl alcohol. Further, its absorption spectrum, after addition of alkali, was identical to the spectrum obtained with the hydrazone of pyruvic acid.

These results strongly point to pyruvic acid as the only acid formed in substantial amounts in thiamineless cultures. Quantitative measurements demonstrated that the amount of pyruvic acid produced is sufficient to account for the lowering of the pH of the medium. Only trace amounts of lactic acid are produced.

5. Induction of primordia formation by thiamine in a pre-formed mycelium

Numerous experiments were designed to induce primordia formation in an undifferentiated mycelium under conditions permitting no net protein synthesis, i.e. in the absence of a nitrogen supply. Such an experimental set-up was thought to offer an ideal opportunity to study differentiation leading to primordia. Thus, any biochemical changes occurring during differentiation would be neither confused by nor interdependent on biochemical changes accompanying growth (i.e. growth of the whole organism). Unfortunately, all these experiments were unsuccessful.

These experiments led us to conclude that differentiation in this stage is strictly dependent on an external nitrogen supply. We therefore determined the minimum concentration of asparagine required in combination with thiamine to induce primordia (as visible to the naked eye). Using mycelium grown in a glucose-citrate-asparagine-calcium carbonate medium, this concentration proved to be 0.4 mg/ml. Much lower concentrations (0.02–0.2 mg/ml) were also sometimes effective but did not yield reproducible results. Moreover, the appearance of primordia was greatly retarded with these low asparagine concentrations.

Fig. 26 shows changes in total nitrogen in an experiment in which primordia were induced in a pre-formed, undifferentiated mycelium in the presence of asparagine concentration of 0.4 and 1.5 mg/ml.

The lowermost curve reflects growth without thiamine in a glucose-citrate-asparagine-calcium carbonate medium. The mycelium will be regarded as undifferentiated because not the slightest indication of primordia or aerial mycelium could be observed. In old cultures, whether or not grown in the presence of thiamine, chlamydospore-like cells can be found scattered through the mycelium and thus the thiamineless mycelia are not entirely undifferentiated with respect to these cells. However, at the moment of replacement these "chlamydospores" are found only sporadically.

If, at the moment indicated in Fig. 26 (arrow), the medium is replaced by a medium containing thiamine, the first primordia become

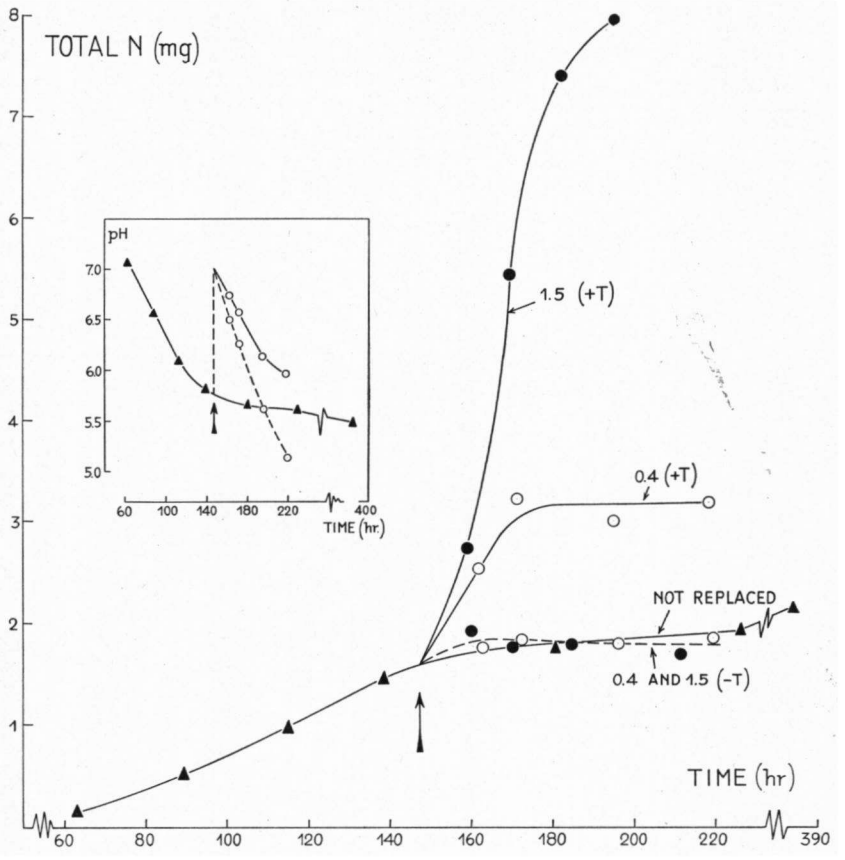


Fig. 26. Induction of primordia by thiamine in a pre-formed undifferentiated mycelium of *S. commune* K.35 and accompanying changes in total nitrogen. Medium for undifferentiated growth: basal medium (high phosphate) plus 2 % glucose, 0.15 % asparagine, 0.05 M sodium citrate, and 2 % $CaCO_3$. Replacement medium: basal medium (high phosphate) plus 2 % glucose and asparagine in a concentration of either 0.04 or 0.15 % (in the graph indicated as mg/ml). Thiamine, if added, 120 mμg/ml. The insert shows accompanying changes in the pH of the medium; symbols are the same as in the major graph.

visible as small white tufts after approximately 24 hours and seem to increase in size and number during the second half of the experiment. This holds whether the asparagine concentration in the replacement medium is 0.4 or 1.5 mg/ml.

An impression of the morphogenetic features during this period of primordia formation can be gained from the micrographs shown in Fig. 2 (A-E). These pictures illustrate development on agar discs in a medium containing 1.5 mg asparagine per ml. If the view expressed in Chapter I concerning primordia initiation is correct, viz that the first visible differentiation is the intertwining of surface hyphae, then these processes must be looked upon as occurring within the first 24

hours after replacement. The curves in Fig. 26 suggest that especially this period is characterized by vigorous nitrogen incorporation. Further processes, e.g. cell multiplication in the primordial region, leading to the production of more clearly recognizable primordia and stroma can evidently proceed in the absence of nitrogen incorporation, as shown in the experiment with the low nitrogen concentration.

Fig. 26 further shows that omission of thiamine prevents any net incorporation of nitrogen from the replacement medium irrespective of the nitrogen concentration. No primordia are initiated under these conditions.

It is unlikely that under the conditions of the experiment thiamine exerts its effect by preventing a lowering of the pH. As can be seen from the insert in Fig. 26, acid is also produced after replacement with a medium containing thiamine. During the first 24 hours, when differentiation occurs, the pH values attained are only slightly different from those attained in a replacement medium lacking thiamine. Furthermore, it was observed that primordia can arise at a pH as low as 5.0.

That only the very first stages of primordia formation are dependent on an external nitrogen supply was also demonstrated in another experiment. Use was made of the fact that older cultures on a medium without thiamine but with trypticase as a nitrogen source bear very small primordia (see section 2). But at the moment at which such a culture enters the stationary phase, the initials are so small that they can only be detected with the microscope. When at this moment (culture age: 5 days; total N: 3.29 mg) a new medium containing glucose and thiamine but no nitrogen source is given, the whole surface bears primordia within 3 days (3 days after replacement: 3.03 mg N). If thiamine is omitted from the replacement medium the initials do not develop any further (3 days after replacement: 2.82 mg N).

Discussion

The results indicate that under certain conditions *Schizophyllum* can dispense with thiamine, although the final yield is always higher with thiamine added to the medium. Our knowledge concerning the intermediary metabolism of this organism is too scanty to permit adequate interpretation of the influence of substances in the medium on thiamineless growth, but some suggestions can be put forward.

It is of course impossible at the moment to make any statements concerning deviating catabolic routes of glucose during thiamine deprivation, but the accumulation of pyruvic acid in the medium suggests that at least part of the glucose is degraded via the glycolytic pathway. The accumulation of pyruvate, which can be readily explained by an inhibition of pyruvate decarboxylase, further suggests an impairment of the operation of the tricarboxylic acid cycle.

The literature contains a number of cases of successful "by-passing" of the thiamine requirement. In *Pityrosporum ovale*, Benham (1947) found that oxaloacetic acid plus α-ketoglutaric acid could be substi-

tuted for thiamine, and in *Trichophyton violaceum* oxaloacetic acid alone was active (GEORGE, 1951). In *Blastocladiella,* CANTINO (1951) was able to support growth with either thiamine or a mixture of acetate, bicarbonate, and pantothenate. SIVA SANKAR (1958) showed that oxythiamine toxicity in *Neurospora crassa* could be completely reversed by acetate, citrate, or succinate as well as by thiamine. It is suggestive that in these cases thiamine deprivation primarily blocks the entry of pyruvate into the tricarboxylic acid cycle and that "by-passing" of this step can be achieved by furnishing cycle intermediates. It therefore seems logical to assume that in our case the effect of substances such as acetate, succinate, and citrate is also due to provision of the cells with cycle intermediates, for example as precursors for amino acid synthesis. The effect of protein hydrolysates is consistent with this concept.

However, there are a few observations which complicate this picture. In the first place, the effect of citrate is only found if calcium carbonate or sodium bicarbonate is added simultaneously. Furthermore, growth experiments not reported in this paper have shown that in the presence of thiamine at pH 5.0 citrate cannot be used as the sole source of carbon, whereas acetate and especially succinate easily serve this purpose. This indicates that it is the permeation of citrate in particular which is hampered at the given pH; nevertheless, this compound exerts a maximum effect on thiamineless growth. It should be admitted, however, that permeation of small amounts of citrate and a more specific effect is not excluded. It should also be kept in mind that thiamineless growth is not increased by using asparagine instead of ammonium as the nitrogen source.

Another explanation for the observed facts is offered by considering that citrate in a concentration of 0.05 M adds to the buffer capacity of the medium around pH 5.4. In consequence, the pH of the medium drops relatively slow and levels off at a pH of 5.7–5.5 (insert Fig. 26), whereas in a medium without citrate the pH falls quickly to a level of 5.2. This difference is important with respect to the bicarbonate concentration in the medium: between pH 6 and pH 5 the concentration of this ion falls quickly to very low values. Now, if the extra bicarbonate stimulates a carboxylation reaction, e.g. the carboxylation of phosphoenolpyruvate, this could provide a dual benefit to thiamineless growth: lowering of a possible toxic intracellular pyruvate concentration and the production of 4C-acids.

Two observations lend some support to this suggestion. Firstly, the effect of calcium carbonate or sodium bicarbonate is only found in the presence of citrate. Secondly, the same mycelial yield as that obtained in the presence of citrate and calcium carbonate can be obtained with a medium lacking these compounds, at least if the medium is replaced a few times, thus maintaining a high pH. After the mycelium has attained a nitrogen yield of about 2 mg, further replacement of the medium causes but little, if any, increment of the nitrogen yield. (Not reported under Results, but compare Fig. 26.)

Whatever the mechanism of stimulated thiamineless growth may

be, a few remarks on mycelial growth under these conditions (with addition of citrate and carbonate) seem to be justified at this point. It has been repeatedly observed that the morphological basis of mycelial growth is that growth is limited to hyphal tips (SMITH, 1923; ZALOKAR, 1959a,b). Interior cells of the mycelium do not normally contribute to the new growth directly, although they supply nutrients to peripheral cells. Not only are external nutrients transported, but the cytoplasm of older cells can also be displaced and used for the growth at the young hyphal tips. Growth is therefore a function not of the total number of cells present, as in bacteria and yeasts, but of the number of hyphal tips and the rate at which these tips are supplied with nutrients.

Fig. 26 shows that after a phase of relatively rapid growth, total nitrogen of the culture continues to increase but at a much slower rate. Especially in this period the number of emptied cells can be seen to increase. At the same time, the total amount of protein and ribonucleic acid contained in the whole organism remains approximately constant, the slow increase of nitrogen being due to an increase of a non-protein component, probably chitin (see following chapter). It therefore seems that active growth continues to occur in these thiamineless cultures, although it is not expressed as an increase of cytoplasmic material of the organism taken as a whole.

It has been shown that thiamine can only induce primordia in a thiamineless, undifferentiated mycelium if a nitrogen source is also present, at least in small quantities. In view of the considerations given above we do not want to over-stress a specific effect of thiamine with respect to primordia initiation. Such a specific effect could be postulated if thiamine induced primordia in the absence of net protein synthesis. To the extent that thiamine induces the production of new (primordium) cells, it seems to make little difference whether these cells are produced from nutrients derived from the medium or at the expense of other cells. It has been shown, on the one hand, that the amount of undifferentiated mycelium formed in the absence of thiamine is greater than that formed prior to the initiation of primordia in cultures that have received thiamine and, on the other hand, that a small amount of external nitrogen is necessary for the very first stages of primordia initiation to occur. Once the initials have been formed, the nitrogen requirement of the newly-produced cells can evidently be provided by cell turnover.

At the moment, the most plausible explanation of the effect of thiamine seems to be that thiamine generally increases the rate of glucose breakdown via respiration. High respiratory activity seems to be a requisite of aerial systems in this fungus (primordia, stroma); the respiratory activity of the submerged undifferentiated mycelium is much lower, even in the presence of thiamine (see Chapter VI). The operation of such a mechanism is also suggested by the finding that low oxygen concentrations support the growth of the undifferentiated mycelium but prevent the formation of aerial systems.

The most important advantage of the thiamine effect lies in its use

as a tool for external control of primordia induction, as has already been shown in connection with the synchronization experiments. In the following chapter we will describe quantitative changes in cellular constituents that become apparent during thiamine-induced formation of primordia under conditions that also control the growth of the organism taken as a whole.

CHAPTER VIII

NUCLEIC ACID AND PROTEIN METABOLISM DURING THIAMINE-INDUCED PRIMORDIA FORMATION

Introduction

The preceding chapter describes experiments concerning the initiation of primordia in a pre-formed undifferentiated mycelium. Experimental control of this morphogenetic act was achieved by first cultivating an undifferentiated mycelium in a thiamine-free medium and then inducing primordia by removing the old medium and introducing a new one containing thiamine.

The attractive features of this system for the study of the biochemistry of primordia formation are obvious:

1. The amount of undifferentiated mycelium produced is larger than the amount normally found prior to the formation of primordia in stationary cultures supplemented with thiamine.
2. The time of primordia induction can be controlled more precisely and synchronism of this process is much better than in stationary culture.
3. During the induction of the primordia, growth of the whole organism can be controlled at the same time.

With regard to the last point it must be recalled that normally the initiation of primordia and growth of the undifferentiated mycelium go on simultaneously. In fact, primordia formation starts already in the very early phases of growth and continues as long as net nitrogen incorporation occurs (cf. Chapter II). It is clear that on the level of proteins or nucleic acids, possible qualitative changes specifically connected with differentiation would be greatly obscured by the simultaneous occurrence of rapid net synthesis of proteins and nucleic acids in non-differentiating cells.

In this chapter we wish to deal in some detail with quantitative aspects of protein and nucleic acid metabolism during undifferentiated growth and primordia formation under controlled conditions. The investigation served two purposes. One was to determine whether indications of morphogenetic significance could be detected in the metabolism of proteins and nucleic acids. Second was to determine whether the quantitative changes in these constituents could provide a basis for further investigations concerned with differentiation at the molecular level during the initial stages of primordia formation.

METHODS

1. *Cultivation, harvest, and homogenization*

Culture vessels, replacement procedure, and details of the media employed are described in Chapter II. For the establishment of a thiamineless, undifferentiated mycelium the following medium was used: basal medium (high phosphate) plus 2 % glucose, 0.15 % asparagine, 0.05 M sodium citrate, and 2 % $CaCO_3$. The replacement medium always contained the basal constituents and 2 % glucose; to this was added asparagine in a concentration of 0.15 % (high nitrogen) or 0.04 % (low nitrogen). To induce primordia, thiamine HCl was also added in a concentration of 120 mμg/ml.

Flasks were inoculated with mycelial fragments from *Schizophyllum commune* K.35 previously grown in the glucose-citrate medium given above for 5-6 days. In this way the carry-over of traces of thiamine by a malt-grown mycelium was avoided.

At various time intervals the fungus was harvested as described in Chapter II. However, prior to the washing procedure, calcium carbonate was removed by repeated suspension and filtration through a fine copper gauze which retained the fungal material and adhering sand but allowed passage of the fine calcium carbonate particles. The washing fluid consisted of 36.5 mM phosphate buffer (pH 7.0).

The harvest of each flask was taken up in about 10 ml ice-cold water and homogenized in a Mickle disintegrator using glass beads (diam. 3 mm). To keep the contents cool during the operations, the shaking flasks of this apparatus were modified in the following way. Double-walled flasks were constructed and ice-cold water was pumped through the outer jacket during homogenization. This procedure was effective in keeping the temperature at about 2–3°C. A 15 minute shaking time caused breakage of all cells, as judged microscopically.

The homogenates were made up to 25 ml with cold water and 5 ml cold 30 % trichloroacetic acid (TCA) was then added, resulting in a final concentration of 5 % TCA. The samples were then stored at —22°C for a maximum time of 7 days. In this way, material collected at different times could be submitted to the fractionation procedures simultaneously.

2. *Extraction of nucleic acids and their estimation*

The two standard procedures most commonly used for the extraction of nucleic acids are those of SCHNEIDER (1945) and SCHMIDT and THANNHAUSER (1945). The initial stages of these procedures are the same, viz. extraction with cold trichloroacetic acid (TCA) or perchloric acid (PCA) to remove acid-soluble materials, followed by a lipid extraction. In the Schneider procedure the nucleic acids (RNA and DNA) are then extracted with hot TCA or PCA, and RNA and DNA are estimated with a colorimetric method for ribose and deoxyribose. In the Schmidt-Thannhauser procedure RNA and DNA are separated by digestion in alkali which hydrolyzes the RNA to mononucleotides and thus makes it acid-soluble, whereas the DNA

and most of the cell protein are precipitated on acidification of the digest. The DNA can be subsequently extracted by alkali or hot TCA or PCA.

These two methods were compared as to their value for extracting nucleic acids from the *Schizophyllum* homogenates. It was found that the Schneider procedure (5 % TCA, 30 min. at 100°C) is completely unsuitable for this material. Based on pentose content of the extract, the "RNA" values found were 3–4 times higher than those based on ultraviolet (UV) absorption (DNA accounts for 5–10 % of total nucleic acid in this material).

Our knowledge of the cell-wall composition in *Schizophyllum* affords a reasonable explanation of this discrepancy. It has been shown that the cell walls contain xylose, which is easily split off by hydrolysis with dilute acid (Chapter III). It is conceivable that the hot-acid extraction of nucleic acids also brings xylose into solution and as a result, since the absorption spectra of the chromogens obtained with xylose and ribose in the colorimetric method used for pentose analysis (see later) are nearly identical, erroneously high "RNA" values are found.

In accordance with the physical properties of the polymer containing xylose, the Schmidt-Thannhauser procedure gave reliable results. It should be recalled that xylose is a component of the S-glucan fraction of the cell wall; one which is soluble in alkali but precipitates when the extract is acidified. Clearly, this explains the fact that RNA determinations in the acidified alkaline extract gave the same results whether based on pentose or UV analysis.

A subsequent alkaline extraction of DNA also brings all the S-glucan and protein into solution and makes these extracts unsuitable for determination of both the UV absorbance and the small amounts of deoxyribose present. However, unlike the alkaline extract, a hot TCA extract is practically free of protein or its breakdown products (as judged from absorption at 280 mμ) and can therefore be used for DNA estimation by UV analysis. Confirmation that the previous alkaline extraction removed all the RNA was obtained by reincubating the acid-precipitated residue (DNA fraction) with alkali and demonstrating that no further RNA could be obtained in the acid-soluble material. Attempts to measure DNA in the hot-TCA extract by deoxyribose content were unsuccessful. The indol method of CERIOTTI (1952) gave values that were too low, probably because of destruction of deoxyribose during the extraction procedure (HUTCHISON *et al.*, 1962).

No indication was found that omission of a lipid extraction influences the recovery or estimation of nucleic acids. Moreover, it has been reported that preliminary lipid extraction increases the amount of acid-soluble protein material (FLECK and MUNRO, 1962). This step was therefore omitted; only ethanol was used to dry the precipitates.

The final procedure adopted for nucleic acid estimation in *Schizophyllum* runs as follows:

The TCA-homogenate was quickly thawed, taking care that the

temperature did not rise much above 5°C. All further operations, including evaporation of ethanol *in vacuo*, were carried out at 0–4°C. Centrifugation was routinely done for 10 minutes at 20 000 *g* (Martin Christ, Omikron).

Five-millilitre aliquots of the 5 % TCA-homogenate were centrifuged and the precipitate washed with 5 ml 5 % TCA. The supernatants were pooled and made up to 10 ml with 5 % TCA for determination of the acid-soluble, UV-absorbing components and nitrogen content (by Kjeldahl analysis). The precipitate was then washed twice with 95 % ethanol and dried *in vacuo* for 1 hour. The dried powder containing the nucleic acids was then treated with 2 ml N KOH for 18 hours at 37°C. The specimens were cooled in ice, neutralized with 0.4 ml 6 N HCl, and acidified with 2 ml 11 % TCA (resulting in a final concentration of 5 % TCA). The mixture was centrifuged and the supernatant containing the RNA-nucleotides saved for estimation of RNA. The precipitate, containing DNA, protein, and cell-wall polysaccharides, was washed once with 5 % TCA and twice with 95 % ethanol and then dried *in vacuo*. To the dry residual powder, 3 ml 5 % TCA was added and DNA extracted by heating the tubes at 90°C for 30 minutes. The insoluble material was spun off and the supernatant saved for estimation of DNA.

All extracts were stored at —22°C. After thawing, a flocculent precipitate was often apparent. Therefore, all extracts were cleared by centrifugation prior to the analysis for pentose or UV absorbancy.

Fig. 27 shows typical UV spectra of the extracts prepared as described above. Routinely, readings were taken at 260 mμ (or 265 mμ in the case of DNA) and 320 mμ, and the difference taken as a

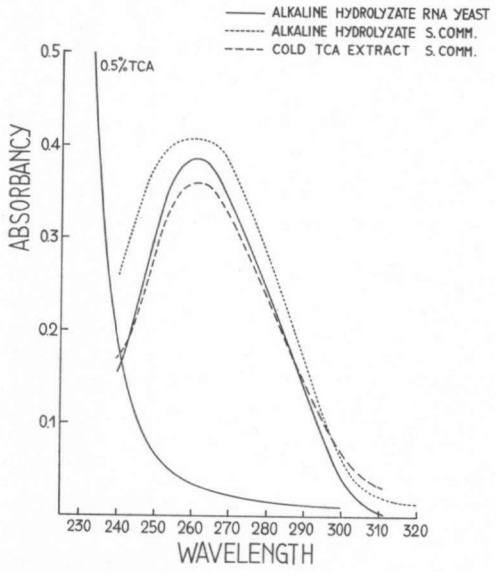


Fig. 27. Ultraviolet spectra of RNA and cold-TCA extracts of *S. commune*. For comparison, the spectrum of alkali-hydrolyzed yeast RNA is also given. Because of the strong absorbancy of TCA below 240 mμ, no records are given for this range.

measure for the amount of nucleotides present. The UV absorption spectrum of the cold TCA-soluble material is nearly identical to that of alkali-hydrolyzed RNA. For the sake of convenience, the material responsible for this absorption spectrum will be indicated henceforth as "free nucleotides". It is realized, however, that this is a rather arbitrary assumption because no further analysis of this material was made.

The acidified alkaline extract contains a considerable amount of polysaccharide material in addition to RNA-nucleotides (cf. Chapter IV). The liberation of glucose makes the orcinol method of MEJBAUM (1939) unsuitable for the estimation of pentose. Therefore, several modifications of the orcinol reaction were tried; the method of DISCHE (1953), using a higher acid concentration and a 3-minute heating time, was found suitable. Analysis of the absorption spectrum of the chromogen showed it to be composed of a ribose-orcinol and a glucose-orcinol chromogen. However, interference of the latter substance with the pentose determination could be eliminated by using a two-wavelength procedure (DISCHE, 1955).

The commercial orcinol was recrystallized according to SCHNEIDER (1957). As standards, yeast RNA (BDH) and herring-sperm DNA (Fluka) were used.

3. *Estimation of protein*

Five-millilitre aliquots of the TCA-homogenate were centrifuged. The precipitate was washed with 5 ml 5 % TCA and the tubes drained. The protein was then solubilized by suspending the precipitate in 5 ml N NaOH and heating in a boiling water-bath for 10 minutes. Prolonged heating did not yield more protein. The residue was spun off, washed with 4 ml N NaOH, and the supernatants combined and made up to 10 ml. Protein was then estimated according to LOWRY *et al.* (1951), using bovine albumin (Sigma, fraction V) as a standard.

RESULTS

1. *Thiamineless growth*

Fig. 28 shows the increment of total nitrogen and the increment of some classes of cell constituents of *S. commune* K.35 growing in a thiamine-free medium.

If the data in Fig. 28 are used to calculate the amount of nitrogen presumably contained in the several nitrogenous compounds and the results compared with total nitrogen of the organism, it is found that the percentage of protein- and RNA-nitrogen drops steadily, whereas the percentage of soluble nitrogen remains approximately constant. For example, using a conversion factor of 0.136 for protein (based on the nitrogen content of the standard), for a young culture (90 hrs.) about 85 % of the total nitrogen is found in protein, whereas in an older culture (230 hrs.) this value amounts to only 54 %. It therefore

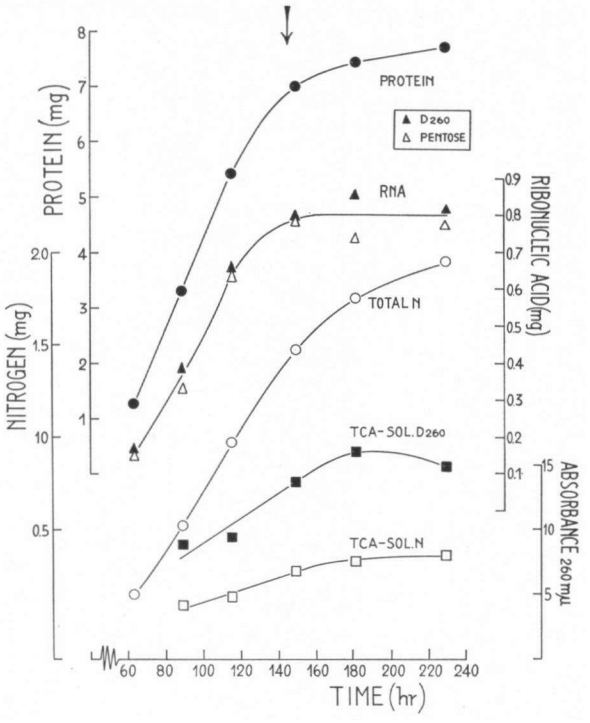


Fig. 28. Total nitrogen, protein, RNA, and the UV absorbance and nitrogen content of the acid-soluble fraction of *S. commune* K.35 during growth in a thiamine-free medium. Composition of the medium: basal medium (high phosphate) plus 2 % glucose, 0.15 % asparagine, 0.05 M sodium citrate, and 2 % $CaCO_3$. The arrow indicates the moment at which the medium was replaced in the experiments shown in Figs. 29–33. Each point represents the average of two cultures.

seems that an increasing amount of nitrogen becomes deposited in an unmeasured fraction, probably chitin. As no direct chitin measurements were made on this material, this can be taken only as indirect evidence for a non-proportional increase of this cell-wall substance. A non-proportional increase of cell-wall material is consistent with the cytological observation that after net protein synthesis slows down the number of emptied cells increases.

The discussion in the preceding chapter contains some considerations concerning mycelial growth. If these apply to the culture described in Fig. 28, then the net increase in cellular constituents yields little information about the actual growth processes in the hyphal tips. Similarly, the evaluation of the relative proportions of the various cell constituents, such as the RNA/protein ratio, probably yields only average values representing those of both growing and non-growing cells.

In Fig. 28 it can be seen that RNA formation is roughly proportional to protein synthesis. The RNA/protein ratios vary somewhat

but do not significantly decrease after net protein synthesis ceases; for the whole growth curve a mean value of 0.118 ± 0.016 was found in this case (11 determinations, RNA measured by UV absorption). Similarly, growing the fungus without thiamine in a medium containing trypticase as the nitrogen source results in a RNA/protein ratio that remains at a high level (about 0.125) for at least 7 days after net protein synthesis ceases.

It has been observed for many biological systems that the rate of protein synthesis and the total amount of RNA are concomitant variables (for a review of the early literature concerned with this topic see BRACHET, 1955). Therefore, it might be assumed that the high RNA/protein ratio in the system described here is due to the occurrence of an active cell turnover. However, even in that case a drop in the RNA/protein ratio would be expected after net protein synthesis slows down. On the other hand, growth without thiamine could feasibly result in the creation of abnormal intracellular conditions that could interfere with protein or nucleic acid metabolism in such a way as to lead to the maintenance of a high RNA/protein ratio even when the rate of protein synthesis slows down.

Fig. 28 also shows the amount of TCA-soluble, UV-absorbing material. Assuming this material to be nucleotides, it can be calculated that 30–50 % of the whole cellular nucleotide material (including RNA) is contained in this fraction.

2. *Shift of a thiamineless mycelium to a high-nitrogen medium in the presence or absence of thiamine*

If, at the moment indicated by the arrow in Fig. 28 a high-nitrogen medium supplemented with thiamine is given, a rapid increase in total nitrogen occurs. After approximately 24 hours the first primordia can be seen macroscopically; the next 24 hours are especially characterized by the further elaboration of the surface layer comprising primordia and stroma (compare also Fig. 2). Figs. 29 and 30 show the complex changes in cell constituents which become apparent after the replacement of the medium.

In the first 24 hours the amount of protein and DNA synthetized is proportional to the increase in total nitrogen. However, the net increase of RNA is much slower, resulting in a continuous drop of the RNA/protein and RNA/DNA ratios.

This is contrary to expectation because the addition of thiamine results in a resumption of net protein synthesis at an even higher rate. In bacteria a shift from slow to fast growing conditions is invariably connected with an immediate adjustment of the RNA content to higher values, creating a higher RNA/protein ratio that is typical for the higher growth rate in the new medium (KJELDGAARD *et al.*, 1958; NEIDHARDT and MAGASANIK, 1960; KJELDGAARD, 1961).

In the preceding section evidence is given that suggests the possibility that there is an abnormally high RNA/protein ratio in a thiamineless mycelium, this ratio bearing no direct relationship to the actual rate of protein synthesis. It can therefore be argued that

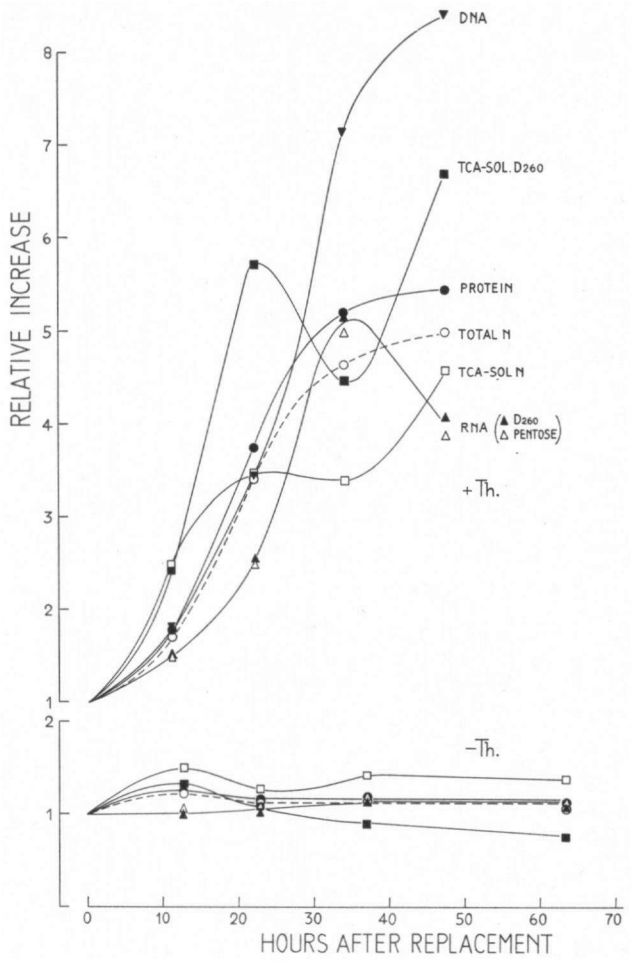


Fig. 29. *S. commune* K.35 shifted from thiamine-free medium to a high-nitrogen medium with or without thiamine at time zero (arrow in Fig. 28). Replacement medium: basal medium (high phosphate) plus 2 % glucose, 0.15 % asparagine, and with or without thiamine (120 mμg/ml). $CaCO_3$ remained in the culture during replacement. Each point represents the average of two cultures.

one of the effects of adding thiamine is the restoration of the normal control of protein and nucleic acid metabolism. The production of protein at a higher rate than RNA should gradually reduce the RNA/protein ratio to lower values.

However, such net changes must be interpreted in relation to the multicellular nature of the organism. For instance, these changes can be satisfactorily explained by assuming a dual effect of thiamine: firstly, the rapid production and proliferation of new cells and secondly, a release of RNA degrading activity in the "old cells" produced in the absence of thiamine. In this sense it is possible to

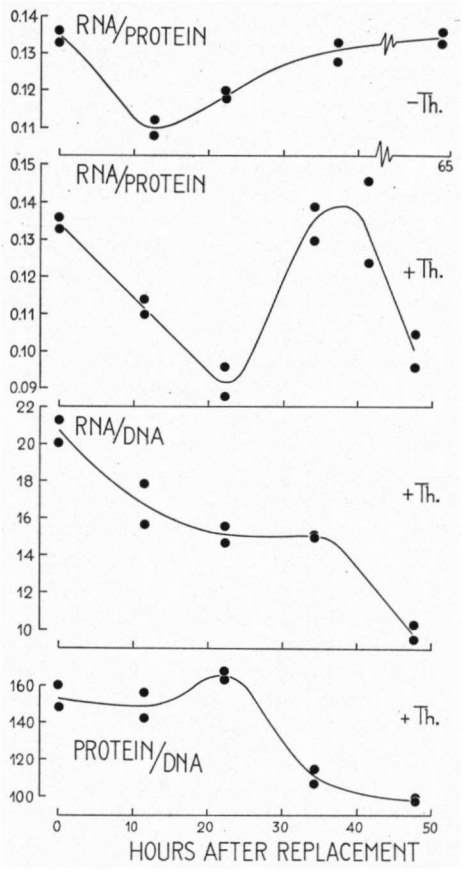


Fig. 30. Calculated ratios of cell constituents from the experiment shown in Fig. 29. Each point represents one culture.

understand a drop in the RNA/protein ratio of the whole organism notwithstanding the constancy of a certain, possibly rather high, ratio in the growing cells. If this view is accepted as a provisional hypothesis, the other complex changes depicted in Figs. 29 and 30 (and the data to be presented in the following section) can be easily explained.

It can be seen that during the first 24 hours after replacement the amount of "free nucleotides" rises much more than any of the other cell constituents. This is what would be expected if during the balanced synthesis of cell constituents from the nutrients in het medium in the growing cells, a preferential degradation of RNA in the preexisting cells occurs. Interestingly, these breakdown products appear to be retained by the cells rather than excreted into the medium.

After approximately 24 hours the release of "free nucleotides" from "old RNA" evidently comes to an end; a rapid increase of total RNA follows, and this increase probably gives a better estimate of

its actual rate of formation. As a consequence, the RNA/protein ratio is no longer lowered by the presumed degradation of RNA in the "old cells" and hence increases rapidly.

It will be recalled that after 24 hours the first primordia become visible. As was shown in the previous chapter, from this moment on, growth at the surface of the culture can draw upon pre-existing cells, i.e. cell turnover now becomes evident.

In the experiment discussed here, after 24 hours the net nitrogen incorporation begins to slow down. Now synthesis of cell material in the upper regions of the culture can only continue at the same rate if, in addition to the uptake of nutrients from the medium, cell constituents from the pre-existing cells are transported and act as substrates for synthetic activity in the growing cells. The changes in the amount of "free nucleotides" suggest this to be true for nucleotides as precursors of RNA synthesis. It is likely, however, that the demand for precursors of protein synthesis can also be satisfied only by cell turnover.

At the cessation of net nitrogen incorporation due to the exhaustion of the nitrogen supply in the medium, the total amount of RNA decreases again with a simultaneous rise of "free nucleotides". This seems to be a general feature of RNA when the nitrogen source of the medium runs out (see following section).

The changes in the amount of "free nucleotides" are also reflected by the shape of the curve depicted for total nitrogen present in the acid-soluble fraction. Subtraction of the nucleotide nitrogen (assuming 15 % N) from this fraction gives an estimate of the other soluble nitrogenous compounds (amino acids etc.). After an initial doubling (within 12 hrs.) this fraction rises only faintly and at the end of the experiment has reached only three times the initial value. As compared with the other cell constituents, this means that after 12 hours the level of this "amino acid fraction" drops steadily. Thus, if cell turnover with respect to protein occurs, the rate of protein degradation is probably slower than the rate of resynthesis in the new cells.

It is interesting that in the second half of the experiment the increase of DNA keeps pace with the rapid increase of RNA and that DNA seems to be more stable with respect to the nitrogen level in the medium. As a result, the amount of DNA increases far more than any other cell constituent. Because all cells contain two nuclei, and assuming the DNA content of these nuclei to be constant, this might indicate that the newly-produced cells in the primordial region are smaller than the undifferentiated cells of the substrate hyphae (i.e. less protein per nucleus). An alternative might be that DNA is much more stable than protein and is not a component of cell turnover. As yet, no direct cytological observations have provided a basis on which to discriminate between these two possibilities.

Summarizing the reasoning given above, it can be said that the complex changes in cell constituents can be largely explained by assuming that in the early phases of growth after addition of thiamine, the growth of new cells is accompanied by a breakdown of RNA to

the level of soluble compounds in the pre-existing cells. Obviously, these compounds can be retained by the cells and are not excreted into the medium. The changes in the later phases of growth are explained by assuming that the formation of new cells depends not only on nutrients from the medium but also draws upon the accumulated nucleotides and protein in the "old cells".

If this view is correct, the increase in the total amount of nucleotides, i.e. the sum of the material specifically absorbing at 260 mμ in the soluble and insoluble (RNA) fractions, can be expected to show a better correlation with the increase of protein than the fractions do individually. Fig. 31 shows the results of such calculation, and it can be seen that the two curves fit together pretty well.

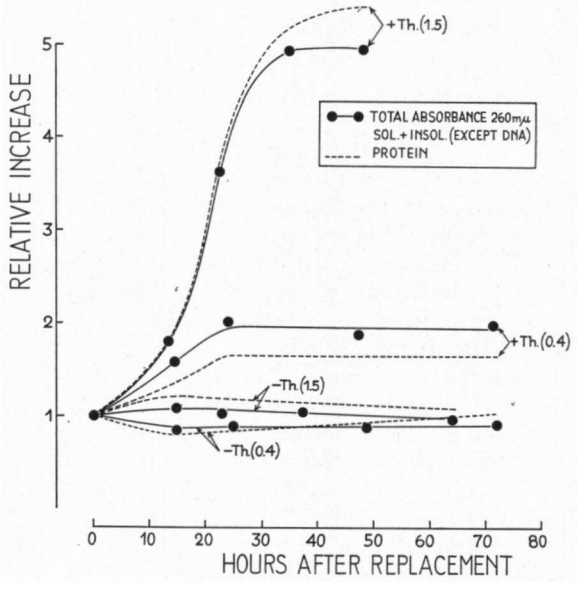


Fig. 31. Changes in total amount of substances absorbing specifically at 260 mμ (acid-soluble and insoluble, except DNA), calculated from the data of Figs. 29 and 32. For purposes of comparison, changes in protein are also drawn (dashed line). Asparagine concentrations are given in parentheses as mg/ml.

Figs. 29 and 30 also give data concerned with changes resulting from the omission of thiamine from the replacement medium. After replacement by such a medium (differing from the pre-shift medium only with respect to the omission of citrate) there is only a very small initial increment of protein, if any. At the same time, the RNA/protein ratio is significantly lowered, accompanied by an increase of the "free nucleotides". Subsequently, the original RNA/protein ratio is re-established and a simultaneous decrease of the "free nucleotides" occurs.

By similar reasoning it can be assumed that the extensive washings

during the replacement procedure temporarily restore normal conditions in the mycelium. For example, the washing procedure might lower the concentration of a substance, accumulated during thiamineless growth, that is responsible for the high RNA/protein ratio. The result would be a temporarily accelerated growth (synthesis of protein and RNA), accompanied by a breakdown of RNA in the preexisting cells (cf. small protein increment; RNA remains constant), which results in an increase of "free nucleotides" and lowering of the RNA/protein ratio. However, since no thiamine is present, the original conditions are gradually re-established: the "free nucleotides" are re-incorporated into RNA with a concomitant rise of the RNA/protein ratio to the original level. Fig. 31 shows that the sum of "free nucleotides" and RNA remains approximately constant.

3. *Shift of a thiamineless mycelium to a low-nitrogen medium in the presence or absence of thiamine*

In the previous section it was suggested that, even in the presence of external nutrients, cells in the deeper layers of the developing system add substantially to the supply of nutrients to the cells of the upper region that constitute the primordia. In this section we will describe what happens to the level of the various cell constituents if the process of cell turnover becomes the only mechanism for primordia formation due to the absence of a nitrogen supply in the medium.

To achieve this, the asparagine concentration in the replacement medium was lowered to 0.4 mg/ml, the lowest concentration found to be necessary for the regular formation of primordia (see previous chapter). The results are shown in Figs. 32 and 33.

It can be seen that in the presence of thiamine the low nitrogen concentration in the medium permits a net protein increment of only 65 % during the first 24 hours after replacement and that subsequently the amount of protein remains constant. At the cessation of protein increase, only the very first indication of primordia can be observed: the further establishment of primordia proceeds in the absence of net protein synthesis.

In contrast to the fate of protein, a quick 40–50 % reduction of the amount of RNA occurs after replacement. This reduction is accompanied by a nearly five-fold increase of "free nucleotides". The total amount of UV-absorbing material (sum of "free nucleotides" and RNA) rises only by a factor of 2 (Fig. 31). Again it can be assumed that the supply of thiamine restores normal conditions in the mycelium, which results in a lowering of the RNA/protein ratio. Apparently, the low nitrogen level in the medium does not allow any appreciable accumulation of newly-formed RNA in the growing cells; total RNA remains at a low level, creating a very low RNA/protein ratio. This could mean either that the newly-produced cells have an extremely low RNA/protein ratio or that, even though the ratio in the growing cells is quite normal, RNA degradation to "free nucleotides" starts as soon as the cells enter non-growing conditions. As yet, no arguments in favour of one of these explanations can be given.

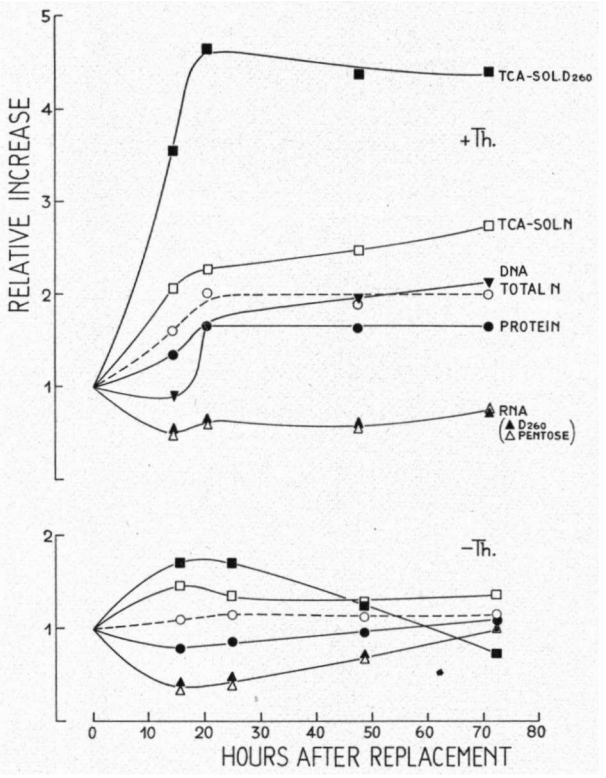


Fig. 32. *S. commune* K.35 shifted from thiamine-free medium to a low-nitrogen medium with or without thiamine at time zero (arrow in Fig. 28). Replacement medium: as for Fig. 29 but asparagine concentration lowered to 0.04 %. Each point represents the average of two cultures.

It can be calculated that the increase of total nitrogen in the acid-soluble fraction is almost completely due to the dramatic rise of the nucleotide material. As in the previous experiment, no soluble intermediates of protein metabolism accumulate.

We do not want to over-stress the initial lag in the increase of the amount of DNA because in another experiment no such a lag was found. However, the general continuous increase in DNA, even in the absence of net protein synthesis, was reproducible. As in the previous experiment, this could point either to a remarkable stability of DNA or to an unproportional increase in the number of nuclei resulting in cells which contain less protein per nucleus.

If thiamine is omitted from the low-nitrogen replacement medium, changes comparable to those recorded for the high-nitrogen medium become apparent (Figs. 32 and 33). However, the initial drop in RNA and the rise of "free nucleotides" are more conspicuous. In addition, there is a small initial loss of protein in this case.

It is remarkable that notwithstanding the size of the initial changes

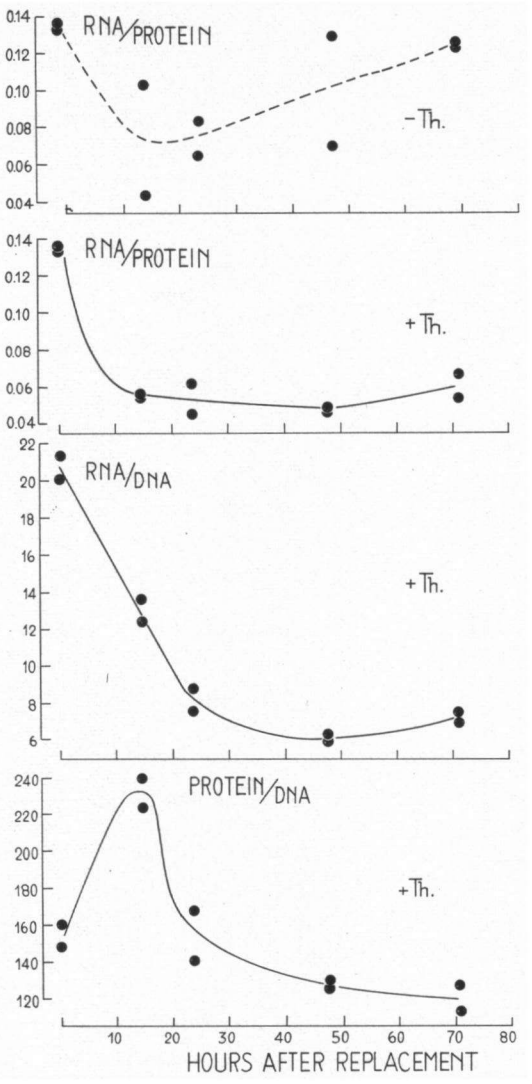


Fig. 33. Calculated ratios of cell constituents from the experiment shown in Fig. 32. Each point represents one culture.

and the low level of nitrogen in the medium, the original state of the thiamineless mycelium is practically restored after 3 days. Fig. 31 shows that in this experiment, too, there is a perfect balance between the amount of RNA and "free nucleotides" in the mycelium.

Discussion

It must be emphasized that the interpretation of the results obtained after replacement of the thiamine-free medium is based upon the

observation that in mycelia grown in the absence of thiamine, a high RNA/protein ratio is maintained even when the rate of protein synthesis slows down. A decrease in this ratio would be expected because of the well-known role of nucleic acids in protein synthesis. Our results show that a considerable decrease of the RNA/protein ratio indeed occurs when *Schizophyllum* is provided with thiamine and net protein synthesis ceases as the result of exhaustion of the nitrogen supply. In *Neurospora* a significant decrease of the RNA/protein ratio in older cultures has also been observed (SUSKIND and BONNER, 1960).

How thiamine deficiency brings about the divergent situation is not known. Clearly, thiamine deficiency must have a profound influence on the over-all metabolism and can change the intracellular concentration of several metabolites. For instance, we have seen that thiamine deficiency leads to the accumulation of pyruvic acid.

Because thiamine deficiency in some way inhibits protein synthesis, it is tempting to compare the effect on the RNA/protein ratio with similar effects of inhibitors of protein synthesis in bacteria. The presence of substances such as chloramphenicol (cf. KURLAND and MAALØE, 1962), puromycin (YARMOLINSKY and DE LA HABA, 1959), and streptomycin or the removal of streptomycin from dependent cells (SPOTTS and STANIER, 1961) cause an inhibition of protein synthesis but permit RNA synthesis, thus effecting the establishment of a high RNA/protein ratio.

It is important to note that the high RNA/protein ratio in thiamineless cultures cannot be explained simply as an inhibition of protein synthesis due to a shortage of one or more amino acids. The fact is that a similarly high RNA/protein ratio is maintained in cultures receiving a tryptic digest of casein (trypticase) as the nitrogen source.

Some support for the view that thiamine deprivation leads to the accumulation of some "inhibitor" of protein synthesis is given by the observation that extensive washing and reincubation in a thiamine-free medium initially decreases the RNA/protein ratio but that after some time the original state is restored. With respect to the removal of citrate it must be noted that this is not the cause of the pattern observed: similar changes are recorded when citrate is omitted from the pre-shift medium and trypticase is substituted for the asparagine in the pre-shift and post-shift medium. However, it must be admitted that there is no direct evidence that the intracellular concentration of an "inhibitor" is actually diminished during the washing procedure.

We have seen that the complex changes in RNA, protein, and "free nucleotides" associated with differentiation in a high- and low-nitrogen medium can be satisfactorily explained by starting from the concept of an artificially high RNA/protein ratio in the thiamineless mycelium and cell turnover after primordia induction.

A remarkable feature is that a high concentration of "free nucleotides" can be built up in the mycelium and that a perfect balance exists between this fraction and the amount of RNA. The fact that these presumed RNA degradation products are retained by the cells could be an expression of the economy of the process of cell turnover

during primordia formation. This is consistent with results already discussed in Chapter II. During the later stages of primordial growth, appreciable quantities of nitrogenous compounds are transported from the mycelium to the primordia. This process can nevertheless proceed without any loss of nitrogen, suggesting that all small nitrogenous degradation products can be retained by the organism.

The changes recorded in the experiment with the low-nitrogen post-shift medium supplemented with thiamine show that it is necessary to allow for some net protein synthesis at least in the phase that differentiation proper takes place. Whether this extra production of proteins from precursors in the medium is a specific feature of differentiation or only an accidental result of the extracellular presence of nitrogen, is not known. The latter case involves the assumption that during differentiation, some extracellular nitrogen must be present to prevent excessive degradation of RNA. Once differentiation has taken place, the undifferentiated cells can evidently supply the wants of the growing differentiated cells that are not surrounded by nutrient solution. It seems likely that the nucleic acid and protein metabolism of these differentiated cells is less dependent on the level of the nitrogen source in the medium because growth of these cells is evidently possible in a nitrogen-free medium.

In all likelihood, one of the features of differentiation in this phase of development is the acquisition of a more aerobic metabolism. Although thiamine is used as a controlling factor in the induction experiments, it seems that such biochemical differentiation also takes place during normal development, with thiamine present from the outset (see Chapter VI). However, it is clear that the acquisition of an active respiratory metabolism can at most be a prerequisite for more specific processes, e.g. the tendency of the hyphae to arrange themselves in multihyphal structures instead of growing individually in all directions. As yet, nothing is known about the biochemical basis of this crucial point of differentiation.

Nevertheless, the results described in this chapter could serve as a starting point for further investigations on the level of proteins and nucleic acids. Following present-day conceptions, the basic processes underlying cell differentiation may be the selective activation in time and space of latent gene DNA and a flow of information via RNA (informational or messenger RNA) to the sites of protein synthesis. For example, an experiment such as that outlined in Fig. 32 might be useful in studying the nature of the newly-produced proteins and RNAs during differentiation of primordial cells, since growth of differentiated and undifferentiated cells proceeds more or less sequentially whereas under normal conditions these processes occur simultaneously.

CHAPTER IX

A BRIEF SURVEY OF SOME BIOCHEMICAL ASPECTS OF THE DISTINCT PHASES OF DEVELOPMENT IN SCHIZOPHYLLUM COMMUNE

For the sake of convenience, the results presented in the previous chapters are grouped according to the various investigated aspects of differentiation and morphogenesis. These results, having been discussed in detail, will now be summarized in relation to the distinct phases of development in order to provide a more comprehensive picture.

The information was obtained by investigating two stocks of *Schizophyllum*, one exhibiting wild morphology (K.8), the other being a morphological mutant blocked in the process of pileus formation (cup mutant K.35). In the following résumé it will generally not be stated which stock provided the results being discussed. Most of the investigations concerning the early stages of development, cell-wall composition, and respiration were done with the cup mutant. However, it is taken for granted that the cup mutant differs from the wild stock only with respect to the last morphogenetic stage and that the results obtained for the earlier stages equally apply to the wild stock. Within the limits of these studies this seems indeed to be the case.

As outlined in the opening chapters of this paper, development can be roughly divided into four phases:

1. undifferentiated growth
2. initiation of primordia
3. growth of primordia
4. formation of pilei

1. *Undifferentiated growth*

The development of the dikaryotic organism starts with the production of a submerged system of undifferentiated hyphae; these hyphae only penetrate the substrate and do not emerge into the air. Normally, at a very early stage of growth differentiation occurs at the surface of the substrate, leading to the establishment of primordia and stroma. From then on, growth of undifferentiated and differentiated hyphae occurs simultaneously during the whole period of growth, i.e. as long as net protein synthesis is possible.

The respiration of the undifferentiated mycelium (measured before differentiation has taken place) is rather low. The requirement for two environmental factors, viz. oxygen and thiamine, was accordingly found to be much lower for undifferentiated growth than for the accomplishment of differentiation. As a matter of fact, the interesting observation was made that, under certain conditions, a considerable amount of undifferentiated mycelium can be produced in the absence of an external thiamine supply. Supplementation of a thiamine-free

medium with citrate and calcium carbonate gave a mycelium with about 30 % of the total amount of nitrogen contained in a mycelium grown in the presence of thiamine. Although this is about twice the amount formed prior to primordia formation in cultures supplied with thiamine, no differentiation can be seen in such a thiamineless mycelium.

Concerning the factors limiting thiamineless growth, a few suggestions were made. Large amounts of pyruvic acid were shown to be excreted and the hypothetical accumulation of a toxic intracellular concentration of this acid and a shortage of amino acids precursors were emphasized. However, there are indications that after the net synthesis of cytoplasm comes to a halt, the mechanism of cell turnover allows the continued (slow) production of new cells.

A remarkable feature of these thiamineless cultures is the maintenance of a high RNA/protein ratio after net protein synthesis ceases, although one would expect this ratio to drop significantly. The hypothesis is put forward that the maintenance of this high RNA/protein ratio is related to abnormal intracellular conditions created by deprivation of thiamine. Although no direct evidence is presented, this hypothesis makes it easy to interprete the complex changes in cell constituents occurring after a shift to fresh media with or without thiamine.

Although it is realized that the thiamineless mycelium probably represents a highly artificial system, it was used in these studies for the establishment of synchronized cultures and as a starting material providing information about the first developmental stage, viz. the initiation of fruit-body primordia.

2. *Initiation of primordia*

No information was obtained concerning the mechanism by which the individual hyphae are directed to aggregate. Whether this is a "hormonal" stimulus or a physico-chemical change in the properties of the cell-wall surface (or both) is not known, but the response to staining of the cell walls in primordia and stroma indicates that it may be worth-while to investigate the latter possibility.

It is obvious that once differentiation leading to the production of aggregating cells has taken place, formative processes cause these cells to arrange themselves into multihyphal systems such as primordia and stroma. Whether or not the differentiated cells are equipped with all the information to do so is not clear. Certainly, environmental factors have a profound influence on these processes. For instance, elevated temperatures or high carbon dioxide concentrations allow the formation of aggregates but inhibit the production of definite primordia; only a stroma and a vigorous aerial mycelium is produced. At 25°C in well-aerated cultures, the early mycelial aggregates invariably lead to the production of definite fruit-body primordia.

It has already been stated that normally the initiation of primordia occurs at a very early moment of the growth phase. In an experi-

mental system, using a pre-formed, undifferentiated mycelium grown in the absence of thiamine, it could be demonstrated that initiation of primordia, as induced by thiamine, can occur if the net increase of protein is severely restricted by a deficiency of the nitrogen supply in the medium. However, attempts to initiate primordia in the complete absence of net protein synthesis were completely unsuccessful. This implies that a minimal amount of growth in the sense of building new material from external nutrients is indispensable for the occurrence of differentiation at this stage. Once the differentiated cells are established (in microscopically-visible aggregates), their growth requirements can be met by a transport of cell material deriving from undifferentiated cells. Broadly speaking, some net protein synthesis from external nutrients seems invariably connected with differentiation at this stage. Further production of protein laid down in newly-produced undifferentiated cells seems only important as far as these cells serve as a store of nitrogenous material claimable for the growth of the small primordia when the nitrogen supply in the medium runs out. During the whole process of primordia formation a carbon and energy source such as glucose must be present.

The measured changes in RNA, DNA, protein, and acid-soluble cell constituents during thiamine-induced primordia formation provide the quantitative basis for further work on biochemical differentiation preceding the observed morphological changes. Of particular interest is the observation that differentiation can take place under conditions permitting only a small increase of protein and even a decrease of total RNA. The uncoupling of differentiation from net synthesis might be of considerable importance in a search for specific changes in proteins and RNAs pertinent to differentiation per se.

Another interesting point which became apparent is that, even during active cell turnover, soluble products of protein breakdown do not accumulate. If RNA is degraded, however, considerable amounts of degradation products accumulate in the cells. Surprisingly, these small molecules can be effectively retained by the cells and, under certain conditions, they are later re-used for the synthesis of RNA.

During the transition from totally undifferentiated to primordia-bearing mycelium, the respiration of the organism increases. This is probably due to a higher respiratory activity of the differentiated cells. Interestingly, high aerobic fermentation, resulting in RQ values as high as 2, can be found at the same time. It was demonstrated that this is not brought about by a limited supply of oxygen and that an interrelation with protein synthesis can explain, at most, only part of the high aerobic fermentation. On the other hand, the very high anaerobic fermentative capacity suggests that only part of the glucose metabolized can be channelled to the respiratory pathway, another part being degraded by a fermentative pathway.

3. *Growth of primordia*

After the primordia are established they increase in size. Apart from the early differentiation of the apical cavity (the initiation of the

hymenium), the morphological appearance of the primordia remains much the same; they only increase in mass.

Whereas the walls of the hyphae of the small primordia initials are still thin, growth of the hyphal filaments in the primordia is accompanied by an early thickening of the walls. In consequence, the fully-grown primordia are mainly composed of thick-walled hyphae except for the region around the apical cavity. During primordial growth, thick-walled hyphae also appear in the stroma.

The process of primordial growth is strictly dependent upon an external carbon supply. The results indicate that only very small amounts of typical reserve carbohydrates, such as glycogen, are formed; by far the greatest part of the glucose assimilated is used as a substrate for the synthesis of the considerable amounts of polysaccharides laid down in the thick walls of the primordial cells.

Analysis of the cell walls of *Schizophyllum* revealed that polyglucoses comprise more than 80 % of the dry-weight. Chitin is also present, but in rather small amounts (3–5 %). It was found that the glucans of the cell walls can be divided into two types: one is an alkali-soluble, acid-precipitable glucan, the other an alkali-insoluble glucan. The alkali-soluble, acid-precipitable polysaccharide is denominated S-glucan; it probably contains a predominance of β-(1→6) linkages. X-ray analysis showed it to be identical to a polysaccharide also present in other fungi that has not yet been investigated in more detail. The alkali-insoluble glucan contains β-(1→3) and β-(1→6) linkages; it is called R-glucan. It is most probably identical to the best-studied component of the yeast cell wall: yeast glucan.

It was shown that the glucose assimilated during primordial growth is primarily used for the synthesis of both the S- and R-glucan of the primordial cell walls. In spite of the striking differences in cell wall thickness, the quantitative distribution of these glucans in mycelium and primordia seems to differ only slightly.

In contradistinction to the need for an external carbon supply, the nitrogenous compounds required for the growth of the primordia can be fully supplied by the nitrogenous compounds contained in the submerged mycelium. In fact, the bulk of the mycelial nitrogen was found to be transported to the growing primordia and, as suggested by cytological evidence, a smaller amount to the developing stroma.

During this process, in which most of the cytoplasm of submerged cells is displaced to primordial cells, a high and constant respiration per unit protein is maintained. This seems to be a rather specific feature of the system in which primordial growth occurs. If primordial growth is inhibited by applying a temperature of 30°C, there is a steady drop in respiration, as seems consistent with an aging culture. Interestingly, in both systems the RQ and the fermentative capacity drop gradually. The hypothesis is put forward that this phenomenon is due to an increasingly hampered access of glucose to the active cells, partly caused by the displacement of cytoplasm from submerged to sub-aerial cells, and regulation of respiration and fermentation at the pyruvate level.

4. *Formation of pilei*

It will be recalled that the accomplishment of all the stages described hitherto depends on the supply of nutrients from the medium. A carbon and a nitrogen supply are necessary for submerged growth and at least for the early stage of primordia formation; a carbon supply is indispensable for the growth of the primordia. Only the last morphogenetic stage, the formation of pilei, proceeds without the uptake of nutrients from the medium.

The production of pilei is allocated to only a limited number of fructifications. However, because these pilei are relatively large structures, special attention was paid to the origin of the material needed for their construction.

As far as nitrogenous compounds are concerned, the results suggest that these are derived from the cell contents of stunted fructifications and probably also from the mycelium. However, more attention was paid to the origin of the carbon used for the construction of the pilei (cell walls) because results obtained with the cup mutant suggest that the breakdown of cellular polysaccharides is an essential prerequisite for pileus formation.

Subsequently, it was found that it is not so much the breakdown of typical reserve carbohydrates as a substantial breakdown of cell-wall polysaccharides that provides the carbohydrates required for pileus formation. In fact, it was found that especially the R-glucan (and chitin) in stunted fructifications and stroma is subject to considerable degradation. In consequence, the S-glucan/R-glucan ratio in the cell walls of stroma and stunted fructifications rises from values around 2–3 to values as high as 17 and 23 respectively. In the cell walls of the pilei synthesized at the expense of cell-wall degradation products, the two glucans are laid down again in a ratio of 2–3.

The correlation between cup morphology and the absence of cell-wall degradation, together with the fact that this process cannot be replaced simply by supplying glucose to the system, suggests that a steady, low concentration of glucose (or another degradation product of the wall glucans) is essential for the process of pileus formation. Strong support for the correctness of such a postulated regulatory mechanism was obtained; it is possible to inhibit pileus formation in the wild stock and to induce pilei in the cup mutant by manipulating the concentration of glucose in the milieu.

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